MICROBIOLOGICAL, IMMUNOLOGICAL AND GENOMIC CHARACTERIZATION OF NONTYPHOIDAL S*ALMONELLA* ISOLATES FROM NAIROBI COUNTY

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(Medical Microbiology)

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2015

Microbiological, Immunological and Genomic Characterization of Nontyphoidal S*almonella* Isolates From Nairobi County

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A Thesis Submitted in Fulfillment for the Degree of Doctor of Philosophy in Medical Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This piece of work is dedicated to my dear wife Liz and Son Alexander Nyangito. To Liz and Alexander: You are the source of my inspiration, thank you for your love, care, patience, endurance and prayers.

ACKNOWLEDGEMENT

First, I am grateful to the almighty God for giving me the courage, wisdom, strength and motivation that enabled me to complete this work.

I would like to sincerely and warmly thank all the persons who participated in the achievement of this work. Specifically, I acknowledge with gratitude my sponsors, European Union Seventh Framework Programme (2007 to 2013) GENDRIVAX project (Grant Agreement no. PIAP-GA-2009-251522) and Kenya Medical Research Institute (KEMRI) for the financial support they provided for this research. I sincerely thank Jomo Kenyatta University of Agriculture and Technology (JKUAT) for having granted me the opportunity to pursue my studies and for providing me with the ever encouranging and ready to assist academic mentors.

I wish to sincerely thank my supervisors Prof. Sam Kariuki, Prof. Anne Muigai and Dr. Simona Rondini for the deep interest they took in this work and for the immense guidance and critical discussion rendered during various stages of my study period. A lot of appreciation goes to my colleagues at Centre for Microbiolgy Research (CMR), KEMRI and the Welcome Trust Enterics laboratory in CMR for their assistance, technical support, cooperation and encouragement rendered during sample collection and my research work. I wish to extend my gratitude to Prof. Allan Saul, Prof. Calman MacLennan and Dr. Simona Rondini for granting me the chance to conduct my PhD research project in their laboratory at Novartis Vaccines Institute for Global Health (NVGH), Italy, for their immense contribution to this work, constructive advice and critically reviewing the manuscripts and progress reports as well as for their encouragement and support during difficult moments. Special thanks go to Yun Shan Goh (a post-doctrate student), Luisa Lanzilao (a technical staff), both of NVGH for their guidance in carrying out Serum bactericidal assays and O-Antigen extraction experiments. I am indeed grateful to Franceca Miccoli of Vaccine Chemistry department at NVGH who helped in the running of study samples for Size-exclusion high-pressure liquid chromatography (HPLC–SEC), ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy and anion-exchange column chromatography with pulsed amperometric detection (HPAEC–PAD) analyses. Special thanks go to Robert Kingsley and Chinyere Okoro of the Welcome Trust Sanger Institute (WTSI), UK led by Prof. Gordon Dougan for helping in the

sequencing of all the study samples and for granting me access to the WTSI bioinformatics computing platform and infrastructure for analysing the sequences. I also extend my sincere and warm thanks to members of staff and all my fellow students and post-doctrate students in NVGH for all the rich discussions that we had during my research work. I thank also Prof. Gunturu Revathi, Aga Khan Hospital for providing additional isolates.

My deepest gratitude goes to my family, first my wife Liz and our son, Alexander who endured periods of my prolonged absence from home and for their patience, love and unfailing encouragement. I remain indebted to my mother, my siblings and friends who have been there for me all this time. To all who are not mentioned but in one way or another contributed towards the successful completion of this work, I say a big thank you.

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LIST OF ABBREVIATIONS

^{1}H NMR	^{1}H nuclear magnetic resonance
Abe	Abequose
AKH	Aga Khan Hospital
AST	Antimicrobial susceptibility testing
CCR7	CC-Chemokine receptor 7
CDC	Centres for disease control and prevention
CFR	Case fatality rate
CFU	Coliform forming units
CMR	Centre for Microbiology Research
CSF	Cerebral spinal fluid
DC	Dendrtitic cells
ERC	Ethical review committee
GENDRIVAX	Genome Driven Vaccine
GGCH	Gertrude's garden children hospital
Hib	*Haemophilus Influenzae* Type b
HPAEC–PAD	Anion-exchange column chromatography with pulsed amperometric detection
HPLC-SEC	Size-exclusion high-pressure liquid

	chromatography
IBD	Invasive bacterial disease
iNTS	Invasive Nontyphoidal *Salmonella*
JKUAT	Jomo Kenyatta University of Agriculture & Technology
KDH	Kilifi District Hospital
KEMRI	Kenya Medical Research Institute
KEPI	Kenya Extended Programme of Immunization
KNH	Kenyatta National Hospital
LB	Luria bertani
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MAMP	Microbe-associated molecular patterns
MDR	Multi-drug resistance
MLST	Multi-locus sequence type
MW	Molecular weight
NTS	Nontyphoidal *Salmonella*
NVGH	Novartis Vaccines Institute for Global Health

OAg	O-Antigen
OD	Optical density
Rha	Rhamnose
***S*. Enteritidis**	*Salmonella* Enteritidis
***S*. Typhimurium**	*Salmonella* Typhimurium
SBA	Serum bactericidal assay
SEn	*Salmonella* Enteritidis
SKT	Solitary intestinal lymphoid tissue
SNP	Single nucleotide polymorphism
SSA	Sub-Saharan Africa
SSC	Scientific steering committee
ST	Sequence type
STm	*Salmonella* Typhimurium

ABSTRACT

Nontyphoidal *Salmonellae* (NTS) of major clinical significance in sub-Saharan Africa (SSA) consist mainly of *Salmonella enterica* serovars Typhimurium and Enteritidis. No vaccine is currently available. In SSA, NTS cause both invasive and gastrointestinal disease and contribute a huge burden of illness especially among young children under five years and HIV-infected adults. In Kenya, this includes sick children presenting to rural district and urban-based facilities. Invasive NTS (iNTS) disease has an associated case-fatality rate of around 20%. Previous reports indicate the importance of the O-antigen of *Salmonella* lipopolysaccharide (LPS) for virulence and resistance to antibody-mediated killing. The objective of the study was to immunochemically and genomically characterize *S*. Typhimurium and *S*. Enteritidis isolates from Kenya implicated with invasive and gastrointestinal NTS disease in order to understand if immunochemical and genomic features correlate with different clinical presentations. The work drew from an existing culture collection of 193 NTS isolates from studies at the Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI) between 2000 and 2011. These included 114 *S*. Typhimurium and 79 *S*. Enteritidis, 70 of which were from blood, 56 from stool, 12 from both blood and stool, three from urine, two from cerebral spinal fluid, and 31 were from stools of healthy carriers. The clinical isolates were mostly from children aged below 10 years admitted to four main hospitals in Kenya. Additional isolates included one from soil, one from a sewer and 17 isolates whose records of their sources of isolation were not available. A complete database detailing clinical metadata of each study isolate was created. Isolates were assigned as invasive (from blood, CSF, urine), gastro-intestinal (from stool), mixed infection (from both blood and stool) or healthy carrier for comparative analysis of results.

The susceptibility of all 193 isolates to antibody-mediated killing was tested by serum bactericidal assay (SBA). Additionally, LPS O-antigen was extracted from each of the 193 isolates for further characterization to evaluate if the different categories of NTS isolates express O-antigen with different features. To estimate the molecular size distribution of O-antigen populations in the samples, all the isolates were analysed by size-exclusion chromatography. Similarly, to confirm the identity of the extracted O-antigen samples and to verify the presence of O-acetyl groups along the O-antigen chain, nuclear magnetic resonance spectroscopy analysis was performed on a subset of isolates and glycosylation levels were determined. Differences in O-antigen profiles were correlated with clinical presentation and serum susceptibility. To genetically characterize the samples, whole-genome sequencing was performed. The population structure of NTS isolates was compared with the available clinical metadata. Antimicrobial Susceptibility Testing (AST) was done and associations between the antibiograms and genomic data were looked for. *S.* Typhimurium isolates were found to be susceptible to antibody-mediated killing, but *S.* Enteritidis showed reduced susceptibility and expressed more O-antigen than Typhimurium ($p<0.0001$ for both comparisons). For *S.* Typhimurium, but not Enteritidis, O-antigen expression correlated with reduced sensitivity to killing ($r=0.29$, 95% CI=0.10 - 0.45, $p=0.002$). Both serovars expressed O-antigen populations ranging 21 - 33 kDa average molecular weight. O-antigen from most Typhimurium were O-acetylated on rhamnose and abequose residues, while Enteritidis O-antigen had low or no O-acetylation. Both Typhimurium and Enteritidis O-antigen were approximately 20-50% glucosylated. Glucosylation levels and amount of O-antigen of *S.* Typhimurium were inversely related. There was no clear association between clinical presentation and antibody susceptibility, O-antigen

level or other O-antigen features. A majority (77%, 88/114) of *S*. Typhimurium and 30% (24/79) of *S*. Enteritidis isolates were MDR, with *S*. Typhimurium isolates having a higher proportion of isolates resistant to at least one antibiotic (97%, 111/114) compared to *S*. Enteritidis isolates (92%, 73/79). Whole genome sequences showed that a majority 78%, (89/114) of *S*. Typhimurium isolates analysed belonged to the sequence type ST313 which also fell within the two previously described lineages, designated lineage I (36%, 41/114) & II (42%, 48/114) while 22% (25/114) isolates belonged to two ST19 lineages, designated lineage IIIA (18/114) & IIIB (7/114). The novel lineage ST19 IIIB has not previously been described. For *S*. Enteritidis, all the 79 isolates belonged to ST11 and fell into two lineages, designated lineage I (30%, 24/79) & lineage II (70%, 55/79). The phylogenetic structure of *S.* Typhimurium isolates was found to be similar to a previously published population structure of invasive *S*. Typhimurium across SSA. A dominant *S*. Typhimurium pathotype, designated ST313, rarely reported outside of Africa, was found to dominate among the MDR *S*. Typhimurium isolates. A majority of these were primarily associated with invasive disease and febrile illness. Clinical records showed that diarrheal patients of *S.* Typhimurium isolates had febrile invasive illness symptoms (fever, vomiting, cough), implying that other *S*. Typhimurium pathotypes (ST19 lineages), previously associated mainly with gastroenteritis are capable of establishing invasive disease. In conclusion, Kenyan *S*. Typhimurium and Enteritidis clinical isolates are susceptible to antibody-mediated killing, with resistance correlating with amount of O-antigen among *S*. Typhimurium ($r=-0.51$, 95% CI -0.69 to -0.27, $p<0.0001$). This supports the development of an antibody-inducing vaccine against NTS for SSA. No clear differences were found in the phenotype of isolates from blood and stool, suggesting that the same isolates can cause invasive

disease and gastroenteritis. Phylogenetic structure of Kenyan *S*. Typhimurium was similar to published structures of NTS in SSA hence NTS disease in Kenya mirrors what is seen in many different SSA countries. Multi-drug resistance observed in a majority of *S*. Typhimurium isolates and a substantial proportion of *S*. Enteritidis isolates confirms widespread MDR among Kenyan NTS as also reported in many other SSA countries. Genomically, invasive and gastroenteric isolates appear to be indistinguishable and so more in-depth genome studies are required to understand whether invasive and gastrointestinal isolates differ.

CHAPTER ONE

1.0 INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) are two *Salmonella enterica* serovars that are classically associated with localised human gastroenteritis (Grimont, 2007; Le Minor and Bockemuhl, 1988). Thus, they are regularly referred to as non-typhoidal *Salmonella* (NTS), in contrast to *S*. Typhi and *S*. Paratyphi A that cause the human restricted systemic disease known as typhoid (typhoidal) (Mastroeni and Maskell, 2006).

Nontyphoidal *Salmonellae* are a major, but neglected cause of invasive disease and therefore referred to as iNTS in Africa (Feasey et al., 2012; MacLennan and Levine, 2013; Reddy et al., 2010). *Salmonella* Typhimurium and *S.* Enteritidis account for nearly 80% of all human isolates reported globally (Vieira et al., 2009). The classification of *Salmonella* isolates into typhoidal *versus* nontyphoidal strains along serotypic lines has held up well, particularly in economically developed countries within Europe and North America (Helms et al., 2005). Nontyphoidal serovars can cause systemic disease in these economically developed countries but the incidence is relatively low and often associated with a compromised condition such as immune deficiency or advanced age (Feasey et al., 2012). While in economically developed countries they predominantly cause a mild self-limiting gastroenteritis (Langridge et al., 2009; Mermin et al., 2005; Hohmann, 2001), in sub-Saharan Africa they are responsible for bacteraemia, often associated with meningitis in young children, with incidence rates comparable to invasive *Streptococcus pneumoniae* disease (Reddy et al., 2010).

Nontyphoidal *Salmonella* serovars, exemplified by classical *S.* Typhimurium strains, cause gastroenteritis by employing two Type III secretion systems (T3SS) (Barthel et al., 2003; Coburn et al., 2005; Tsolis et al., 1999). The invasion-associated T3SS encoded by Salmonella pathogenicity Island 1 (SPI-1) enables *S.* Typhimurium to enter the intestinal epithelium (Zhou, 2001), while the T3SS encoded by SPI-2 is subsequently used to promote survival within macrophages (Abrahams and Hensel, 2006). The SPI-1 and SPI-2 T3SS are structurally related to the archetypal flagellar T3SS. Invasion of epithelial cells *in vivo* is observed within 10 to 15 minutes after introduction of *S.* Typhimurium into the intestinal lumen, but by 4 hours after infection the majority of bacteria are located in the lamina propria, with bacteria no longer observed within epithelial cells by electron microscopy (Santos et al., 2002). In sub-Saharan Africa, cases of NTS, frequently complicated by bacteremia, are now more numerous than cases of enteric fever (Gordon et al., 2008). Although invasive NTS bacteremia was first documented over 20 years ago (De Wit et al., 1988), the magnitude of this emerging problem has only belatedly been appreciated (Morpeth et al., 2009). The incidence of NTS has risen in association with predisposing infections with HIV and *Plasmodium falciparum* (Bronzan et al., 2007). NTS isolates are now among the most common blood culture isolates in many parts of Africa, comprising as much as 50% of cases of bacteremia (Brent et al., 2006; Gordon, 2008; Gordon et al., 2008). The distribution of pediatric NTS bacteremia in African children corresponds almost precisely to the prevalence of malaria (Gordon et al., 2008), although the reasons for this correlation are poorly understood.

The true burden of iNTS disease is uncertain due to the absence of a characteristic clinical presentation. Patients often present with nonspecific fever (Brent et al., 2006; Gordon et al., 2002; Graham et al., 2000a) and blood culture is necessary for diagnosis. Even where blood culture facilities are available, rapid clinical progression of NTS bacteremia results in many patients dying before a microbiological diagnosis can be made (Graham et al., 2000a). No vaccine is available for NTS, and clinical management is made difficult by widespread multi-

drug resistance (MDR) and the need for late-generation expensive antibiotics (Gordon et al., 2008; Kariuki et al., 2006b; Kingsley et al., 2009; Kariuki et al., 2005; Kariuki and Dougan, 2014).

In Kenya, iNTS disease is particularly common in rural areas (Tabu et al., 2012), with incidence rates as high as 568/100,000 person-years (Talbert et al., 2010). A recent study from western Kenya found an association between NTS diarrhoea and mortality in hospitalized children (O'Reilly et al., 2012), indicating that NTS isolates in the region cause both invasive and gastrointestinal disease, but it is currently unknown whether there are any specific phenotypic or genotypic characteristics associated with each clinical presentations.

Previous whole genome sequencing studies demonstrate that invasive African *S*. Typhimurium is genetically distinct and characterized by a novel genotype, known as Multi-Locus Sequence Type (MLST), commonly reffered to as Sequence Type (ST) 313 which is a major cause of invasive NTS disease in many parts of sub-Saharan Africa, including Kenya (Okoro et al., 2012). Currently there is a focus on characterization of the genome of ST313 isolates. Similarly, equivalent studies are ongoing for *S*. Enteritidis, which has been reported to be more prevalent than *S*. Typhimurium in some parts of Africa (Ikumapayi et al., 2007; Paglietti et al., 2013) and globally (Galanis et al., 2006). Although information on the genome structure of different *Salmonella* sp., is increasingly becoming available, relatively little is known about the key genomic differences that are present in diarrhoea-associated strains and those from blood stream infections.

These previous whole genome and phylogenetic studies have further shown that many of the invasive African *S*. Typhimurium are genetically different (Wain et al., 2013) from other *S*. Typhimurium lineages and belongs to the novel MLST group, ST313, rarely found outside Africa. Two distinct lineages of the ST313 pathovar have been defined and their emergence may be associated with antibiotic usage and the spread of HIV (Okoro et al., 2012). The ST313 pathovar is characterized by marked genome degradation, usually associated with human restricted pathogens

(Kingsley et al., 2009) that has a striking resemblance to that observed in *S*. Typhi. However, little is known about the relationship between the genotypic and phenotypic features of ST313 that may be associated with their ability to cause invasive disease (MacLennan and Levine, 2013).

Studies on the genotype/phenotype relationship of NTS isolates, and the characterization of whole genomes of diarrheagenic versus invasive *S*. Typhimurium could help in the understanding of whether the same strains are responsible for both clinical presentations or whether invasive disease is the consequence of different, potentially more virulent clades of NTS (Onsare and MacLennan, 2014). Whole genome analysis could also provide valuable insights into routes of transmission. Indeed, evidence has accumulated that the major transmission route of ST313 may be human to human rather than from a classical zoonotic source normally associated with NTS spread (Parsons et al., 2013).

O-antigen constitutes the outermost part of the lipopolysaccharide (LPS) of Gram-negative bacteria (Rietschel, 1992). In pathogenic bacteria such as *Salmonella* spp, LPS plays an important role in the interaction between the bacterium and its host and it is required for colonization and host resistance (Park et al., 2008). While the lipid A moiety is the predominant cause of the endotoxic effects of LPS, OAg is the most immunodominant portion of the molecule, and is responsible for serovar-specificity (Raetz and Whitfield, 2002).

O-antigen protects bacteria from the environment and from serum complement (Joiner et al., 1982; Saxen et al., 1987; MacLennan et al., 2008), which can lead to bacterial killing by membrane attack complex formation. The OAg structure plays a role in bacterial virulence, with longer OAg chains associated with increased complement and antibody resistance (Bravo et al., 2008; Joiner et al., 1986; Murray et al., 2006) and protection against host antimicrobial factors (Holzer et al., 2009).

1.1 Problem statement

Nontyphoidal *Salmonella* serovars Typhimurium and Enteritidis are important agents of invasive disease, associated with sepsis and meningitis in young children and in adults with HIV and remain a public health concern in Kenya and in sub-Saharan Africa (Feasey et al., 2012; Kankwatira et al., 2004). The increasing antibiotic resistance of NTS pathogens and high case fatality of NTS disease are stimulating the quest for control, management and preventive strategies such as finding effective vaccines to assist in disease control (Clasen et al., 2007; Crump et al., 2011a; Graham et al., 2000b). No vaccine against NTS is currently available for use in humans (Goh and MacLennan, 2013). Nontyphoidal *Salmonella* infecting humans are under strong selection pressure and that this has major impacts on vaccine strategies (Waldner et al., 2012). Vaccines design needs to address issues of antigenic diversity to ensure that a novel vaccine covers the major circulating serovars of the pathogens (MacLennan et al., 2014). Since target of protective immunity are under strong pressure, understanding the diversity of pathogen components such as OAg gives important information about targets of natural immunity (MacLennan et al., 2014). A successful vaccine against NTS will put new selection pressure on the pathogens and understanding the population dynamics of pathogens will help design vaccines that minimize the risk of the emergence of vaccine resistant pathogens (MacLennan et al., 2014).

However, relatively little is known about the key phenotypic or genotypic differences that are present in NTS strains associated with diarrhoea and those from blood stream infections and whether there are any immunological, genotypic or phenotypic features that may be associated with their ability to cause invasive disease.

1.2 Justification

In Kenya, with the introduction of a conjugate vaccine in 2004 against invasive *Haemophilus influenza* type b (Hib*), Haemophilus influenza* disease has reduced by over 80%. In addition, the introduction in 2011 of a conjugate pneumococcal vaccine into the national Kenya Expanded Program on Immunization (KEPI) is expected to

lead to a major fall in invasive *S. pneumoniae* disease (Scott and English, 2008). Nontyphoidal *Salmonella* may therefore become the principal cause of community - acquired bacteremia in Kenya and in other African countries as they introduce similar vaccination schedules.

In Kenya it is clear that invasive NTS disease is of great public health importance especially in poor-resource settings where water supply and sanitation conditions are less than optimal (Okeke et al., 2007). Since currently there is no vaccine against iNTS (Goh and MacLennan, 2013), for rational efforts towards iNTS preventive measures, including vaccine development, elucidation of facets of the pathogen biology, microbiological and immunological characterization of iNTS is important (Wick, 2011).

A detailed genotypic and phenotypic comparison between invasive and diarrhoeal clinical NTS strains from Kenya has not been conducted. An understanding of the relevant protective immune mechanisms against NTS bacteremia is essential if a vaccine is to be developed (Nyirenda et al., 2010). There is also a need to understand the relationship between phenotypic and genetic characteristics of NTS strains and whether they contribute to determining if invasive or non-invasive disease occurs in humans.

Although data from previous studies has described the importance of antibodies both for serum killing and intracellular oxidative killing of invasive NTS (MacLennan et al., 2008; Goh and MacLennan, 2013), no data is available yet describing the susceptibility of sub-Saharan Africa clinical NTS isolates to serum killing when tested in the same conditions and whether there are any specific factors that may account for serum susceptibility.

In addition, although previous studies conducted at Novartis Vaccines Institute for Global Health (NVGH) have shown that OAg from different *S.* Typhimurium strains are characterized by two populations at different MW, and differences in acetylation

and glycosylation levels (Micoli et al., 2014), those differences have not been put in the context of strain pathogenicity, yet.

This combination of microbiological-immunological approaches, linked to whole-genome characterization would therefore provide important insights into the current knowledge on NTS, most important on pathogenicity factors.

1.3 Research questions

The present study addressed the following research questions:

I. Based on the pateints' recorded clinical metadata on clinical sources of isolation and sample type, what are the categories of NTS isolated in Kenya?
II. What are the susceptibility profiles of the NTS isolates to antibody (serum)-mediated killing?
III. What are the lipopolysaccharide O-antigen expression features and structural characteristics including O-acetylation and glucosylation of the NTS isolates?
IV. What is the whole-genone Single nucleotide (SNP)-based population structure and Multi-Locus Sequence Types (MLST) of the NTS isolates?
V. What are the antimicrobial susceptibility profiles of the NTS isolates from Kenya and is there any association between antimicrobial susceptibility profiles and whole-genome based population structure of the NTS isolates?

1.4 Null hypothesis

Invasive isolates are by nature, not more resistant to survival in the blood than gastrointestinal isolates and the phylogenetic structure of Kenya NTS isolates is not similar to that of geographically diverse NTS isolates from across sub-Saharan Africa.

1.5 Objectives

1.5.1 General objective

To investigate microbiological, immunological and genotypic characteristics of NTS isolates from Nairobi County responsible for invasive and enteric infection.

1.5.2 Specific objectives

1. To characterize archived NTS isolates as invasive, enteric, mixed (invasive/enteric) or healthy carrier isolates based on the clinical sources and sample type data as recorded in the available patient records.

2. To characterize NTS isolates for *in vitro* susceptibility/resistance to antibody-mediated killing.

3. To characterize NTS isolates' lipopolysaccharide O-antigen expression and structural characteristics including O-acetylation and glucosylation.

4. To deduce the SNP-based population structure of the NTS isolates using whole-genome based phylogenetic methods
5. To determine antimicrobial susceptibility of the NTS isolates

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Nontyphoidal *Salmonella* classification and nomenclature

Nontyphoidal *Salmonella* spp. belongs to the genus *Salmonella*, family *Enterobacteriacae*. *Salmonella* spp. are separated into two species, *Salmonella enterica* and *Salmonella bongori* (Haeusler and Curtis, 2013), with the former being further classified into six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae, indica*) (Haeusler and Curtis, 2013). *Salmonella* spp. are Gram-negative, motile, facultative anaerobic bacilli, which rarely ferment lactose (Nataro et al., 2011). *Salmonella* spp. are classified according to antigenically diverse surface antigens: polysaccharide O (somatic) antigens, H (flagellar) antigens and Vi (capsular) antigens (Nataro et al., 2011). Agglutination reactions based on the O-antigen are commonly used to divide *Salmonella* into serogroups which include, but are not limited to, A, B, C1, C2, D and E (Table 1) (Grimont, 2007).

Salmonella enterica subspecies *enterica* consists of almost all the serotypes pathogenic for humans (Nataro et al., 2011). In regard to human disease, although many *Salmonella* serovars exits, *Salmonella* infections are broadly divided into typhoidal *Salmonella* or Nontyphoidal *Salmonella* (Haeusler and Curtis, 2013) (Table 1).

Table 1: Examples of clinically important Salmonella serotypes and relevant classical clinical diseases in respect to serogroups.

Serogroup	**Serovar example ***	**"O" antigens**	**Phase 1 "H" antigens**	**Phase 2 "H" antigens**	**Clinical disease**
A	Paratyphi A	1,2,12	a	no phase 2 antigen	Enteric fever
B	Paratyphi B	1,4,5,12	b	1,2	Enteric fever
	Typhimurium	1,4,5,12	i	1,2	NTS
	Heidelberg	1,4,5,12	r	1,2	NTS
C1	Paratyphi C	6,7,	c	1,5	Enteric fever
	Choleraesuis	6,7	c	1,5	NTS
	Virchow	6,7	r	1,2	NTS
C2	Newport	6,8	e,h	1,2	NTS
D1	Typhi	9,12,Vi	d	no phase 2 antigen	Enteric fever
	Enteritidis	1,9,12	g,m	no phase 2 antigen	NTS
	Dublin	1,9,12	g,p	no phase 2 antigen	NTS

**** All examples of the serotypes given belong to Salmonella enterica subspecies enterica (Grimont, 2007; Kauffmann, 1966; Le Minor and Bockemuhl, 1988).***

2.2 Disease management of Nontyphoidal *Salmonella*

2.2.1 Challenges in disease diagnosis

Invasive nontyphoidal *Salmonella* (iNTS) disease especially in sub-Saharan Africa lacks a clear pathognomonic clinical presentation and therefore may kill without the patient, family or healthcare provider knowing or suspecting the disease (MacLennan and Levine, 2013). The most common clinical feature is fever and gastrointestinal symptoms which occur in less than half of the patients (MacLennan and Levine, 2013). Indeed, a proper diagnosis is only possible where blood culture is available

but unfortunately this facility is rare in large parts of sub-Saharan Africa especially in Kenya (MacLennan and Levine, 2013).

The disease falsely presents as severe pneumonia with rapid breathing (MacLennan and Levine, 2013) thus posing a management challenge since the common empirical treatment algorithms for severe pneumonia involve therapy with penicillin, with added chloramphenicol or gentamicin for very severe pneumonia (Graham and English, 2009). Inadequate availability of affordable and reliable diagnostic microbiological investigation equipment in most sub-Saharan Africa settings makes accurate documentation of the incidence of NTS difficult (Clemens, 2009). The disease burden is often not fully appreciated in a range of neglected tropical diseases such as NTS because of patchy diagnostics and reporting (Feasey et al., 2010; Hotez and Kamath, 2009).

Clinical associations with iNTS disease in children in sub-Saharan Africa also adds to diagnosis and treatment challenges (MacLennan and Levine, 2013). For example coinfection of iNTS and malaria can lead to the scenario where a child is successfully treated for malaria, yet dies from undiagnosed iNTS. The same can occur in children with severe anemia or malnutrition, as these are also risk factors for iNTS. In adults with HIV infection, recurrent iNTS disease has also been reported and may occur through recrudescent or *de novo* infection (MacLennan and Levine, 2013).

2.2.2 Disease burden of Nontyphoidal *Salmonella*

There is increased recognition of NTS as a major cause of severe febrile illness in sub-Saharan Africa. Nontyphoidal *Salmonella* infection represents a considerable burden mainly in developing countries. Reports of the huge burden of disease due to NTS in Kenya include sick children presenting to rural district facilities as well as urban-based hospitals (Graham and English, 2009). In a single site study in a rural area of Kilifi, Kenya the minimal incidence of community- acquired NTS bacteremia was estimated at 175 per 100,000 per year for children less than 2 years of age (Berkley et al., 2005). However, since many cases of illness in such a community

never reach hospital and blood culture is relatively insensitive, the true disease incidence is most likely considerably higher. In these studies Gram-negative bacilli were associated with an in-patient mortality of 62% in children less than 14 days old and 31% in older children (Bejon et al., 2005; Brent et al., 2006). In other site-based studies in rural Kilifi, Kenya, in 2000 - 2005, the local minimum incidence of community-acquired NTS was estimated at 166 per 100,000 per year for children less than five years of age (Berkley et al., 2005; Mwangi et al., 2002), whereas in another rural site in western Kenya from 2006 to 2009 among 55,000 persons, incidence was estimated at 568/100,000 person-years of observation (Tabu et al., 2012). The true incidence in both sites was however thought to be underestimated owing to incomplete blood culturing of febrile patients, community cases that never reach the hospital, and the general insensitivity of blood culturing (Kariuki and Dougan, 2014). At the national referall hospital, Kenyatta National Hospital (KNH) in Nairobi, among over 1300 patients over the period 1988 - 1997, an incidence of 13.7% was reported among HIV-positive adult patients, compared to 3.1% among HIV-negative patients of NTS-associated IBD, accounting for half of the bacteremic cases, with mortality ranging between 18.5% and 40%. At the Kenyatta National Hospital, the largest referral hospital in Nairobi, Kenya, 13.7%, among HIV-positive adult patients, compared with 3.1% among HIV-negative patients had invasive bacterial disease, with NTS accounting for half of the bacteremic cases and mortality ranging between 18.5-40% (Arthur et al., 2001).

Other studies have shown that NTS are the second-commonest cause of neonatal meningitis and the third-most-common cause of bacterial meningitis in children over two months of age in Malawi and Kenya (Bejon et al., 2005; Brent et al., 2006). They are also an important cause of septic arthritis and neonatal sepsis (Lepage et al., 1990; Milledge et al., 2005). In Malawi, the overall case-fatality rate associated with NTS bacteremia is estimated to be 22.3% (Gordon et al., 2008).

The exact total burden of disease attributable to iNTS in Africa has not been measured, but annual incidence is estimated at 175-388 cases per 100,000 children aged between 3 - 5 years and 2000-7500 cases per 100,000 HIV-infected adults

(Feasey et al., 2012). Several other studies investigating the cause of bloodstream infection in febrile adults and children in Africa have shown that NTS are among the most common isolates from febrile presentations in adults and children across sub-Saharan Africa, especially where HIV prevalence is high (Figure 1) (Reddy et al., 2010).

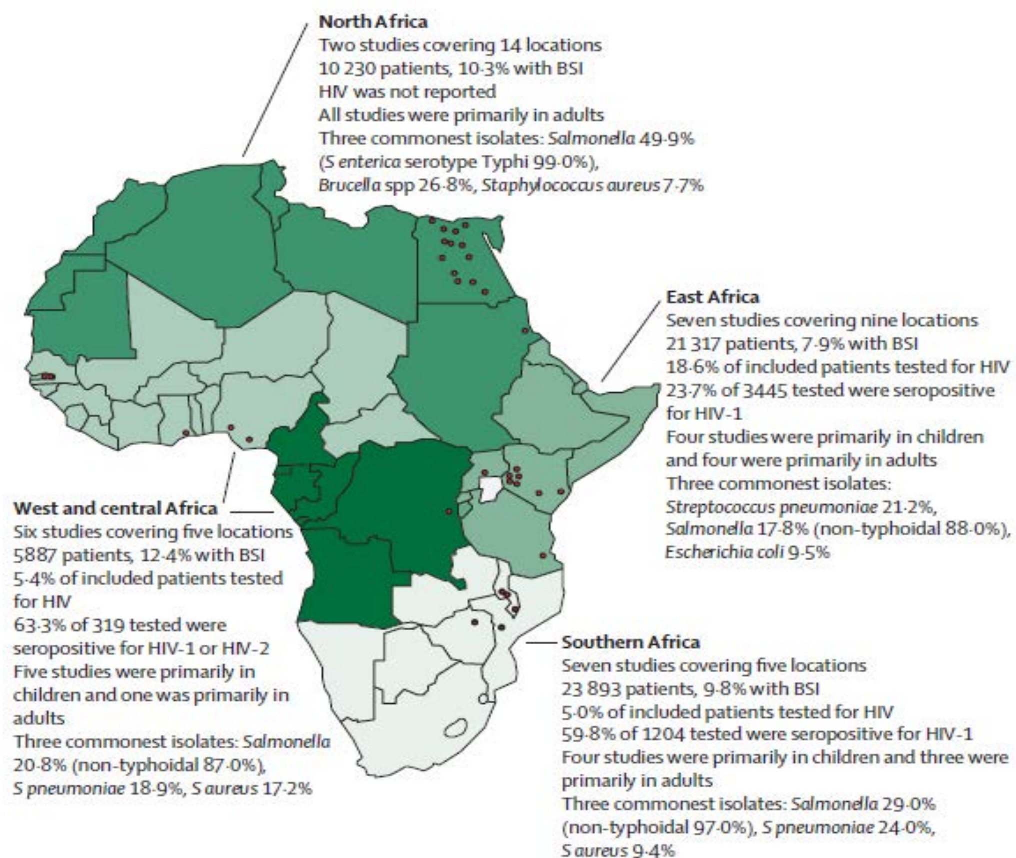

Figure 1: Map of Africa showing results of an analysis of studies investigating the cause of bloodstream infection in febrile adults and children in Africa (Reddy et al., 2010);

BSI=bloodstream infection

A combined data from the 11 study sites across sub-Saharan Africa gave NTS incidence rates of 478 and 530/100,000 children/year for the two cohorts recruited (Agnandji et al., 2011). These incidence rates are similar to those reported from blood culture-based studies of iNTS in individual sites in sub-Saharan Africa. A single-site study from Mozambique is such an example (Sigauque et al., 2009). The

study gave an incidence rate for iNTS bacteremia of 388/100,000 children/year in children under 1 year of age.

In Mali, where known NTS risk factors such as HIV prevalence is low, severe iNTS disease accompanied by high case-fatality rates is also a common cause of bacteremia and continues to be a problem in children not infected with HIV (Tennant et al., 2010).

Although the global human health impact of *Salmonella* infections has not been estimated, gastroenteritis is a major cause of morbidity and mortality, worldwide, both in children below five years old (Bern et al., 1992; Kosek et al., 2003) and in the general population (Scallan et al., 2005). While the true global incidence of NTS is still unknown, it is estimated that 93.8 million cases of gastroenteritis due *Salmonella* spp. occur worldwide leading to 155,000 deaths each year (Majowicz et al., 2010). According to Salm-Surv (a World Health Organization (WHO) supported food-borne disease surveillance network) data from 2001 to 2005, *S.* Enteritidis was the most common serotype world-wide (65% of the isolates), followed by *S.* Typhimurium (12%) and *S.* Newport (4%). In these data, for Africa, *S.* Enteritidis and *S.* Typhimurium represented 26% and 25% of the isolates, respectively (Galanis et al., 2006). In Asia, Europe and Latin America/Caribbean, *S.* Entiritidis was the most frequent isolate (38%, 87% and 31%, respectively) (Galanis et al., 2006). In North America *S.* Typhimurium was the most frequently reported (29%) followed by *S.* Enteritidis (21%) and other *Salmonella* spp. (21%) (Galanis et al., 2006). Sub-Saharan Africa hospital-based studies reported blood stream *Salmonella* spp. infections to be more frequently associated with particularly *S.* Enteritidis and *S.* Typhimurium, than *S.* Typhi or *S.* Paratyphi (Galanis et al., 2006). In this region, iNTS is endemic and has elevated morbidity and mortality in children less than three years and adults with HIV infection (Crump et al., 2011b; Mandomando et al., 2009; Morpeth et al., 2009). In contrast, NTS invasive disease is infrequent in Asia except in subjects with severe immunosuppression (Dhanoa and Fatt, 2009; Khan et al., 2010).

The estimated increasing incidence of NTS in economically developed countries has become a public health concern (Weinberger and Keller, 2005; Majowicz et al., 2010; Maraki and Papadakis, 2014). The estimated NTS-associated illnesses incidence in Europe is 690 per 100,000 inhabitants per year (Sanchez-Vargas et al., 2011). This incidence varies between regions from 240 per 100,000 in Western Europe to 2390 per 100,000 person-years in Central Europe (Majowicz et al., 2010). The annual incidence in Israel ranges from 52 to 102 per 100,000 (Weinberger and Keller, 2005). The incidence of NTS bacteremia in Finland, Australia, Denmark and Canada during 2000 to 2007 was estimated at 0.81 per 100,000 per year (Laupland et al., 2010). In the US the Foodborne Diseases Active Surveillance Network (FoodNet) found that NTS infections were the most commonly reported (17.6 cases per 100,000 inhabitants) among foodborne infections and the incidence has not declined since 1996, when the surveillance was initiated (Sanchez-Vargas et al., 2011). FoodNet data from 1996 to 2005 reports that NTS infections have been the leading cause of death (39%) among foodborne bacterial pathogens with highest mortality among adults more than 65 years and highest incidence among children less than five years of age (69.5 infections per 100,000 children) (Barton Behravesh et al., 2011). Childhood infections have been associated with daycare center attendance and contact with cats or reptiles (Younus et al., 2007).

FoodNet reported an incidence of invasive salmonellosis of 0.9 cases per 100,000 inhabitants with the highest risk among infants (7.8 cases per 100.000 inhabitants) (Vugia et al., 2004). From 2005 to 2010 the CDC reported an unusually high number of cases in the US as a result of several independent *Salmonella* outbreaks (Lynch et al., 2006). Frequent factors associated with outbreaks included improper storage or incomplete cooking of food products among other factors (Lynch et al., 2006). A January 2010 multistate outbreak in USA led to significant concern among health authorities, consumers and farm owners and lasted one entire year (Sanchez-Vargas et al., 2011). This outbreak caused by *S.* Enteritidis-contaminated eggs affected 16 states and resulted in an estimated number of 1939 cases (Sanchez-Vargas et al., 2011). The estimated 380 million contaminated chicken eggs shipped across the US

led to a massive egg recall recommended by the US Food and Drug Administration to prevent further spread of the infection (Sanchez-Vargas et al., 2011). Another published *Salmonella* epidemiological data reported that in 1999, in USA alone there were an estimated 1.4 million cases of NTS infection, resulting in approximately 600 deaths (Mead et al., 1999).

2.3 Clinical presentation and transmission of Nontyphoidal *Salmonella*

2.3.1 Clinical presentation of Nontyphoidal *Salmonella*

The clinical presentation of NTS infections differs in manifestation between sub-Saharan region and the rest of the world. In a classical view, NTS is known to mainly cause a self-limiting enterocolitis in immune-competent individuals, in which patients present with nausea, vomiting, profuse watery diarrhoea, and abdominal pain (Hohmann, 2001). This is the common clinical presentation of NTS in many countries outside of the sub-Saharan region. Up to 5% of patients will develop secondary bacteraemia (Hohmann, 2001; Cherubin et al., 1974; Saphra and Winter, 1957; Todd and Murdoch, 1983), but attributable mortality is probably low (1-5%). Similar reports have indicated that NTS infections in man are usually associated with a self-limiting gastroenteritis, with very low case-fatality rates (CFR), in most parts of the world (Zhang et al., 2002). Nontyphoidal *Salmonella* infections can persist in the gastrointestinal tract after diarrhoeal illness, and this risk is heightened by antibiotic therapy (Sirinavin and Garner, 2000). This type of bacteraemia without associated diarrhoea occurs in at-risk groups such as patients who are immune-compromised because of HIV infection, steroid use, malignancy, chronic renal or liver disease, diabetes, or sicke-cell disease, and elderly and newborn patients (Gordon et al., 2008). Consistent with this view, data from studies in developed countries have shown that NTS infection is mainly foodborne and presents as gastroenteritis, with bacteremia a rare complication often associated with immunodeficiency (Hohmann, 2001). Focal or metastatic disease also occurs in individuals with structural abnormalities, such as valvular heart disease, aneurysms or atherosclerosis, biliary or urinary abnormalities, or prostheses (Cohen et al., 1987).

Invasive NTS disease can be a complication of several inherited immunodeficiencies. For example chronic granulomatous disease, in which phagocytes are unable to kill ingested organisms (Lazarus and Neu, 1975); sickle-cell disease, in which dysfunctional macrophages might cause susceptibility; and B-cell deficiencies (Leen

et al., 1986). It is reported that children with rare inherited deficiencies of components of the interleukin 12-interleukin 23 pathway have a particularly high incidence of recurrent invasive NTS in keeping with the significance of these proinflammatory cytokines in the clearance of intracellular infections (MacLennan et al., 2004).

In contrast to the above classical view, the clinical presentation of invasive NTS disease in sub-Saharan Africa is typically febrile systemic illness (Gordon et al., 2002; Graham et al., 2000a; Okoro et al., 2012; Peters et al., 2004) resembling enteric fever. Diarrhoea is often lacking and other clinical presentations are diverse and non-specific (Figure 2).

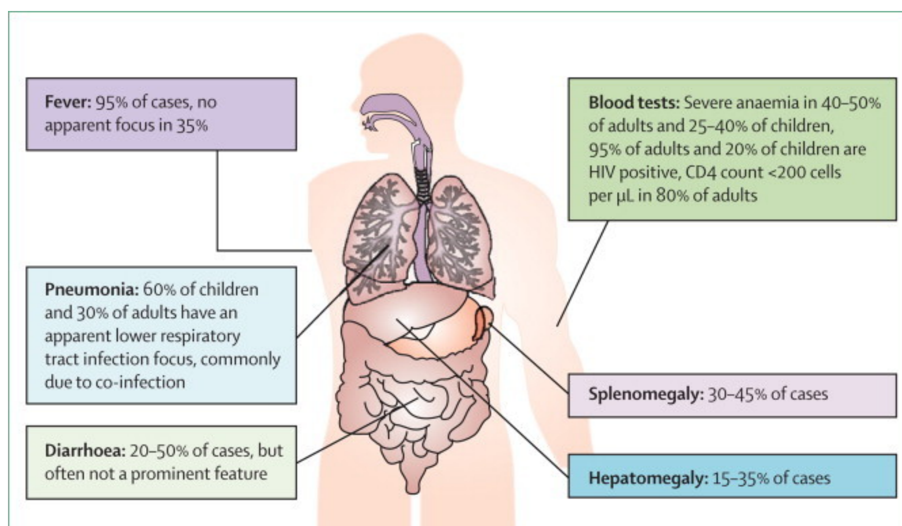

Figure 2: Clinical features of invasive Nontyphoidal Salmonella disease in adults and children in sub-Saharana Africa (Feasey et al., 2012).

In studies carried out in sub-Saharan Africa, highly invasive NTS infections, the majority of which are caused by *S.* Typhimurium have been reported as the most cause of bacteraemia in this region (Reddy et al., 2010), and especially in younger children. In this population, invasive NTS disease is often not associated with diarrhoea and gastroenteritis (Kariuki et al., 2006a). Case-fatality rates are 20-25% in

children (Brent et al., 2006; Graham et al., 2000a; Vandenberg et al., 2010), up to 50% in adults (Molyneux et al., 2009) and even higher in the HIV-positive population (Gordon et al., 2002; Kankwatira et al., 2004).

Another similar study of bacteraemia in adults in Malawi observed that a combination of high fever and splenomegaly suggested invasive disease, but that diagnosis cannot reliably be made without microbiological investigation (Peters et al., 2004). In young children particularly, a problematic clinical features overlap exists with the presentations of pneumonia and malaria, and paediatric guidelines for empirical diagnosis and treatment in low-income settings fail to identify or treat invasive NTS disease (Graham and English, 2009; Graham et al., 2000a; Nadjm et al., 2010).

Patients with invasive disease in sub-Saharan Africa frequently present with apparent focal infection, especially of the lower respiratory tract, which is commonly attributable to co-infection with other pathogens e.g. *Mycobacterium tuberculosis* (Martinson et al., 2007) and *Streptococcus pneumonia* (Gordon et al., 2002). Notably, and of great concern is that studies in sub-Saharan Africa have reported that even microbiologically identified invasive NTS disease treated with appropriate antibiotics has a case fatality of 22-47% in African adults and children (Gordon et al., 2002; Gordon et al., 2008). This may partly be attributable to co-infection with other pathogens such as *Mycobacterium tuberculosis* (Martinson et al., 2007) and *Streptococcus pneumonia* (Gordon et al., 2002) or antimicrobial resistance.

The contribution of NTS to diarrhoeal illness in sub-Saharan Africa is poorly described and published work suggests a confusing description of the clinical presentation of NTS disease. Culture-based studies of diarrhea in sub-Saharan Africa have shown that NTS are isolated in 2-27% of culture-postive diarrhoeal illness but are also in the stools of 2-7% of asymptomatic controls (Germani et al., 1998; Obi et al., 1997; Valentiner-Branth et al., 2003). Thus, to attribute illness to stool positivity is difficult. Additionally, it is not known whether the same strains of NTS cause both invasive and diarrhoeal disease, or if not, whether the modes of transmission are

different (Feasey et al., 2012). Basic questions about the environmental reservoirs and host ranges of invasive strains in Africa remain unanswered. Food quality studies have isolated 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 done and the relevance to invasive disease is uncertain. A study looking into the households of index cases of paediatric invasive NTS disease in Kenya showed that 6.9% of human contacts carried NTS in their stools, and 66% of isolates were similar to the invasive isolate in the index patient by molecular analysis (Kariuki et al., 2006b). By contrast, only unrelated strains of *S*. Typhimurium, *S*. Enteritidis, *S*. Agona, *S*. Choleraesuis, *S*. Derby, and *S*. Anatum were isolated from livestock or the household environment, thus raising the possibility that human-to-human transmission occurs (Kariuki et al., 2006b). A comprehensive study of the epidemiology of invasive and non-invasive NTS strains in Africa could clarify these issues.

2.3.2 Transmission of Nontyphoidal *Salmonella*

Classically, NTS transmission to humans can occur by consumption of food animal products, non-animal food products, contaminated water, or by contact with animals (Sanchez-Vargas et al., 2011). Food products mass production and distribution disseminates pathogens rapidly to communities (Sanchez-Vargas et al., 2011). The complex ecologies linking NTS to animals and plants implies the need for concerted efforts in contamination prevention throughout the food chain supply (Fisher and Enter-net, 2004; Majowicz et al., 2010; Stevens et al., 2009). Farm animals are the major reservoir for NTS in economically developed countries with transmission through contaminated animal products (Sanchez-Vargas et al., 2011). NTS are naturally found in chickens, ducklings, sheep, goats, pigs, reptiles, amphibians, birds, pet rodents, dogs, cats, and in a variety of wild animals making infection control a challenge to public health authorities (Dione et al., 2011; Jardine et al., 2011; Kikuvi et al., 2010; Siembieda et al., 2011; Swanson et al., 2007; Wacheck et al., 2010). Nontyphoidal *Salmonella* pet transmission to humans easily occurs by contact with animal feces (Aiken et al., 2010; Mermin et al., 2004). Non-typhoidal *Salmonella*

infections associated with pet transmission may affect infants, and result in invasive disease and severe complications (Aiken et al., 2010; Mermin et al., 2004).

2.4 Risk factors associated with Nontyphoidal *Salmonella*

Risk factors for predicting invasive NTS disease are currently based on clinical findings (Crump et al., 2011b), while little is known about pathogen predictors in this respect. In Africa, NTS bacteremia particularly occurs in HIV-infected adults (Gordon et al., 2002) and in children under five years of age, the majority of whom are not HIV infected (Brent et al., 2006; Graham, 2002; Graham et al., 2000a). Nontyphoidal *Salmonella* bacteremia frequently occurs in the absence of gastrointestinal symptoms (Brent et al., 2006; Graham, 2002; Graham et al., 2000a). Other risk factors for invasive NTS in children include malnutrition (Graham et al., 2000a), recent or acute malaria, severe malarial anemia (Berkley et al., 2009; Brent et al., 2006; Bronzan et al., 2007; Mabey et al., 1987) and sickle-cell anaemia (Williams et al., 2009).

In addition to sepsis, invasive NTS can seed to the meninges, especially in children (Feasey et al., 2012). In Malawi for example, *S*. Typhimurium has been the second most common cause of bacterial meningitis since the introduction of the *Haemophilus influenza* B vaccine, and accounted for 15% of culture-confirmed meningitis in 2008 (Feasey et al., 2012). In addition, invasive NTS infection in African children has been associated with active schistosomiasis (Gendrel et al., 1994; Gendrel et al., 1984; Neves and Martins, 1967; Rocha et al., 1971; Swindells et al., 1996) because the bacteria can adhere to the adult helminth's tegument, where they can evade antibiotic therapy (LoVerde et al., 1980; Melhem and LoVerde, 1984). Schistosomiasis has not been reported to contribute to the invasive disease in HIV-infected adults (Dowling et al., 2002; Gordon et al., 2003).

2.5 Nontyphoidal *Salmonella* infection and immune responses

2.5.1 Steps leading to *Salmonella* infection and subsequent adaptive immune response by the host

The current understanding of NTS pathogenesis and immune responses by the host is based on studies with *S.* Typhimurium in murine infection models (Wick, 2011). Although this mouse model, like any nonprimate model used to study human diseases, is not perfect, it has indeed provided valuable information regarding *Salmonella* infection. An advantage of murine models is that they allow mechanistic aspects of immunity to infection to be systematically studied (Wick, 2011). Such studies have contributed greatly to the understanding of the host response required to clear *Salmonella* infection, from the events that occur in the innate response to limit bacterial replication through development of adaptive immunity that ultimately eradicates the infection (Wick, 2011).

2.5.1.1 The first step of Nontyphoidal Salmonella infection: Bacterial penetration into host tissues

Physical barriers and resident immune cells in the intestine comprise the first line of defense against infection by *Salmonella* acquired orally (Wick, 2011). An important physical barrier preventing ingested bacteria from accessing host tissue is the single epithelial cell layer that separates the intestinal lumen from the underlying cells in the lamina propria (Wick, 2011). Several routes may contribute to Salmonella penetration across the intestinal epithelial layer (Tam et al., 2008a).

However, bacteria crossing the epithelium overlying Peyer's patches *via* M cells is a predominant pathway, particularly for invasive, pathogenicity island-1-proficient bacteria. The M cells overlying solitary intestinal lymphoid tissues (SILTs), which are organized lymphoid tissue in the intestine other than Peyer's patches, also contribute to bacterial entry (Halle et al., 2007). Finally, non-M cell-mediated 'cell sampling' routes whereby *Salmonella* can access the lamina propria have also been described (Halle et al., 2007; Hapfelmeier et al., 2008; Tam et al., 2008a). This pathway involves predominantly invasion-deficient *Salmonella* and is mediated by

CX 3 CR1 + phagocytes (Halle et al., 2007; Hapfelmeier et al., 2008; Tam et al., 2008a). Although the cell sampling pathway involving CX 3 CR1 + phagocytes can contribute to penetration of invasion-deficient *Salmonella* into the lamina propria (Hapfelmeier et al., 2008), M cell-mediated pathways dominate for invasion-proficient *Salmonella*.

Once invasive *Salmonella* use M cells to cross the epithelial barrier, the bacteria encounter resident cells in the subepithelial dome. This region is located beneath M cells in Peyer's patches and solitary intestinal lymphoid tissues (SILTs), and is rich in Dendritic cells (DCs), particularly of the CD8 α^- subset (Halle et al., 2007; Hapfelmeier et al., 2008; Iwasaki and Kelsall, 2000; Schulz et al., 2009; Tam et al., 2008a; Varol et al., 2010). Moreover, although other myeloid lineage cells such as macrophages and neutrophils can be found in low numbers in Peyer's patches of naive mice using sensitive, high-throughput techniques such as flow cytometry (Rydstrom and Wick, 2007; Wick, 2004), cells expressing Gr-1 and F4/80 are not readily detected in the subepithelial dome by immunohistochemistry (Halle et al., 2007; Hapfelmeier et al., 2008; Iwasaki and Kelsall, 2000; Kelsall and Strober, 1996; Rydstrom and Wick, 2007; Schulz et al., 2009; Varol et al., 2010; Wick, 2004; Tam et al., 2008a). Thus, the organization of Peyer's patches, with DCs being locally enriched in the subepithelial dome, allows these cells ready access to bacteria that penetrate through M cells. Given the pivotal role of DCs in priming naive T cells to start an immune response, DCs in intestinal lymphoid tissues are important players in initiating immunity to the oral bacterial pathogen (Wick, 2011).

2.5.1.2 Dissemination of the orally acquired Salmonella to mesenteric lymph nodes (MLN) and other tissues

Once *Salmonella* penetrate the intestinal epithelial layer after oral infection they seed the intestinal lymphoid organs, Peyer's patches and SILTs, and the lamina propria (Wick, 2011). They also spread to the MLN draining the intestine, and to extraintestinal tissues, particularly the spleen and liver (Halle et al., 2007; McSorley et al., 2002; Sundquist and Wick, 2005; Voedisch et al., 2009). CC-chemokine

receptor 7 (CCR7) plays an important role in the dependent bacterial transport from the intestine to the MLN via DCs migrating in lymph, while alternate pathway(s) allow the bacteria to reach systemic tissues (Wick, 2011). Phagocytes in the intestine allow S*almonella* to rapidly access extraintestinal tissues without necessarily relying on transport to the MLN in lymph-borne DCs (Wick, 2011). Intestinal bacteria in phagocytes can directly access the blood, which can rapidly seed systemic tissues (Vazquez-Torres et al., 1999; Worley et al., 2006). MLN plays an important protective role in limiting the spread of intestinal bacteria in both commensals (Macpherson and Uhr, 2004) and pathogens (Voedisch et al., 2009), to systemic tissues. Intestinal cells and lymphoid tissues therefore plays specialized roles in controlling the dissemination of luminal bacteria while different processes dominate the colonization of the MLN and systemic tissues after oral *Salmonella* infection (Wick, 2011).

2.5.1.3 Dendritic cell responses to Salmonella infection

The innate immune response is rapidly activated after oral infection, and significant changes in cell number are apparent within two days of intragastric *Salmonella* administration (Rydstrom and Wick, 2007; Rydstrom and Wick, 2009). Changes that occur include recruitment of DCs, inflammatory monocytes and neutrophils (Wick, 2011). Although DCs accumulate in Peyer's patches in response to infection, their increase is relatively modest (Sundquist and Wick, 2005). Given the unique role of DCs in priming T cells and their limited role in keeping bacterial replication in check, it is likely that their functional features rather than their number per se is most important (Wick, 2011). For example, DCs expressing CC-chemokine receptor 6 (CCR6) are rapidly recruited to the sub epithelial dome region, and these cells have a key role in activating *Salmonell*a-specific T cells (Salazar-Gonzalez et al., 2006). In addition to the slight increase in DC number, another important aspect of the early DC response to infection is their maturation. This process, which involves changes in DCs as detected phenotypically by increased surface expression of MHC class II and the co-stimulatory molecules Cluster of Differentiation (CD) 86, CD80 and CD40, is necessary for efficient activation of naive T cell (Reis e Sousa, 2006). Given the role

of intestinal DCs in maintaining intestinal homeostasis (Coombes and Powrie, 2008), the maturation process of intestinal DCs, perhaps coupled with recruitment of new 'nontolerized' DCs, is particularly important for their ability to prime naive T cells to orally acquired pathogens (Coombes and Powrie, 2008; Reis e Sousa, 2006). Indeed, DCs harbor *Salmonella* after oral infection (Sundquist and Wick, 2005; Tam et al., 2008b) and are critical to priming bacteria-specific T cells (Salazar-Gonzalez et al., 2006). Thus, although recruitment of CD11c ^{hi} DCs to Peyer's patches during *Salmonella* infection is modest, the accumulation of particular subsets, such as DCs expressing CCR6 and/or cells less conditioned in a tolerogenic environment, may be important (Coombes and Powrie, 2008). The initiation of DC maturation is an essential early event in the response to *Salmonella* infection and is a key step in starting an adaptive immune response to this pathogen (Wick, 2011).

Interestingly, *Salmonella* preferentially induces death of one subset of CD11c^{hi} conventional DCs during infection, the CD8 α^{+} subset, while CD8 α^{-} DCs and recruited inflammatory monocytes are resistant (Sundquist and Wick, 2009). This bacteria-induced DC death depends on myeloid differentiation primary response gene (88) (MyD88), an adaptor protein that controls signaling through many Toll-like receptors (TLRs), and on signaling through TNFR1 (Wick, 2011). DCs have a fairly short life span of a few days, which could serve as a general mechanism to minimize undesired conditions such as chronic lymphocyte activation or autoimmunity (Wick, 2011). However, DCs must survive long enough to activate T cells, and an insufficiently short life span negatively influences the magnitude of the T cell response (Wick, 2011). It is still not clear what could be the consequences of infection-induced DC death or if this is a virulence factor aimed to undermine adaptive immunity and reduce the host's ability to clear the infection and whether this promotes or impedes bacterial spread (Wick, 2011). It is however known that conventional DCs are required to activate *Salmonella* -specific T cells *in vivo* (Salazar-Gonzalez et al., 2006). The CD8 α^{+} DC sub-set also has some special features of its antigen presentation capacity (Wick, 2011). For example, CD8 α^{+} DCs efficiently take up dead cells, cross-present antigens on major histocompatibility

complex - I (MHC-I) and produce interleukin 12P70 (IL-12p70) and thus influence CD4 α^+ T helper cell 1 (Th1) T cell responses (Shortman and Heath, 2010). As Interferon (IFN)- γ-producing CD4 $^+$ Th1 T cells are critical to surviving *Salmonella* infection (Hess et al., 1996), selective death of this subset could be a way for bacteria to avoid clearance. In this respect, it is also intriguing to note that recruited inflammatory monocytes have a limited capacity to process *Salmonella* for antigen presentation (Rydstrom and Wick, 2007; Sundquist and Wick, 2009). However, CD8 α^- DCs from infected mice have the capacity to present *Salmonella* antigens on MHC-II for CD4 $^+$ T cell recognition (Sundquist and Wick, 2009). The consequence of *Salmonella*-induced death of CD8 α^+ DC during infection is not known (Wick, 2011). However, it could be a way for *Salmonella* to slow down effective antigen presentation and dampen an effective Th1 T cell response, which may favor bacterial replication and spread within an infected host (Wick, 2011).

2.5.1.4 Dendritic cell maturation during infection

Dentritic cells in the steady-state intestine have an important role in maintaining tolerance to intestinal flora and potential food antigens, and the process of maturation is important in overcoming this tolerogenic bias (Wick, 2011). Infection with intestinal pathogens must therefore trigger DC maturation (Wick, 2011). How is DC maturation triggered in vivo? It is known that an important initiating event occurs when conserved microbial structures, so-called microbe-associated molecular patterns (MAMPs) such as LPS and flagella, engage pattern recognition receptors on host cells (Wick, 2011). Indeed, host cells express different pattern recognition receptor families that recognize MAMPs, which act as common denominators indicating an infection. The receptor families involved in sensing bacterial infection include C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors (NLRs) and Toll-like receptors (TLRs) (Elinav et al., 2011; Kawai and Akira, 2010; Robinson et al., 2006). Distinct functions of these receptor families create a complimentary approach to detecting and controlling infections (Elinav et al., 2011; Trinchieri and Sher, 2007). For example, C-type lectin receptors often act as phagocytic receptors while NLRs are intracellular sensors of microbial

components, particularly peptidoglycan structures, and also detect cell injury (Elinav et al., 2011; Robinson et al., 2006). Ligand recognition by NLRs results not only in production of cytokines and chemokines, but also in assembly of inflammasomes that results in production of the active forms of IL-1β and IL-18 (Davis et al., 2011; Elinav et al., 2011). The inflammasome is indeed important in the innate immune response to *Salmonella* (Broz et al., 2010; Davis et al., 2011). TLRs are also important in controlling *Salmonella* infection (Weiss et al., 2004). A main function of TLRs, which are expressed by intestinal epithelial cells and immune cells, is to initiate signaling cascades that lead to pro-inflammatory cytokine and chemokine production by nuclear factor-kappa B (NF- B) activation (Kawai and Akira, 2010). Several of the cytokines induced by bacterial triggering of pattern recognition receptors, particularly IL-1β and tumor necrosis factor alpha (TNF-α) can influence MHC-II and costimulatory molecule expression and contribute to DC maturation during *Salmonella* infection (Sundquist and Wick, 2005; Tam et al., 2008b). However, phenotypic maturation as assessed by altered expression of MHC and co-stimulatory molecules does not necessarily reflect functional maturation of the DCs (Wick, 2011). That is, full DC maturation must result in DCs that elicit effector functions in T cells (i.e. IFN-γ production) (Joffre et al., 2009). An important component of achieving fully functional DC maturation is signals delivered by direct DC interaction with microbial structures (Joffre et al., 2009). In contrast, signals delivered indirectly by cytokines in the absence of direct microbial contact results in phenotypic but not necessarily functional DC maturation (Joffre et al., 2009). Like studies using purified bacterial products, infection studies have revealed that direct DC activation by *Salmonella* also influences phenotypic and functional DC maturation (Tam et al., 2008b). However, differences in bacterial killing by DCs lacking TLR pathways that were used to remove the effect of pro-inflammatory cytokines (and thus indirect DC maturation) underscores the complexity of mechanistic studies in infection models (Tam et al., 2008b). Thus, DCs respond differently to direct bacterial contact relative to sensing cytokines in the environment (Wick, 2011). However, little is known about the role of direct versus indirect DC

maturation during infection with a live pathogen, and given the complexity of studying cellular responses during infection with a replicating pathogen, understanding direct versus indirect DC maturation during infection requires further investigation (Wick, 2011).

2.5.1.5 Recruitment and activation of inflammatory monocytes and neutrophils

In contrast to the modest increase in DCs in response to oral *Salmonella* infection, neutrophils and inflammatory monocytes rapidly infiltrate Peyer's patches and mesenteric lymph nodes (Rydstrom and Wick, 2007; Sundquist and Wick, 2005; Tam et al., 2008a). Inflammatory monocytes can be distinguished from conventional CD11c ^{hi} DCs and other myeloid cells by, for example, their CD11c^{int} CD11b^{hi} Ly6C^{hi} Ly6G^{low} phenotype. Indeed, the accumulation of these cells in infected tissues results in their numeric dominance relative to neutrophils and DCs in the gut-associated lymphoid tissue during the acute phase of infection (Rydstrom and Wick, 2007; Sundquist and Wick, 2005). Although the importance of bacterial uptake and killing by neutrophils during *Salmonella* infection has been known for some time, inflammatory monocytes are a relatively recently described myeloid lineage cell whose function during infection is not fully understood. These cells, however, are predominant producers of products important in fighting bacterial infections, particularly TNF-α and inducible nitric oxide synthase (iNOS), in infected tissues (Tam et al., 2008a). This feature also distinguishes them from conventional (CD11c^{hi}) DCs, which produce neither iNOS nor TNF-α in *Salmonella*-infected mice (Rydstrom and Wick, 2007; Sundquist and Wick, 2005). The ability of inflammatory monocytes to produce TNF-α, iNOS and IL-1β combined with their ability to take up and kill *Salmonella* (Rydstrom and Wick, 2007), suggests that they contribute to holding initial bacterial replication in check (Wick, 2011). Although this may very well be the case, inflammatory monocytes are not as efficient at internalizing *Salmonella* as neutrophils (Rydstrom and Wick, 2007).

Another possible function of inflammatory monocytes during infection is to act as antigen-presenting cells (Wick, 2011). Indeed, inflammatory monocytes have several

features that prompted investigation of their ability to activate T cells. For example, in addition to their ability to take up and kill *Salmonella*, they also have relatively high expression of MHC-II and co-stimulatory molecules, particularly CD80 (Rydstrom and Wick, 2007; Sundquist and Wick, 2005). These are all important features of antigen-presenting cells (Wick, 2011). In addition, inflammatory monocytes express the integrin CD11c at intermediate levels, suggesting a phenotypic, and possibly functional, relationship to CD11c^{hi} conventional DCs (Wick, 2011). However, experimental data show that infection-induced Ly6C^{hi} inflammatory monocytes have a poor capacity to stimulate T cell proliferation when exposed to *Salmonella ex vivo* in an antigen-specific system (Rydstrom and Wick, 2007; Sundquist and Wick, 2009). Inflammatory monocytes can, however, induce T cell proliferation when exposed to peptide and can stimulate T cell proliferation in a mixed lymphocyte reaction (Rydstrom and Wick, 2007; Tam et al., 2008a). Inflammatory monocytes have therefore a more likely role in containing bacterial replication rather than making a major contribution to activating bacteria-specific T cells during infection (Wick, 2011). Indeed, inflammatory monocytes may even inhibit or alter T cell function (Delano et al., 2007).

2.5.1.6 Mechanisms of cell recruitment to intestinal tissues during Salmonella infection

The inflammatory monocytes and neutrophils that accumulate in *Salmonella*-infected tissues are released into the blood early during the response to infection (Rydstrom and Wick, 2007). These cells enter Peyer's patches by a mechanism that can occur independently of the selectin mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) (Rydstrom and Wick, 2007), which is required for T cell homing into Peyer's patches. Insight into the signals that drive monocyte and neutrophil homing to Peyer's patches and MLN during infection were provided by analyzing chemokine receptor expression on cells released into the blood in response to infection combined with kinetic studies of *Salmonella*-induced chemokine expression in Peyer's patches and MLN (Rydstrom and Wick, 2007; Rydstrom and Wick, 2009).

Triggering of pattern recognition receptors by their ligands is important in initiating a large number of events critical to fighting infection, and indeed TLR signaling and the inflammasome are important in controlling *Salmonella* infection (Broz et al., 2010; Weiss et al., 2004). Complementarity in the TLR signaling pathways ensures recruitment of myeloid cells to infected tissues (Wick, 2011). That is, simultaneous removal of both MyD88 and TLR4, whose combined absence removes bacteria-mediated TLR signaling, is required to significantly decrease both monocyte and neutrophil influx into Peyer's patches and MLN (Wick, 2011). In contrast, removing signaling through TLR4 alone, removing flagella-mediated signaling by infecting wild-type mice with flagella-deficient *Salmonella*, or infecting TLR4 knockout mice with flagella-deficient *Salmonella* has no effect on monocyte or neutrophil recruitment to gut-associated lymphoid tissue, while MyD88 deficiency alone partially affects recruitment (Rydstrom and Wick, 2009). In contrast, release of monocytes and neutrophils from the bone marrow into the blood is not significantly reduced even in infected mice (Rydstrom and Wick, 2009).

Overall, TLR signaling is important from the host's perspective to survive infection (Wick, 2011). Given that a rapid and efficient response is required to contain infections, it is not unexpected that several complimentary and overlapping mechanisms, such as multiple receptors in the same family and distinct receptor families, are in place to ensure host survival (Wick, 2011). One such example is the multiple TLRs that sense bacterial infection and contribute to phagocyte recruitment to infected lymphoid tissues as described above. However, TLR signaling can also be exploited by *Salmonella* to enhance virulence (Wick, 2011). For example, *Salmonella*-induced TLR4 signaling impairs expression of homeostatic chemokines involved in organizing lymph node architecture, which in turn reduces the efficiency of the host's adaptive immune response and compromises bacterial clearance (St John and Abraham, 2009). Furthermore, TLR signaling is necessary for acidification of *Salmonella*-containing vacuoles, which is required for induction of *Salmonella* pathogenicity island II and *Salmonella* pathogenicity island II-regulated virulence genes (Arpaia et al., 2011) that are critical for intracellular survival of *Salmonella*.

That is, in the absence of TLR signaling, *Salmonella* could not replicate intracellularly and thus had reduced virulence (Arpaia et al., 2011).

While the murine infection models have provided much in-depth insight into the pathogenesis of *Salmonella* infection and indeed have paved the way for mechanistic studies allowing detailed understanding of the innate immune response to this oral pathogen, a number of questions in *Salmonella* pathogenesis still remain unanswered. There are still gaps in the understanding of *Salmonella* pathogenesis, such as the mechanisms of functional DC maturation *in vivo* and how TLRs as well as other pattern recognition receptors contribute to recruitment and activation of innate cell populations. Moreover, despite the current understanding of how the host responds to combat *Salmonella*, this bacterium continues to evolve. For example, recent studies have revealed how *Salmonella* exploits TLR signaling to reduce the host's adaptive immune response by impairing expression of chemokines involved in organizing lymph node architecture (Wick, 2011). TLR signaling is also required for *Salmonella* to express virulence genes when it resides in phagocytic vacuoles (Wick, 2011). Thus, the TLR system, which has traditionally been considered a host defense mechanism, also contributes to *Salmonella* virulence. This new information on *Salmonella* pathogenesis highlights that Salmonella pathogenesis and innate immune response is yet to be fully understood.

2.6 Role of O-antigen in immune response

O-antigen (OAg) can block the access of complement to lipid-A-core and thus block the initiation of the alternative complement pathway (Reeves, 1995). O-antigen can also initiate this pathway at a distance from the membrane and so deflect it (Reeves, 1995). O-antigen is, in general, highly immunogenic, and antibody induced can initiate either the classical or the alternative complement pathway, which may lead to cell death or phagocytosis (Reeves, 1995).

Many bacterial species have extensive variation in OAg structure and this polymorphism must be maintained by some form of balanced selection, such as niche-specific selection (Reeves, 1992) with each clone having an OAg that is

appropriate to its niche (Reeves, 1995). However, the role of OAg specificity has been studied in relatively few cases (Reeves, 1995). Now that many gene clusters involved in OAg biosynthesis have been cloned and some sequenced, and as advances in NMR and other techniques give us more OAg structures, it is possible to investigate the role of OAg and their variation in pathogenicity (Reeves, 1995).

2.6.1 Lipopolysaccharide O-antigen is both a virulence factor and a B cell antigen capable of offering protection

Lipopolysaccharide (LPS) is the major component of the surface of most Gram-negative bacteria including NTS and is recognized by immune cells as a pathogen-associated molecule (Micoli et al., 2013; Freudenberg et al., 2001). The exposed location of LPS on the bacterial surface makes it an ideal molecule for recognition of bacteria by the immune system (Freudenberg et al., 2001). Lipopolysaccharide is therefore a highly potent activator of the innate immune system and as such constitutes a major marker for the recognition of intruding Gram-negative pathogens such as NTS by the host (Freudenberg et al., 2008). The interaction of LPS with cells, such as macrophages, granulocytes, dendritic cells or mast cells leads to the formation and release of a large spectrum of inflammatory mediators which are essential for the early innate and subsequent adaptive anti-bacterial defense (Beutler et al., 2003; Freudenberg et al., 2001). Antibodies against the OAg may confer protection against infections (Carlin et al., 1987) and vaccines have been developed by conjugation of O-antigen to carrier proteins to enhance immunogenicity (Micoli et al., 2013). O-antigen-based conjugate vaccines have been proposed for many human pathogens including Salmonellae (Konadu et al., 1996; Simon et al., 2011; Watson et al., 1992), Shigella species (Chu et al., 1991; Passwell et al., 2003; Taylor et al., 1993), and *E. coli* (Konadu et al., 1994).

A study to investigate the role of anti-lipopolysaccharide (LPS) antibodies in serum bactericidal activity (SBA) against *S.* Typhimurium in healthy adults and children in USA found that healthy individuals can have circulating antibodies to LPS (Trebicka et al., 2013). These antibodies can either mediate or inhibit killing of *S.* Typhimurium. These findings contrast with the observations from Africa, which

linked bactericidal activity to antibodies against a *S*. Typhimurium outer membrane protein and correlated the presence of inhibitory anti-LPS antibodies with HIV infection (MacLennan et al., 2008). These fundings thus support the hypothesis that *S*. Typhimurium from sub-Saharan African differs from those from the rest of the world.

2.6.2 Structure of Lipopolysaccharide O-antigen

Structurally LPS consists of two parts: a variable, antigenically active polysaccharide and a lipid (lipid A) (Figure 3). Lipopolysaccharide consists of a polysaccharide chain of repeating units, the OAg, linked to a core oligosaccharide containing 10–12 sugar units (Micoli et al., 2013). The core is covalently bound through 2-keto-3-deoxyoctonate (KDO) to lipid A (Micoli et al., 2013). Lipid A is highly conserved and exerts endotoxic activity, while the OAg chain differs between serovars and is a major contributor to the serological specificity of bacteria (Micoli et al., 2013).

Lipid A is the biologically active centre of the LPS molecule and is responsible for the toxic and other biological effects of LPS (Galanos et al., 1972; Luderitz et al., 1973). The structure of lipid A is highly conserved among many Gram-negative bacteria, especially among *Enterobacteriaceae* (Freudenberg et al., 2001). Consequently, LPSs generally have comparable activity regardless of the pathogenic properties of the corresponding parent bacteria (Luderitz et al., 1973; Rietschel et al., 1998). In contrast to exotoxins, LPS is usually released in larger amounts only when bacteria undergo disintegration (Freudenberg et al., 2001). Isolated, purified LPS causes a number of pathophysiological reactions in experimental animals and human volunteers, reactions which are strikingly similar to those seen during infections with Gram-negative bacteria (Freudenberg et al., 2001). These include fever, headache, nausea, diarrhoea, changes in leukocyte and trombocyte counts, disseminated intravascular coagulation, multiorgan failure, shock and death (Freudenberg et al., 2001). All these reactions are induced by cytokines and other endogenous mediators which are produced after interaction of the LPS with the humoral and cellular targets of the host (Morrison and Ryan, 1987; Rietschel et al., 1998). Experiments with animals have demonstrated that LPS sensitivity may increase considerably during

infection with certain microorganisms, including the Gram-negative bacteria such as *S*. typhimurium (Matsuura and Galanos, 1990). This leads to a state of LPS hypersensitivity (Freudenberg et al., 2001).

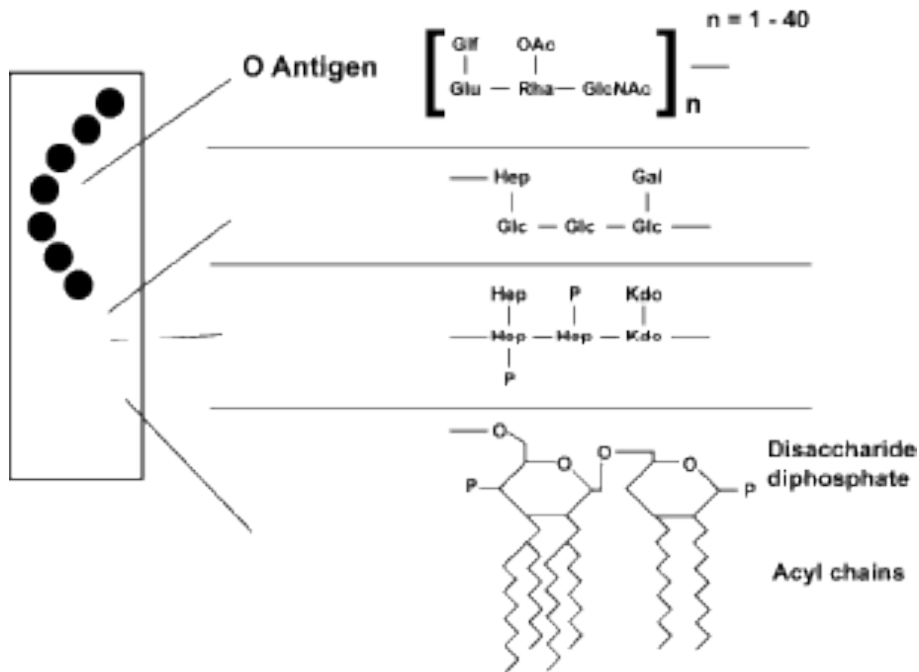

Figure 3: Lipopolysaccharide (LPS) structure (Marolda et al., 2006).

A representative scheme of the structure of the LPS molecules indicating its various regions: O Antigen, Outer Core, Inner Core, and Lipid A regions. Glf, galactofuranose; Glc, glucose; Rha, rhamnose; GlcNAc, N-acetylglucosamine; OAc, O-acetyl; Hep, heptose; Gal, galactose; KDO, 2-keto-3-deoxyoctulosonic acid; P, phosphate.

Lipopolysaccharide plays an important role in maintaining the structural integrity of the bacterial outer membrane by interacting with outer membrane proteins as well as divalent cations (Marolda et al., 2006). Phosphate groups covalently attached to heptose residues in the inner core participate in these ionic interactions, which provide a barrier preventing the passage of hydrophobic substances such as detergents, dyes, and antibiotics across the outer membrane (Nikaido, 1994; Nikaido and Vaara, 1985).

The biosynthesis of LPS is a complex process involving various steps that occur at the plasma membrane, followed by the translocation of LPS molecules to the bacterial cell surface (Marolda et al., 2006). The core oligosaccharide (OS) is

assembled on preformed lipid A by sequential glycosyl transfer of monosaccharides, while the OAg is assembled on undecaprenol-phosphate (Und-P), a polyisoprenoid lipid to which OAg is linked via a phosphodiester bond (Marolda et al., 2006). These pathways eventually converge by the ligation of the OAg onto the outer core domain of the lipid A-core OS acceptor, with the concomitant release of Und-P (Raetz and Whitfield, 2002; Valvano, 2003). Und-P is also required as a lipid intermediate for the biosynthesis of other cell-surface structures, including peptidoglycan and the enterobacterial common antigen (Marolda et al., 2006).

Mutations in the core biosynthesis genes (*waa*, formerly *rfa*) lead to “rough” mutants with an incomplete core, which lacks the site for the attachment of O-polysaccharides (Reeves et al., 1996). Bacterial mutants lacking heptose in the LPS display a more dramatic phenotype known as “deep rough.” This phenotype is characterized by hypersensitivity to novobiocin, detergents, and bile salts, as well as defects in F plas-mid conjugation and generalized transduction by the bacteriophage P1 (Valvano, 2003). Mutations in *wb** (formerly *rfb*) genes, which are involved in the synthesis of the O-polysaccharide, give rise to rough mutants that have a complete core structure (Marolda et al., 2006). Typically, *wb** gene clusters encode nucleotide sugar synthases (for biosynthesis of the nucleotide sugar precursors specific to OAgs), and glycosyltransferases (for the sequential and specific addition of sugars that make the O-repeating unit) (Marolda et al., 2006). Additional genes encoding functions involved in the assembly of the O-polysaccharide are also present in these clusters, such as *wzy* (OAg polymerase) and *wzx* (putative OAg *flippase*) in some systems, and *wzm* (membrane component of ABC transporter) and *wzt* (adenosine triphosphate-binding component of ABC transporter) in others (Raetz and Whitfield, 2002; Valvano, 2003). In systems containing *wzx* and *wzy*, the average size distribution of the O-polysaccharide chain is modulated by the product of *wzz*, a gene usually located in the proximity of *wb** clusters (Marolda et al., 2006). An O-ligase activity encoded by a gene located within the core OS cluster, *waaL*, is required for the transfer of the O-polysaccharide onto lipid A-core OS (Marolda et al., 2006).

2.6.3 Interaction of Lipopolysaccharide with complement

The serum proteins together known as complement act in a cascade to form complexes culminating in the membrane attack complex (MAC) (Reeves, 1995). The MAC can integrate into lipid bilayers to form pores that lead to cell death and often cell lysis (Reeves, 1995). The 'classical' cascade is initiated by complement component C1q binding to antibody-antigen complexes (Reeves, 1995). However, complement component C3, in conjunction with other proteins, can react directly with many microbial polysaccharides, including lipid-A-core and OAg, to generate MAC by the 'alternative' pathway in the absence of antibody (Reeves, 1995). For many pathogens, complement itself only damages mutants with altered LPS (Reeves, 1995). Under natural conditions, the alternative complement cascade is a relatively nonspecific non-induced defense against the enormous number of bacteria that could otherwise be opportunistic pathogens (Reeves, 1995).

Rough mutants of pathogenic species are generally serum sensitive, due to complement reacting with lipid A, which leads to cell death (Frank et al., 1987; Morrison and Kline, 1977). OAg is thought to block the access of complement to lipid A (Pluschke et al., 1983a), which is probably why clinical isolates are generally smooth, although there are exceptions: in one study, up to half the *E. coli* strains present in the stools of patients with chronic inflammatory liver disease were rough (Hopf et al., 1989). In contrast, a rough strain of *S.* Choleraesuis was virulent only when inoculated into a vein or the peritoneum, but not when given orally, suggesting that OAg is required for survival in the gastrointestinal tract (Marolda et al., 2006).

The involvement of OAg in protecting the cell from serum killing has been shown in many cases; for example, in *Klebsiella pneumoniae*, the protective effect was found to be correlated with the observed thickness of the OAg layer (McCallum et al., 1989). Species with the lipooligosaccharides (LOS) structure lack polymeric OAg, but are nonetheless generally resistant to serum killing (Reeves, 1995). However, some *rfc* mutants of species with typical OAg that have only a single O unit and resemble LOS are serum sensitive. The precise requirements for OAg to protect

against complement interaction with lipid-A-core are not known, but are affected by OAg specificity (Kusecek et al., 1984).

2.6.4 Direct binding of complement to O-antigen and the role of O-antigen specificity in pathogeniclty

O-antigen can also bind to complement in the absence of antibody (Reeves, 1995). This may vary with OAg specificity and, in *E. coli*, may correlate with the sensitivity of cells to serum killing (Pluschke and Achtman, 1984). Leive and colleagues showed that activation of the alternative pathway by S. *enterica* led to killing (Liang-Takasaki et al., 1983) and found that complement bound preferentially to long-chain molecules (Joiner et al., 1986). Grossman *et al* (Grossman et al., 1987) regulated the amount of OAg produced and so modified the chain-length distribution by limiting the mannose supply to a mutant strain of *S*. Montevideo (group Cl) that could not synthesize GDP-mannose. They showed that if $<20\%$ of the LPS molecules had ≥ 14 ''O'' units, or if the average length fell below 4-5 ''O'' units, then complement fixation led to cell death. They argued that, in wild-type organisms, OAg activates complement away from the cell surface and the MAC decays before causing damage, but that reduction of the chain length reduces the barrier to diffusion so that the MAC reaches the outer membrane and kills the cell (Reeves, 1995). An alternative explanation is that the reduced OAg barrier allows complement to reach the cell surface and trigger the cascade on lipid-A-core (Reeves, 1995). In either case, a major role of Cld (the *cld* gene product) is probably to ensure that OAg chain length is optimal for preventing complement-mediated cell lysis (Reeves, 1995). Chain length and serum sensitivity have also been shown to be correlated in *S*. Choleraesuis (Nnalue and Lindberg, 1990).

Investigations into the role of complement in serum killing, complement deposition, LPS types, resistance to complement killing protein (*rck*), or binding ability of anti-*Salmonella* antibodies have been conducted (Dougan et al., 2011; Goh and MacLennan, 2013; Gondwe et al., 2010; MacLennan et al., 2008). The *rck* protein is an outer membrane protein reported to confer resistance to serum killing in *Salmonella* (Heffernan et al., 1992). When the protein was expressed in either *E.*

coli or *S*. Typhimurium, it conferred serum resistance independent of LPS length. The current work particularly focused on the characterization of OAg present on bacterial cell surface.

In relation to the role of O-antigen specificity in pathogeniclty, of the 170 forms of OAg known for *E. coli*, some are correlated with specific forms of pathogenesis (Reeves, 1995). For *E. coli* O1, O7 and O18, there is evidence that the OAg differences account for the differences in the nature of pathogenicity (Achtman and Pluschke, 1986; Pluschke et al., 1983b). It has also been shown that the virulence of *E. coli* Flexneri is reduced if the OAg is changed (Gemski et al., 1972).

The virulence of *S*. Typhimurium (group B), is reduced if the OAg is substituted by those of groups D or C1(Valtonen et al., 1977; Valtonen, 1970); this has been shown to be due to increased activation of the alternative complement pathway, leading to increased phagocytosis (Liang-Takasaki et al., 1983; Jimenez-Lucho and Foulds, 1990; Liang-Takasaki et al., 1982; Valtonen et al., 1975). The group B OAg that is naturally present in this clone seems in some way to subvert natural immunity (Reeves, 1995). However, the difference in virulence is not great and strains of reduced virulence were used to facilitate the detection of the difference (Reeves, 1995). In this context, it is relevant that natural selection can act on differences so small that they are difficult to detect in the laboratory (Reeves, 1995). It is estimated that a selective advantage of only 0.1% for one OAg over another in a given niche is sufficient to maintain different alleles in different clones (Reeves, 1992), but such an advantage would be extremely hard to demonstrate in a laboratory assay. These effects may well explain why *S.* Typhimurium has the group B OAg (Reeves, 1995).

2.6.5 Protection by antibody to O-antigen

Not many studies on protection offered by antibodies to OAg have been published. O-antigen is highly immunogenic after immunization with whole cells or with LPS (Reeves, 1995). However, OAg separated from the lipid-A-core is not very immunogenic and is often referred to as a hapten (Beckmann et al., 1964). The immunogenicity of OAg may depend on the adjuvant effect of the lipid-A

component of LPS (Reeves, 1995). O-antigen, as it is a polysaccharide, elicits only T-cell-independent, that is, essentially antibody, responses (Reeves, 1995). The antibody-antigen complex then activates the complement system, leading to the production of the MAC or to phagocytosis (Reeves, 1995).

Antibodies against OAg can protect against infection; for example, this has long been reported for *Vibrio cholerae*, and has recently been demonstrated directly in an experimental model of infection with this pathogen (Apter et al., 1993). Likewise, the protection against ty- phoid given by immunization with S. *enterica ty21a* (*sv. Typhi*) has been proposed to be due to immunoglobulin A (IgA) directed against OAg (Kantele et al., 1991). This protection against otherwise-effective pathogens is generally due to antibody and complement-induced phagocytosis, rather than MAC-induced damage (Reeves, 1995). A particularly informative study (Lindberg et al., 1993) used a strain of *S.* Dublin with genes encoding O4, O12, in addition to O9, O12. It gave good protection against *S.* Typhimurium (O4, O5, O12) in mice, whereas the parent *S*. Dublin strains did not. This confirms that the protection seen in mice is directed against the immunodominant O4 and O9 epitopes of the OAg (Reeves, 1995). However, the same strain in calves protected only against *S*. Dublin (Segall and Lindberg, 1993), showing that, in this case, other factors are involved.

Studies using monoclonal antibodies (mAbs) have been particularly informative (Reeves, 1995). For example, a mAb to *E. coli* O18 confers passive protection to baby rats after oral challenge with an *E. coli* O18:K1 strain. Pluschke *et al*. (Pluschke et al., 1989) have made immunoglobulin switch variants starting with an IgM mAb, which retained the same specificity, but were of different immunoglobulin classes. IgM and the four IgG subclasses all triggered lysis, but IgM was a thousandfold more effective than were any of the lgG subclasses.

Pollack et al (1990) used mAbs that reacted with different parts of the LPS of *E. coli* and *S*. Minnesota. They found that O-reactive mAbs bound well to intact bacteria and protected against live intra- peritoneal challenge, whereas an anti-lipid-A mAb was more crossreactive, but in general did not protect (Pollack et al., 1990). Carlin et al

(1987) used a series of mAbs specific for different epitopes of the OAg of *S. enterica* group B, and found that IgG3 mAbs of four different specificities varied 2500-fold in their protective effect against an *S.* Typhimurium strain. Antibody against the exposed dideoxyhexose (epitopes 4 and 5) was the most effective and that against the less- exposed epitope 12 was the least effective. However, for IgM, protection was high for all epitope specificities. IgA did not protect, probably because the challenge was intraperitoneal and not oral (Reeves, 1995).

The protective role of antibody has been studied in detail for *E. coli* O111:B4 (Reeves, 1995). Some O111 strains of *E. coli* have an unattached, high-molecular-mass OAg capsule, in addition to OAg forming part of the LPS. The role of the OAg capsule is not clear, as strains that lack it are more resistant to killing by serum in the presence of antibody against OAg (Goldman et al., 1984). Selection for resistance to serum in the presence of such antibody produced mutants with no OAg capsule, but with increased amounts of LPS-associated OAg (Reeves, 1995). In the presence of antibody, the MAC seems to be directed to the membrane surface, but increasing the amount of LPS-associated OAg (but not the capsular form) prevents this from occurring (Reeves, 1995). Oishi et al (Oishi et al., 1992) used mAbs to O111:B4 to measure C3 binding. Although C3 was deposited on bacteria in the absence of antibody, mAbs against LPS increased C3 attachment in a dose-dependent manner. Bacterial killing occurred only in the presence of mAb and required an intact alternative pathway. IgM was more effective than lgG, and killing correlated with the fixation of C3. A separate study used mouse IgG2a mAbs, against either the OAg or against the core of an O111:B4 strain (Hoffman et al., 1994). The anti-OAg mAb protected dogs in a septic-shock model, but anti-core mAb was effective only in animals also treated with antibiotics.

2.6.6 Relation of O-antigen to host components

Some OAg resemble host components and probably facilitate invasion by mimicry of the host (Reeves, 1995). For example, some LPS of *Haemophilus influenzae* and *Neisseria gonorrhoeae* mimics epitopes of human glycosphingolipids (Mandrell et al., 1992). Other OAg lead to specific interactions and perhaps act as adhesins

(Reeves, 1995). Studies of hybrids with different OAg (Gemski et al., 1972) and competition studies using free LPS (Izhar et al., 1982) suggest that *E. coli* Flexneri requires its own or a related OAg for cell invasion (Reeves, 1995). Other examples include *P. aeruginosa* strains that cause corneal infection (Gupta et al., 1994) and *Actinobacillus pleuropneumoniae*, which causes pleuropneumonia in pigs (Paradis et al., 1994).

2.6.7 Role of O-antigen chain length

As already discussed, modification of OAg chain length by restricting the supply of mannose can affect the outcome of the interaction of complement with OAg (Reeves, 1995). In *E. coli*, S. *enterica* and *Yersinia pseudotuberculosis*, the strongly modal chain-length distribution has been shown to be determined by Cld (Bastin et al., 1993; Batchelor et al., 1992). No studies have been done yet on the effect on complement action or virulence of the change in chain-length distribution caused by mutation in the *cld* gene (Reeves, 1995). However, an observation about *S*. Typhi is probably relevant: strains O901 and H901, which are known to be less to serum bactericidal activity than is strain Ty2, have a chain-length distribution (Jimenez-Lucho and Foulds, 1990) that is characteristic of mutations in the *cld* gene, suggesting that the distribution conferred by Cld is required for serum resistance (Reeves, 1995). Furthermore, the fish pathogen *Vibrio anguilarum* has increased OAg chain length when grown on fish-blood agar, which correlates with increased resistance to non-immune fish sera (Mutharia and Amor, 1994).

Previous studies have found that a plasmid of *E. coli* Flexneri carries a *cld* gene (Stevenson et al., 1995) that acts independently of the chromosomal *cld* gene to give LPS molecules with OAg chain lengths of about 90-100, in addition to molecules with chain lengths of about 15 due to the chromosomal cld gene. The modal value imparted by the plasmid-borne *cld* gene is very high and, although it is not known what effect this has, it might speculatively be thought to affect the pathogenicity of the strain (Reeves, 1995).

2.7 Role of O-acetyl groups on the immunological properties of Lipopolysaccharide O-antigen

Serological distinction between OAg is one of the criteria in the Kauffmann-White scheme for classification of the salmonellae (Kauffmann, 1966; Le Minor and Bockemuhl, 1988). The OAg of *S*. Typhimurium consists of a polymer of a tetrasaccharide composed of galactose, rhamnose, and mannose in the main chain and abequose (3,6-dideoxy-galactose) attached to the mannose residue (Hellerqvist et al., 1968). Serologically, *S*. Typhimurium is classified as O4, O5, and O12. The O12 antigen is complex but involves the trisaccharide of the main chain (Jorbeck et al., 1979; Svenson and Lindberg, 1978), the abequose residue confers the O4 serotype (Jorbeck et al., 1979), and, if the abequose residue is acetylated on the 2-hydroxyl group, then this confers the O5 serotype (Hellerqvist et al., 1968).

The structure, length, and the integrity of the OAg are important virulence factors in *Salmonella* infections as the OAg is involved in resistance to phagocytosis, antimicrobial peptides and serum complement (Joiner et al., 1982; Ilg et al., 2009; Murray et al., 2003). The lack of abequose O-acetylation does not diminish the virulence of *S*. Typhimurium in a murine infection model (Michetti et al., 1992). However, its importance in immune response can be deduced from the fact that a monoclonal secretory IgA that recognizes O-acetylated abequose as the determining part of the antigen can protect mice against oral challenge with *S.* Typhimurium (Michetti et al., 1992; Slauch et al., 1995). In addition, in other bacterial species, the presence of O-acetyl groups in the OAg has been shown to influence the OAg antigenicity and thus, the immunogenicity of vaccines developed using the polysaccharide part of the LPS as immunogen (Konadu et al., 1996; Berry et al., 2002; Kubler-Kielb et al., 2007). The knowledge of the exact repeating unit of the OAg, including the occurrence and position of O-acetyl groups, is therefore a major prerequisite for the development of vaccine candidates based on LPS (Ilg et al., 2013).

Salmonella OAg has been studied on extracted LPS using solution NMR spectroscopy (Bock et al., 1984; Olsthoorn et al., 2000; Olsthoorn et al., 1998; De Castro et al., 2013).

Studies have indeed shown O-acetyl groups have a critical influence on the immunological properties of the LPS (Berry et al., 2002; Ellerbroek et al., 2004). In a study undertaken to determine whether the presence of O-acetyl groups on meningococcal serogroup A polysaccharide (PS) is critical for the induction of a protective antibody response (Berry et al., 2002), in 17 of 18 postimmunization human sera, inhibition enzyme-linked immunosorbent assay (ELISA) indicated that majority of antibodies binding to serogroup A PS were specific for epitopes involving O-acetyl groups. Studies with mice also showed an essential role for O-acetyl groups, where serum bactericidal titers following immunization with de-O-acetylated (de-O-Ac) conjugate vaccine were at least 32-fold lower than those following immunization with O-Ac PS-conjugate vaccine and 4-fold lower than those following immunization with native capsular PS (Berry et al., 2002). Inhibition studies using native and de-O-Ac PS confirmed the specificity of murine antibodies to native PS (Berry et al., 2002). The dramatic reduction in immunogenicity associated with removal of O-acetyl groups indicates that O acetylation is essential to the immunogenic epitopes of serogroup A PS (Berry et al., 2002). Since levels of bactericidal antibodies are correlated with protection against disease, O-acetyl groups appear to be important in protection (Berry et al., 2002).

The antigenic importance of the O-acetyl groups is strongly influenced by their position on the PS backbone (Berry et al., 2002). The O-acetyl groups are bulky and they cover most of the reactive moieties of the PS as can be seen for the *Vi* PS of *S.* Typhi and are readily available to interact with components of the immune response (Szu et al., 1991). Other studies have also evaluated the effect of O acetylation of *Staphylococcus aureus* PS on immunogenicity and found that although the *S. aureus* PS is heavily O acetylated, stretches of bare PS were able to elicit backbone-specific, bactericidal antibodies (Fattom et al., 1998). In the Berry et al (2002) study, only 1 of 18 human sera contained antibodies that bound equally well to both native PS and

de-O-Ac PS in the inhibition assay. For this individual, antibodies specific for the PS backbone may be present.

O-acetyl groups have been shown to affect the immunogenicity or antigenicity of several bacterial PSs (Berry et al., 2002). A competitive inhibition ELISA study to evaluate the specificity of antibodies elicited in response to vaccination with an O-acetyl-positive serogroup C *N. meningitidis* vaccine showed that antibodies from vaccines had greater affinity for the O-acetyl- positive PS than for the O-acetyl-negative variant (Arakere and Frasch, 1991). However, the O-acetyl groups on the meningococcal serogroup C PS are not necessary for induction of protective antibodies (Richmond et al., 2001). Similarly, O acetylation of the pneumococcal type 9V PS is not required for induction of opsonic antibodies (McNeely et al., 1998). In contrast, removal of O-acetyl groups from the *Vi* PS of *S*. Typhi resulted in complete loss of immunogenicity, and O-acetyl-negative variants of the *E. coli* K1 capsular PS were less immunogenic than the O-acetyl-positive PS (Orskov et al., 1979; Szu et al., 1991). The immunogenicity of *N. meningitidis* serogroup A PS, like that of the *Vi* PS, has been shown to depend on the presence of O-acetyl groups (Berry et al., 2002). In this study, the bactericidal titers in the groups immunized with unconjugated PS were of interest. The groups that received native PS produced high bactericidal titers relative to ELISA titers, possibly due to Ig class differences between PS and PS conjugate groups. The relative predominance of IgM and IgG3 seen in the PS group in the study was consistent with previously reported murine responses to PS (Muller and Apicella, 1988; Rubinstein et al., 1998) and may result in more efficient complement fixation and killing. However, the higher proportion of functional antibody induced by unconjugated PS relative to the total anti-PS antibody also raises the concern that the conjugation process affected epitopes of the PS, resulting in relatively high levels of antibodies that are not bactericidal. This data emphasized the need to anticipate and evaluate the effects of conjugation on potential protective epitopes of serogroup A PS and the importance of using an assay that measures functional antibody.

Investigations of the importance of O-acetylation and xylose for interference with neutrophil migration in *Cryptococcus neoformans* have found that de-O-acetylation causes a dramatic reduction of the inhibitory capacity of the capsular polysaccharide glucuronoxylomannan (GXM) in the *in vitro* assays for neutrophil chemokinesis, rolling on E-selectin and firm adhesion to endothelium (Ellerbroek et al., 2004). *In vivo*, chemical deacetylation of GXM significantly reduced its ability to interfere with neutrophil recruitment in a model of myocardial ischemia (65% reduction vs a nonsignificant reduction in tissue myeloperoxidase, respectively), thus indicating that 6-O-acetylated mannose of GXM is a crucial motive for the inhibition of neutrophil recruitment (Ellerbroek et al., 2004).

GXM consists of a linear (1→3)-α-D-mannan backbone that is variably O-acetylated and substituted with glucuronic acid and variable amounts of xylose. O-acetyl has been recognized as the major epitope for recognition of GXM by various monoclonal and polyclonal Abs (Belay and Cherniak, 1995; Cherniak et al., 1980; Eckert and Kozel, 1987; Kozel et al., 2003) and as important for clearance of GXM from serum (Kozel et al., 2003).

Characterization of the interaction between a series of monoclonal immunoglobulin A antibodies and the LPS of *S*. Typhimurium has shown that recognition of LPS by the monoclonal antibodies is affected by acetylation of the OAg on the abequose moiety, the determinant of the O5 epitope (Slauch et al., 1995). In this characterization, although recognition of LPS by several of the monoclonal antibodies was completely dependent on acetylation, the antibodies recognized clearly separable epitopes, thus suggesting that acetylation of OAg affects the three-dimensional structure of the molecule and thus creates and destroys a series of conformational antigenic determinants (Slauch et al., 1995). The results demonstrated that a change in the acetylation state of LPS has no effect on virulence (Slauch et al., 1995). However, acetylation has important consequences for the mucosal immune response and thus could potentially have profound implications for

the ability of an immune host to respond to a subsequent infection (Slauch et al., 1995).

2.8 Effect of glucosylation on immunogenicity

Antibodies directed against the OAg of the LPS molecule of *Salmonella* mediate bacterial killing and are protective, and conjugation of the O-polysaccharide to a carrier protein represents a possible strategy for vaccine development (Micoli et al., 2014). Bacterial LPS can demonstrate high levels of heterogeneity, particularly LPS from *S*. Typhimurium (Micoli et al., 2014). O-antigen can vary in chain length and other modifications, like glucosylation and O-acetylation of the repeating units, which can constitute additional specific O-polysaccharide factors (Helander et al., 1992). This structural variability could have an impact on the immunogenicity of corresponding glycoconjugate vaccines and different isolates need to be evaluated in order to identify the appropriate source of OAg to use for the development of a candidate conjugate vaccine with broad coverage against *S*. Typhimurium (Micoli et al., 2014).

2.9 Antibiotic resistance in Nontyphoidal *Salmonella* infections

In developing countries where the burden of infectious diseases is high, patients with antimicrobial-resistant infections may be unable to obtain or afford effective second-line treatments (Vandenberg et al., 2010). In sub-Saharan Africa, the situation is worsened by poor hygiene, unreliable water supplies, civil wars, and increasing numbers of immunocompromised people, such as those with HIV, which facilitate both the evolution of resistant pathogens and their rapid spread in the community (Takem et al., 2014). Antimicrobial therapy is essential for NTS infections to save life in these resource-poor settings, particularly where HIV infection is common, and mortality rates can be extremely high (Mastroeni and Maskell, 2006). Although NTS infections can be treated with antibiotics, these are often not readily available in endemic areas and antibiotic resistance is on the rise (Wick, 2011). Both plasmid and chromosomally mediated multi-antimicrobial resistance have developed in NTS and continue to spread throughout the world.

Although there is paucity of data on multidrug resiatnce attributable to NTS in Africa, a number of studies available across Africa have provided important insights on the major challenges MDR iNTS poses in the treatment and management of the disease. Multidrug resistant iNTS has been reported in various sub-Saharan Africa countries including Kenya and Malawi (Gordon, 2008; Kariuki et al., 2006a; Kingsley et al., 2009; Bachou et al., 2006; Ikumapayi et al., 2007; Vandenberg et al., 2010). In a population-based surveillance study among 55,000 persons in malaria-endemic, rural and malaria-nonendemic, urban sites in Kenya from 2006–2009, overall, 80% of *S*. Typhimurium isolates from blood were MDR in the rural site (Tabu et al., 2012). The use of chloramphenicol as a drug of choice for treatment for suspected severe bacterial infections and cases of iNTS infection confirmed by blood culture in endemic countries such as Malawi led to the emergence of resistance around 2001 - 2004 (Kariuki and Dougan, 2014). The acquisition of chloramphenicol resistance may have afforded resistant clones a greater opportunity to survive treatment and transmit disease (Okoro et al., 2012).

Investigatation of aetiology and anti-microbial resistance patterns of bacterial isolates in blood stream infections in Kenya have revealed high level resistance among the Gram positive organisms and also amongst extended spectrum beta lactamase (ESBL) producing *E.coli* and *Klebsiella* spp. (Kohli et al., 2010). In this study, *Salmonella* spp. showed multiple resistant patterns to co-trimoxazole, chloramphenicol and ampicillin with resistance rates of greater than 35%. In addition, one hundred and twenty three patients (11%) tested positive for HIV.

Multiple resistance has been reported to be common and mediated by a variety of resistance genes but not by phage DT104 in a study to identify clinically isolated *S*. Typhimurium in western Kenya and to assess antimicrobial resistance profiles and strain inter-relatedness (Onyango et al., 2009). Twenty *S*. Typhimurium strains were resistant to ampicillin, streptomycin and sulfamethoxazole. Ciprofloxacin resistance and phage DT104 were not detected.

More than 50% multi-drug resistance prevalence in *Shigella* spp*., Salmonella* spp. and diarrheagenic *E. coli* to ampicillin, tetracycline and trimethoprim/sulfamethoxazole has also been reported in a case-control surveillance study for enteric pathogens of acute diarrhea from Kericho and Kisumu District Hospitals in western Kenya between september 2009 to September 2011 (Swierczewski et al., 2013). Several enteroaggregative and enterotoxigenic *E. coli* isolates producing extended-spectrum beta-lactamases were also isolated in this study.

Presence of almost full MDR among NTS isolates from blood cultures in Central Africa has been reported in a surveillance study in DR Congo to investigate NTS isolates recovered from bloodstream infections over the years 2007 - 2011 (Lunguya et al., 2013). Resistance to fluoroquinolones, azithromycin and third generation cephalosporins was reported as being still low, but emerging. MDR, decreased ciprofloxacin susceptibility, azithromycin and cefotaxime resistance were 80.7%, 4.3%, 3.0%and 2.1% respectively. ESBL production was noted in three (1.3%) isolates (Lunguya et al., 2013).

A hospital based retrospective analysis of blood cultures from infants to children up to 14 years of age with preliminary diagnosis of sepsis and admitted to the Neonatal Intensive Care Unit (NICU) and Paediatric Wards of the Teaching Hospital Tamale, Ghana from July 2011 to January 2012 reported that while Gram-positive and Gram-negative bacteria showed low susceptibility to Ampicillin, Tetracycline and Cotrimoxazole, the GNR were susceptible to Gentamicin and third-generation cephalosporins (Acquah et al., 2013).

Antimicrobial susceptibility testing and whole-genome sequencing of two cases of extremely drug-resistant *Salmonella enterica* serovar Senftenberg isolated from patients in Zambia found that the isolates were resistant to, and harbored genes toward, nine drug classes, including fluoroquinolones and extended-spectrum cephalosporins, contained two plasmid replicons, and differed by 93 single-nucleotide polymorphisms (Hendriksen et al., 2013).

In Nigeria, eighteen *S.* Typhimurium ST313 isolates were characterized by antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) with six of the isolates being characterized by whole genome sequence typing (WGST) (Leekitcharoenphon et al., 2013). All *S.* Typhimurium ST313 isolates harbored resistant genes encoded by *blaTEM1b*, *catA1*, *strA/B*, *sul1*, and *dfrA1*. Additionally, *aac(6')1aa* gene was detected.

Investigation of prevalence and characteristics of extended-spectrum β-lactamase-producing clinical *Salmonella enterica* isolates from 1999 to 2009 in Dakar, Senegal reported a significant increase in the prevalence of resistance to amoxicillin (0.9% in 1999 to 11.1% in 2009) and nalidixic acid (0.9% in 1999 to 26.7% in 2009) observed in nontyphoidal *Salmonella* serovars (Harrois et al., 2014). For ciprofloxacin and extended-spectrum cephalosporins (ESCs), the rates of resistance were reportedly low: 0.3% and 0.5%, respectively.

In sub-Saharan Africa countries, inaccessibility to healthcare facilities and the often poor laboratory capacity for diagnostic services promotes antimicrobial resistance by hindering ability to detect and control foodborne and other enteric infections, including antimicrobial resistant pathogens (Tomson and Vlad, 2014). Most healthcare facilities in these countries have inadequate diagnostic capacity, and the population in these settings has limited access to formal healthcare services, as evidenced by the prevalence of self-medication and inadequate knowledge about the appropriate use of antibiotics by both healthcare providers and the general population (Kariuki and Dougan, 2014). Inadequate laboratory diagnosis implies that antibiotic resistance frequently goes unrecognized and may only be detected as clinical treatment failure (Kariuki and Dougan, 2014). Few peripheral health facilities have laboratory facilities that can provide diagnostic services, and most diagnosis is based on physician assessments on patients (Tomson and Vlad, 2014). Where few laboratory services may be available, reliable supply of reagents is a major challenge, and often lack of precision in diagnosis leads to overprescribing, particularly of broad-spectrum agents, promoting emergence and spread of potentially epidemic resistant clones (Kariuki and Dougan, 2014). Sources include itinerant street vendors

and kiosks in many informal settlements in cities, whose main intention is to make a quick profit and have every incentive to encourage antibacterial drug misuse (Okeke et al., 2007).

Retail pharmacies, often operating unlicensed, appear to be more accessible to patients: they are located within the community, do not charge consultant fees, have shorter waiting times, and are willing to negotiate treatment protocols to meet the financial needs of clients (Goel et al., 1996; Okeke et al., 2005). Huge populations, especially in rural settings in sub-Saharan region, use retail pharmacies as the first site for outpatient care (Kariuki and Dougan, 2014). A number of pilot studies in Kenya estimated that 65% of pharmacies dispense antibiotics without a doctor's prescription (Kakai and Wamola, 2002; Kwena et al., 2008). In fact, drug retailers also appear to be an important source of information about illness in general in these settings (Goel et al., 1996; Grigoryan et al., 2008; Kakai and Wamola, 2002; Kwena et al., 2008; Okeke et al., 2005). Caregivers of young children rely on both chemists and healthcare workers to guide them in diagnosing and preparing treatments for diarrhea at home (Kariuki and Dougan, 2014). In addition, pharmacy employees are often relied upon for advice on childhood diarrhea and case management. This creates a potential ground for inappropriate use of antibiotics, which promotes resistance (Grigoryan et al., 2008).

Inappropriate use of antibacterials has also contributed to antibiotic resistance in sub-Saharan Africa. Although the total amount of antibiotic consumption is an important issue, perhaps of greater concern in sub-Saharan Africa region, where the need for antibiotics exceeds available stocks, is the way these drugs are used (Kariuki and Dougan, 2014). Inappropriate antibiotic use in settings in sub-Saharan Africa is complex and requires an understanding of the incentives behind prescribing, dispensing, and consuming drugs in order to design effective policies to curb resistance (Esimone et al., 2007). Some of the factors that drive irrational antibiotic use relate to motivation to save money by purchasing insufficient dosages and avoiding the consultant fees required at formal healthcare facilities (Tomson and Vlad, 2014). Other factors involve the geographic inaccessibility of formal

healthcare facilities, noncompliance with prescribed regimens by prematurely ending treatment when symptoms subside, sharing of medication with family members and friends who may be unwell, and generally hoarding drugs for future use (Kariuki and Dougan, 2014). Lack of laboratory confirmation for diagnosis, improper training, and legitimate fears of bad treatment outcomes in cases of critical illness are also important in promoting inappropriate antibacterial use (Lunguya et al., 2013).

Providers' incentives for antibacterial use are also an influencing factor for antibiotic resistance in sub-Saharan Africa. Drug distributors, including not only pharmacists but also pharmacy attendants, patent medicine stallkeepers, and itinerant drug sellers, often sell drugs without prescription and are an important source of primary care for people in many developing sub-Saharan Africa countries (Igun, 1994; Indalo, 1997). Patients in search of convenient and accessible health care often seek treatment at drug retail premises. Many drug sellers have not been formally trained in diagnosis and prescription but often have financial incentive to perform those tasks, with varying degrees of competence. Despite the potential loss of business to storekeepers, educational interventions have been successful in increasing prescription requirement demands and promoting referral advice (Chuc et al., 2002). A few studies in kenya have examined the extent of providers' knowledge in different contexts and the prescription or recommendation of antibiotics in practice (Kariuki and Dougan, 2014). However, other influences appear to drive supply, since even practitioners who understand the correct treatment may inappropriately prescribe antibiotics (Duse, 2011; Esimone et al., 2007; Grigoryan et al., 2008; Kwena et al., 2008; Mukonzo et al., 2013). The widespread use of antimicrobial agents in the treatment of infections in the tropics, inadequate or underutilized diagnostics which can cause uncertainty about the best treatment and fear about the outcome if the healthcare provider withholds antibiotics, leading not only to overuse of antibacterials in general, but also to a heavy reliance on broad-spectrum antibiotics (Sang et al., 2012).

The exact prevalence of antibiotic resistance in NTS on a global basis is difficult to ascertain (Mastroeni and Maskell, 2006) but MDR invasive NTS remains a major

health challenge. Though sufficient data to paint a clear picture is not available (Matheson et al., 2010), few available publications indicate that in UK, MDR in *S*. Typhimurium can be traced as far back as in 1960s (Anderson, 1968). To expound on this, for over a decade an MDR strain of *S*. Typhimurium definitive pahe type 29 (DT29), resistant to ampicillin, streptomycin, sulfonamides, tetracyclines and furazolidone, caused numerous infections in cattle and humans. Attempts to treat and control the disease with a range of antibiotics were ineffective, but resulted in the acquisition of transferable multiple drug resistance by type 29. The transmission of drug-resistant type 29, directly or indirectly, from bovines to man resulted in many human infections (Anderson, 1968). Resitance in *S*. Typhimurium in the UK remained relatively low until the emergence of strains DT204. 193 and 240c (Mastroeni and Maskell, 2006). These strains became epidemic in calves and humans in the UK and caused infections in France, Germany, Belgium and Italy following the export of infected livestock (Rowe et al., 1979). In another study to review all *Salmonella* infections diagnosed in the Cambridge area, UK within a 10-year period (1.1.1999 and 31.12.2008), a trend was observed towards increasing chloramphenicol resistance among *Salmonella enterica* over the study period (Matheson et al., 2010). Ciprofloxacin resistance in NTS from stool was not seen until 2003, with sporadic incidence since this time and a small yet marked increase in the final year of the study. Trimethoprim and amoxicillin resistance remained relatively low. Since chloramphenicol is not widely used in the UK for treatment of *Salmonella* infections, the increase in resistance is likely to have reflected the introduction of new, resistant strains into the community, or co-selection for chloramphenicol resistance in strains carrying mobile genetic elements conferring resis-tance to multiple antibiotics (Matheson et al., 2010).

Characterization of antimicrobial susceptibility phenotypes, types of extended-spectrum β-lactamase gene plasmids and serological relationships of 21 NTS isolates from patients who attended three different hospitals from 2006 to 2008 in India, reported that 48% possessed the extended-spectrum cephalosporin resistance phenotype, which represents a real and immediate challenge to the effective

antimicrobial therapy of *Salmonella* spp. infections associated with systemic manifestations (Menezes et al., 2010).

Therefore, antibacterial resistance among NTS, a key group of bacterial pathogens of major public health significance globally including sub-Saharan Africa remains a major huddle to providing basic health care needs to communities. Although the paucity of data and lack of effective systems for routine surveillance and reporting make estimations of the magnitude of the problem of antibacterial resistance difficult in sub-Saharan Africa, there is enough evidence of the effects of resistance, especially in rising cases of treatment failure in severe infections (Kariuki and Dougan, 2014). Strategies aimed at the prevention of transmission of infectious diseases could lead to reduced disease burden and hence lower prevalence of resistance. The increasing emergence of multidrug resistance to NTS and a lack of new targets for drug development indicate that the absence of NTS vaccines should be urgently addressed. Other measures such as public health directed efforts aimed at improving the supply of clean water and environmental sanitation will also contribute significantly to reducing the burden of NTS infections in most of the sub-Saharan countries. Surveillance studies are also vital to provide data showing resistance trends among the bacterial pathogens, monitoring rational use of antibiotics, and developing clinical management guidelines to standardize therapy in healthcare facilities aimed at curtailing development of resistance against the remaining effective antibacterials (Kariuki and Dougan, 2014).

2.10 Genomic, molecular and evolutionary studies of Nontyphoidal *Salmonella*

The presence of high levels of genome degradation observed in the *S*. Typhimurium pathovar ST313 has a striking resemblance to what observed for *Salmonella enterica* serovar Typhi (Kingsley et al., 2009). Evidence suggests that, as well as undergoing gene degradation, *S.* Typhimurium has also recently acquired genes, such as those encoding the *Vi* antigen in *S*. Typhi, by horizontal transfer events (Baker and

Dougan, 2007; Kingsley et al., 2009), raising the possibility that ST313 strains have undergone microevolution to become human adapted (Feasey et al., 2012).

More recent genome sequencing data of a broad collection of African isolates have revealed that the *S*. Typhimurium ST313 clonal group of bacteria, sharing common ancestry and which has been circulating for around 50 years now has split into two lineages that have acquired similar resistance genes on separate occasions (Wain et al., 2013). *Tn21* transposon element carried on the virulence plasmid pSLT, associated with chloramphenicol resistance (*cat* gene) has been implicated in the clonal displacement of the invasive isolates in the two lineages identified (Okoro et al., 2012).

It is still not clear whether these ST313 strains cause a significant burden of gastroenteritis in sub-Saharan Africa (Feasey et al., 2012). However, the finding that NTS isolates from healthy people match in clonal identity with index cases of invasive NTS disease living together in Kenya suggests that healthy carriers could be important in the transmission of these pathogens (Kariuki et al., 2006b). While molecular epidemiological study of invasive and diarrhoeal strains of *S*. Typhimurium and *S*. Enteritidis could be helpful to understand the transmission of invasive ST313 strains in sub-Saharan Africa, the host immune status and genetic background could be the dominant factor affecting susceptibility to invasive NTS (Feasey et al., 2012).

With the rapid technological advances in science, whole-genome sequencing is emerging as the method of choice in bacterial typing (Pallen and Loman, 2011; Parkhill and Wren, 2011). Success in tracking worldwide epidemics (Beres et al., 2010; Chin et al., 2011; Harris et al., 2010; Mutreja et al., 2011), regional outbreaks (Beres et al., 2010; Gardy et al., 2011), food-borne outbreaks (Lienau et al., 2011; Rasko et al., 2011a), and bioterrorism agents (Rasko et al., 2011b) has demonstrated that the fine resolution provided by whole-genome sequencing facilitates our understanding of the spread of infectious agents (Snitkin et al., 2012). The continued improvements in turnaround time and accessibility of DNA sequencing technologies

are now approaching a point where genomic data can be generated in a clinically relevant time frame (Snitkin et al., 2012). Genome sequencing has been applied for example to nosocomial strains (Eyre et al., 2012; Koser et al., 2012; Lewis et al., 2010; Lieberman et al., 2011) but with limited study size or scope in reconstructing transmission links during the course of the outbreak (Snitkin et al., 2012).

Whole genome sequencing has also been used to improve phylogenetic resolution within serotype Typhimurium, particularly within the ST313 lineage and it is therefore possible to link haplotypic clusters to individual countries, suggesting that particular phylogenetic signatures may map countries or geographical regions (Okoro et al., 2012).

A systematic characterization of endemic *S*. Typhimurium and *S*. Enteritidis isolates from one endemic region and an investigation on the relationship between genotypic and phenotypic features of NTS isolates has not been conducted. The cost of DNA sequencing has steadily gone down, by roughly 10-fold every five years (Zankari et al., 2012). As a consequence, DNA sequencing is becoming increasingly accessible for routine use and has for example been utilized for complete characterization of antimicrobial resistance and virulence gene content during the safety evaluation of 28 strains intended for use in human nutrition (Bennedsen et al., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Origin and categorization of bacterial isolates and serum samples

The study culture collection contained 193 archived NTS isolates (114 *S.* Typhimurium and 79 *S.* Enteritidis) collected between 2000 and 2011 at the Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya. Study isolates were selected from the archived bacterial culture collection based on availability of unequivocal serovar identification and availability of clinical records and metadata. The isolates were stored in Tryptic Soy Broth with 15% glycerol at -80°C. The isolates were from blood and stools with a few additional samples from urine (3 samples), cerebrospinal fluid (CSF, 2 samples) and environmental sources (2 samples). The isolates were mostly from children admitted to three main hospitals in Nairobi County, Kenya namely: Kenyatta National Hospital (KNH), Gertrude's Garden Children's Hospital (GCCH) and Aga Khan Hospital (AKH). This collection also included isolates from healthy carriers (31 samples). Before use in the present study, the archived isolates were re-sub-cultured and the serotype was confirmed by antibody-based agglutination (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Based on the clinical sources and sample type, the NTS isolates were divided into four main groups. Those isolates from body fluids that are normally sterile such as blood and CSF were grouped as ‘invasive’, those from stool samples of patients were grouped as ‘gastroenteric’, those from stool samples of healthy individuals were grouped as ‘healthy carriers’ while those from soil and sewer water sources were grouped as ‘environmental’. The three isolates from urine samples were considered clinically significant invasive sterile site isolates since *Salmonellae* are not part of the normal perineal skin flora and the isolates were from patients with symptoms of urinary tract infection.

Undiluted sera from ten subjectively healthy and HIV-uninfected Malawian adults were used to generate a pooled serum to assess sensitivity to antibody-mediated killing of the study *Salmonella* isolates. Serum from African (Malawi) adults was preferred over that from children because it has been shown that almost all Africans acquire bactericidal antibody to NTS in childhood (MacLennan et al., 2008). Each serum was tested prior to pooling to ensure that killing of the index *S*. Typhimurium ST313 strain, D23580, was within the 0.9 to 3.0 Log_{10} range previously described for such sera (MacLennan et al., 2010). Sera were handled at 4°C during the pooling process to ensure preservation of endogenous complement activity and frozen in aliquots at -80°C.

3.2 Ethical and scientific approvals

Ethical approval for preparation of this serum was granted in Malawi by the College of Medical Research and Ethics Committee, College of Medicine, University of Malawi (Goh and MacLennan, 2013). Scientific and Ethical approval for the use of bacterial isolates in Kenya was granted by the Scientific Steering Committee (SSC) and the Kenya National Ethical Review Committee (ERC) respectively based at KEMRI (SSC No. 2115 of 2011, Approval letters: Appedix 1 and 2). Scientific approval to carry out the study was also granted by the College of Health Sciences (CoHS), Jomo Kenyatta University of Agriculture and Technology (JKUAT), University admission/registration number TM 402-2301/2011.

3.3 Study design

This was a laboratory based retrospective study involving investigations of microbiological, immunological and genomic characteristics of NTS isolates from Kenyan patients in relation to their clinical presentations. Respective patient records were reviewed to provide differences in presentation of invasive and enteric isolates**.**

3.4 Statistical analyses

Basic statistical analysis was performed for the quantitative study data. Bivariate analysis involving serum susceptibility and OAg amounts of the two *Salmonella*

serovars examined (*S*. Typhimurium and *S*. Enteritidis) was compared using the Mann-Whitney U test. Possible correlations (i.e. OAg amount and serum susceptibility, glucosylation levels and OAg production) were analysed by Spearman rank. Univariate descriptive and exploratory analyses involving O-acetylation levels of OAg samples and antimicrobial susceptibility test profiles were done by use of proportions.

3.5 Laboratory based methods

3.5.1 Immunoassay and O-antigen characterization methods

3.5.1.1 Serum Bactericidal Activity (SBA) assay

The study isolates were tested for their susceptibility/resistance to killing against the pooled adult human serum pool, using a protocol involving whole serum and endogenous complement activity as described by MacLennan *et al*., (2008). Briefly, 5 µl washed bacteria at two hour log-growth phase at an OD of approximately 0.2, with shaking at 180 rpm was added to 45 µl undiluted serum at a final bacterial concentration of 1×10^6 colony forming units (CFU/ml) and incubated at 37 °C with the number of viable bacteria count determined by serial dilution on Luria Bertani (LB) agar after 0 and 180 min. As a negative control, reference sera were heat-inactivated at 56°C for 45 min to inactivate endogenous complement and included in each SBA experiment. Additional internal controls included testing the representative endemic African *S*. Typhimurium isolate D23580, previously shown to be susceptible to serum killing (MacLennan et al., 2008) and hyperimmune mouse serum containing high levels of anti-OAg antibodies (Rondini et al., 2013). Susceptibility to serum killing was defined as any reduction in viable bacterial count compared with the initial *Salmonella* concentration.

3.5.1.2 O-antigen extraction and quantification

O-antigen extraction was performed by acid hydrolysis (Micoli et al., 2013). Bacterial isolates were grown overnight in LB medium. The bacterial OD was measured and the bacterial cultures were concentrated in PBS to OD: 35. Acetic acid

(2% v/v) was then added to the concentrated growth bacterial cultures (pH 3) which were incubated for three hours at 100 °C. The reaction was stopped by the addition of 14% ammonium hydroxide, increasing the pH to around six. Bacterial debris was removed by centrifugation and OAg in the supernatant was 0.22 µm microfiltered and desalted by HiTrap® Desalting five ml columns (GE Healthcare Company, LC, UK). Phenol sulphuric acid assay, using glucose as standard, was used for quantification of total sugar content (Dubois, 1956; Micoli et al., 2013).

3.5.1.3 ^{1}H Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

^{1}H NMR analysis was performed as a confirmation of the identity of the OAg samples (typical signals of the OAg chain can be detected, confirming the presence of the characteristic sugars) and to verify the presence of O-acetyl groups along the OAg chain as previously described (Micoli et al., 2013).

Briefly, a spectrum of a supernatant from a widely studied *S.* Typhimurium strain SL1344 was used as reference for identifying the O-acetyl peaks.

Dried OAg samples were solubilized in 650 µl deuterated water (D_2O; Aldrich) and transferred to 5 mm NMR tubes. Proton NMR experiments were recorded at 25 °C on a Varian VNMRS-500 spectrometer, equipped with a Pentaprobe. Acquisition time of 5 s, relaxation delay of 15 s, and number of scans of 256 were set for recording the spectra. For data acquisition and processing VNMRJ version 2.2 revision C and Mestrenova 6.1 (Mestrelab Research) were used, respectively. One-dimensional proton NMR spectra were collected using a standard one-pulse experiment. Chemical shifts were referenced to hydrogen deuterium oxide (HDO) at 4.79 ppm (^{1}H) (Micoli et al., 2013).

3.5.1.4 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Glucosylation level of OAg samples was estimated by HPAEC–PAD after acid hydrolysis of the OAg to release the monosaccharides constituting the sugar chain as described previously (Micoli et al., 2012; Micoli et al., 2013). Commercial glucose was used for building the calibration curve (0.5-10 µg/ml). Glucose (Glc) level is

calculated as molar ratio of Glc to rhamnose (Rha), sugar present in each OAg repeating unit.

Briefly, standards and OAg samples, diluted to have sugar monomer in the range 0.5–10 μg/ml, were hydrolyzed at 100 °C for 4 h in 2 M trifluoroacetic acid (TFA). This hydrolysis condition was optimal for release of all monomers (including rhamnose, galactose and mannose) without their degradation.
After the hydrolysis, samples were stored at 2 - 8 °C for about 30 min, dried by SpeedVac overnight, reconstituted in water, and filtered using 0.45-μm Acrodisc (PALL) filters before chromatographic analysis. HPAEC–PAD was performed with a Dionex ICS3000 equipped with a CarboPac PA10 column (4 x 250 mm) coupled with a PA10 guard column (4 x 50 mm). Separation of the sugars was performed with a flow rate of 1 ml/min, eluting in a gradient from 10 to 18 mM NaOH over 20 min. After being washed for 20 min with 100 mM AcONa in 28 mM NaOH, the column was re-equilibrated with 10 mM NaOH for 20 min. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. The Dionex standard quadruple-potential waveform for carbohydrates was used.
The resulting chromatographic data were processed using Chromeleon software 6.8. The analysis was also used as confirmation of the OAg samples identity verifying the correct monosaccharides ratios in the repeating unit (Micoli et al., 2012; Micoli et al., 2013).

3.5.1.5 Size-exclusion High-Performance Liquid Chromatography - Size Exclusion Chromatography (HPLC-SEC)

HPLC–SEC analysis was used to estimate the molecular size distribution of OAg populations (Micoli et al., 2012; Micoli et al., 2013). Samples were run, without pre-treatment, on a TSK gel G3000 PWXL column (30 cm x_7.8 mm; particle size 7 μm; cod. 808021) with a TSK gel PWXL guard column (4.0 cm _x 6.0 mm; particle size 12 μm; cod. 808033) (Tosoh Bioscience, Tokyo, Japan). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2, at the flow rate of 0.5 ml/min

(isocratic method for 30 min). O-antigen peaks were detected by differential refractive index (dRI). Void and bed volume calibration was performed with λ-DNA (λ -DNA molecular weight (MW) Marker III 0.12–21.2 kb; Roche) and sodium azide (Merck, New Jersey, USA), respectively. O-antigen average MW was estimated on standard dextrans (Sigma) calibration curve.

3.5.2 Whole-genome based phylogenetic analyses

3.5.2.1 Genomic DNA preparation of the study Nontyphoidal *Salmonella* isolates

Genomic DNA was prepared by growing the archived isolates in Luria Bertani (LB) medium followed by taking single colonies of grown cultures and incubating in LB broth overnight at 37 °C. Bacterial cells were then pelleted by centrifugation (4300 rpm) for 5 min), and DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. Briefly, the bacterial cells were harvested from the sample by pelleting 1.5 mL of an overnight bacterial broth culture by centrifugation. The cells were then resuspended by resuspending the pellet in 180 µl of a lysis buffer. Cell lysis preparation was then performed by adding 20 µl proteinase K solution to the sample. Lysis of cells was then performed by adding 200 µl of a lysis solution. This was then followed by column preparation by adding 500 µl column preparation solution to the sample to maximize membrane binding of DNA. The column was then prepared for binding by adding 200 µl of ethanol (95%-100%) to the lysate followed by a through vortexing for 5-10 sec. The entire sample mixture (lysate) was then loaded into the binding column. This was then followed by 2 runs of washing using 500 µl of a wash solution followed by centrifugation. DNA sample was eluted by pipetting 200 µl of sterile water onto the centre of the column followed by centrifugation. DNA quality and quantity were evaluated by gel electrophoresis and the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). After ascertaining the quality and quantity of all the 193 NTS study samples, 20 ng/µl DNA from each isolate was submitted for sequencing. Whole-genome sequencing of all the submitted study NTS isolates was performed by Illumina sequencing

technology at the Wellcome Trust Sanger Institute, UK. Briefly, illumina sequencing technology involves the following major steps: Genomic DNA preparation involves randomly fragmenting genomic DNA and ligating adaptors to both ends of the fragments. DNA sample is then attached to surface by binding single-straned fragments randomly to the inside surface of the flow cell channels. Bridge amplification then follows by adding unlabeled nucleotides and enzymes to initiate solid-phase bridge amplification. Fragments then became double stranded by enzyme incorporating nucleotides to build double-straned bridges on the solid-phase substrate. Denaturation of the double-straned molecules follows which leaves single-stranded templates anchored to the substrate. Complete amplification is achieved when several million dense clusters of double-straned DNA are generated in each channel of the flow cell. The first sequencing cycle (the first base) begins by adding four labeled reversible terminators, primers, and DNA polymerase. After laser excitation, the emitted fluorescence from eah cluster is captured and the first base is identified. The next cycle that determines the second base repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase. Image second chemistry cycle, which occurs after laser excitation involves capturing the image as previously and the identity of the second base is recorded. The sequencing cycles are repeated to determine the sequence of bases in afragment, one base at a time. Finally, data are aligned and compared to a reference, and sequencing differences were identified.

3.5.2.2 Genomic library preparation and sequencing

This involved preparation of multiplex libraries with a 200-bp insert size using 12 unique index tags and sequencing to generate 54- or 76-bp paired-end reads. Cluster formation, primer hybridization and sequencing reactions were based on reversible terminator chemistry using the Illumina Genome Analyzer II system according to standard protocols (Harris et al., 2010; Bentley et al., 2008). Sequence data were submitted to Sanger Institute central sequence reads repository available for analysis.

3.5.2.3 MultiLocus Sequence Type (MLST) analyses

The MLST determination and analysis was done using the Sanger Institute (Cambridge, UK) S. *enterica* database seven house keeping genes (*aroC, dnaN, hemD, hisD, purE, sucA* & *thrA*) allele coordinates alignments and confirmed by manual checks of detected alleles with the *Salmonella* MLST database hosted by the University of Warwick (http://mlst.warwick.ac.uk/mlst/, (formally hosted by University of Cork- http://mlst.ucc.ie/).

3.5.2.4 Read alignment and SNP detection.

Mapping of the sequence data of each isolate was done by an automated mapping pipeline maintained by the Sanger Institute (Cambridge, UK) by first splitting the fastq files into chunks and mapping each chunk to the respective reference genomes, *S*. Typhimurium isolate SL1344 (Hoiseth and Stocker, 1981) and *S*. Enteritidis PT4 isolate P125109 (Thomson et al., 2008) using SMALT mapper (http://www.sanger.ac.uk/resources/software/smalt/). SNPs were identified using SAMtools mpileup and were filtered for a minimum mapping quality and a quality ratio cut-off (Croucher et al., 2011; Harris et al., 2010; Li et al., 2009; Mutreja et al., 2011; Ning et al., 2001). SNPs called in phage sequences and repetitive regions of the *S*. Typhimurium and *S*. Enteritidis reference genomes were excluded. Recombinant segments of the genome were removed from the whole-genome alignment as described previously (Croucher et al., 2011). After the removal of recombinant segments, mobile elements and repetitive sequences, a concatenated alignment composed of 3838 informative SNP sites from the sequenced *S*. Typhimurium isolates and 1652 informative SNP sites from the sequenced *S*. Enteritidis isolates were produced. Small insertions and deletions (indels) were also identified from the mapping result output but were not used for subsequent phylogenetic analyses.

3.5.2.5 Whole genome Single Nucleotide Polymorphism (SNP)-based phylogenetic analyses.

Maximum-likelihood phylogenetic trees, Figure 21 (*S*. Typhimurium) and Figure 23 (*S*. Enteritidis) were constructed from resulting SNP alignments with RAxML v 8.0.19, SSE3 (Stamatakis, 2006) using a general time-reversible (GTR) substitution model with γ correction for among-site rate variation. Previously characterized invasive *S*. Typhimurium isolates A130 (Kingsley et al., 2009) and D23580 (Kingsley et al., 2009) and gastroenteritis-associated SL1344 (Okoro et al., 2012) and *S*. Enteritidis PT4 isolate P125109 (Thomson et al., 2008) were added to this analyses as comparators for the respective serovars. Support for nodes on the trees was assessed using 100 bootstrap replicates.

3.5.3 Antimicrobial susceptibility testing (AST)

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion technique (Bauer et al., 1966) for 9 antimicrobials commonly prescribed for bacterial infections. The antimicrobials (Oxoid, Basingstoke, United Kingdom) included: ampicillin (10µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (1 µg), nalidixic acid (30 µg), tetracycline (10µg), trimethoprim (2.5µg), trimethoprim/ sulphamethoxazole 1:19 (co-trimoxazole) (25µg), nitrofurantoin (300 µg). Briefly, the isolates were grown in LB broth inoculated from LB plates (picking one colony each for inoculation) and incubated overnight at 37 ^{o}C with shaking. Next 30 µL of each overnight culture were pipetted into 3 mL of PBS (1:100 dilution), thoroughly mixed by vortexing and the density of the cell suspension adjusted using a 0.5 McFarland standard. Next 100 µL of the cell suspension was pipetted unto the surface of pre-dried iso-sensitest agar plates (Difco) (Becton, Dickinson and Company, New Jersey, USA) and evenly spread on the agar surfaces using sterile spreaders (this was done within 15 min. of preparing cell suspensions). The inoculated plates were allowed to dry for 5 min. before applying antibiotics discs. Antibiotics discs (Oxoid, Basingstoke, United Kingdom) were applied to the dry surface of the inoculated plates using an 8-cartridge antibiotics disc dispenser (Oxoid, Basingstoke, United Kingdom). The ninth antibiotic disc was applied on the

surface of a separate iso-sensitest agar plate. *E. coli* ATCC 25922 was included in each run for quality control purposes. Plates were incubated within 15 min. of disc application at 37 ^{o}C for ~18 h. Plates with acceptable semi-confluent growth of colonies had the diameters of zones of inhibition measured to the nearest millimetre with a ruler, and the measurements recorded. Results interpretation was based on BSAC interpretative tables for *Enterobacteriaceace*. (http://bsac.org.uk/wp-content/uploads/2012/02/Version-12-Apr-2013_final.pdf)

CHAPTER FOUR

4.0 STUDY FINDINGS AND DISCUSSION

4.1 Results

4.1.1 Categorization and distribution of the study NTS bacterial collection

Of the 193 NTS isolates in the study, 114 (59%) were *S*. Typhimurium and 79 (41%) were *S.* Enteritidis. Appendix Table 2 and Appendix Table 3 a.i, Appendix Table 3 a.ii, Appendix Table 3 b.i, Appendix Table 3 b.ii describes the study isolate collection obtained from blood, stools (patients and healthy contacts), CSF, urine and environmental/animal sources. Nairobi county where the isolates were obtained from is an urban setting. Clinical information on patients' age was available for 67% of cases (73 with *S*. Typhimurium, 57 with *S.* Enteritidis). The age ranged from 1 month to 59 years for *S*. Typhimurium isolates and less than 1 month to 64 years for *S.* Enteritidis isolates, the majority (65%, 85 isolates) of these patients being below five years (53 *S*. Typhimurium, 32 *S.* Enteritidis). Overall, the median age of subjects from whom *S*. Typhimurium and *S*. Enteritidis strains were isolated was 2 and 3.5 years, respectively. Data on the age of patients was not available for stool samples from healthy carriers (Table 2 and Appendix Table 3 a.i, Appendix Table 3 a.ii, Appendix Table 3 b.i, Appendix Table 3 b.ii).

Table 2: Number of study NTS isolates by serovar and origin.

	***S*. Typhimurium (% of total *S*. Typhimurium, n=114)**								***S*. Enteritidis (% of total *S*. Enteritidis, n=78)**				**Total (% of total NTS)**
	Blood	**Stool**	**Blood & Stool**	**Urine**	**CSF**	**Soil**	**Sewer water**	**∗ND**	**Blood**	**Stool**	**Blood & Stool**	**∗ND**	
Invasive	48 (42%)	–	7 (6%)	3 (3%)	2 (2%)	–	–		22 (28%)	–	5 (6%)	–	87 (45%)
Gastrointestinal	–	24 (21%)	–	–	–	–	–	–	–	32 (41%)	–	–	56 (29%)
Healthy carriers	–	21 (18%)	–	–	–	–	–	–	–	11 (14%)	–	–	32 (17%)
Environmental	–	–	–	–	–	1 (1%)	1 (1%)	–	–	–	–	–	2 (1%)
∗ND	–	–	–	–	–	–	–	7 (6%)	–	–	–	9 (12%)	16 (8%)
Total	**114**								**79**				**193**

∗ND= Isolates for which no documentation on clinical form was available; n=Total number

Gastrointestinal and healthy carriers isolates were derived from stool samples

4.1.2 Serum Bactericidal Activity (SBA) assay

Serum bactericidal assay results using the serum pool demonstrated a marked difference in susceptibility to antibody-mediated killing among individual isolates and a clear difference in susceptibility between *S*. Typhimurium and *S*. Enteritidis isolates (Figure 4, Appendix Table 4 a.i, Appendix Table 4 a.ii, Appendix Table 4 b.i and Appendix 4 b.ii). All except one *S*. Typhimurium isolate (0.9%, 1/114) underwent a reduction in viable bacterial counts over the 180 min. time course of the assay, and so were designated 'sensitive' to antibody-mediated killing. Overall median Log_{10} reduction in viable bacteria after 180 min was 2.9 for *S*. Typhimurium. Indeed 50% (57/114) *S*. Typhimurium were highly susceptible to killing, undergoing a 3.0 Log_{10} reduction. In contrast *S*. Enteritidis as a group were less susceptible to antibody-mediated killing than *S*. Typhimurium, being killed by a median of 1.2 Log_{10} ($p<0.0001$, Mann Whitney test). However, all *S*. Enteritidis were sensitive to killing. A small proportion (10.3%, 6/78) was killed by a full 3.0 Log_{10}.

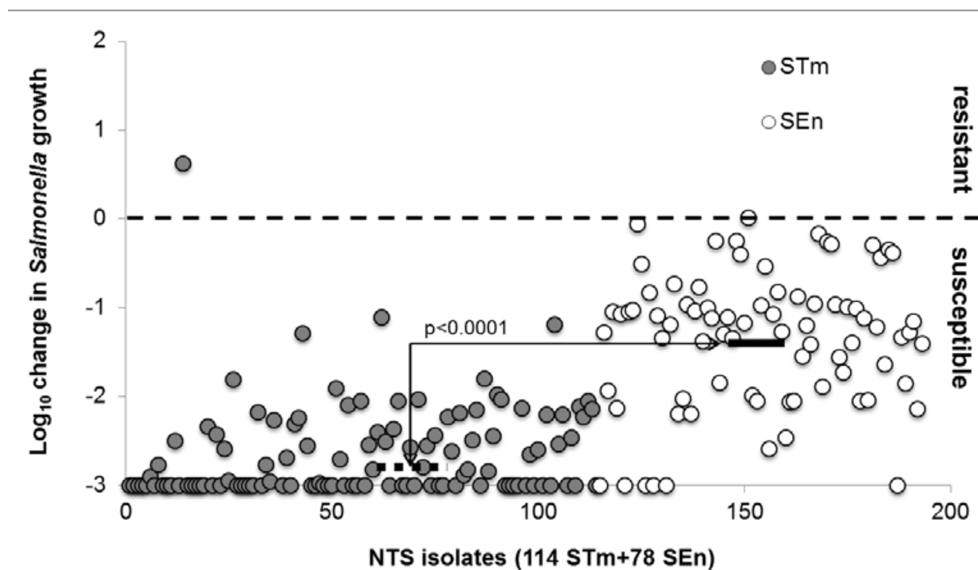

Figure 4: Serum bactericidal assay (SBA) results of S. Typhimurium and S. Enteritidis isolates with adult human serum

Bacterial colony forming units (CFU) were counted at time 0 (T0) and after 3 h (T180) incubation of bacteria in 45 µL undiluted serum (final bacterial concentration 1×10^6 CFU/ml). Killing/growth was determined by Log_{10} (CFU at T180)-Log_{10} (CFU at T0). Strains that could grow (Log_{10} change >0) were considered "resistant", strains that were killed (Log_{10} change <0) were considered susceptible. Dashed/continuous line indicates median Log_{10} change of* S. *Typhimurium (-2.9) and* S. *Enteritidis (-1.2) isolates, respectively.

4.1.3 O-antigen characterization results

4.1.3.1 O-antigen quantification and molecular size distribution analysis by Size-exclusion High-Performance Liquid Chromatography - Size Exclusion Chromatography (HPLC-SEC)

Results from the O-antigen that was extracted from all the 193 NTS study isolates and normalized to the same final OD and total amount quantified by phenol sulphuric acid assay (Micoli et al., 2013) showed that NTS isolates produced a range of OAg amounts, with low (<10 µg/ml/OD) and high producers (>25 µg/ml/OD) (Figure 5). On average, *S.* Enteritidis isolates expressed more OAg than *S*. Typhimurium, with

70.9% of the isolates producing more than 15 µg/ml/OD OAg compared to only 42.1% *S*. Typhimurium (Figure 5). Median OAg production for *S.* Enteritidis isolates was 16.8 µg/ml/OD compared to 14.4 µg/ml/OD of *S*. Typhimurium ($p<0.0001$ by Mann Whitney test) (Figure 5). All study isolates contained one single main OAg population with average MW of 21-33 kDa, kDa represented by the major peak (Figure 6). A small proportion of the study isolates, <5% contained OAg population with average MW < 6 kDa represented by the minor peak as shown in Figure 6.

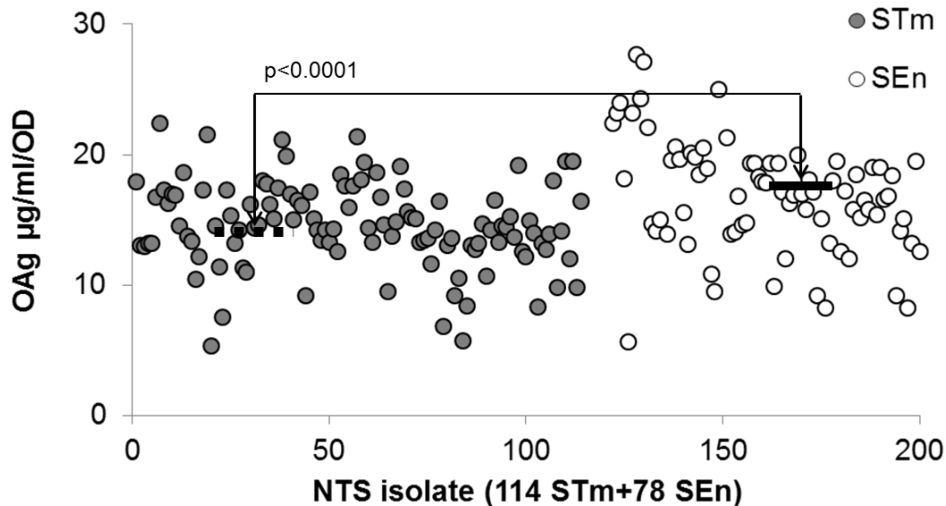

Figure 5: O-Antigen production of S. Typhimurium (STm) and S. Enteritidis (SEn) isolates after growth and normalization to the same final OD: 35

Dashed/continuous line indicates median OAg production of S. Typhimurium (STm) (14.4 µg/ml/OD) and S. Enteritidis (SEn) (16.8 µg/ml/OD) isolates, respectively.

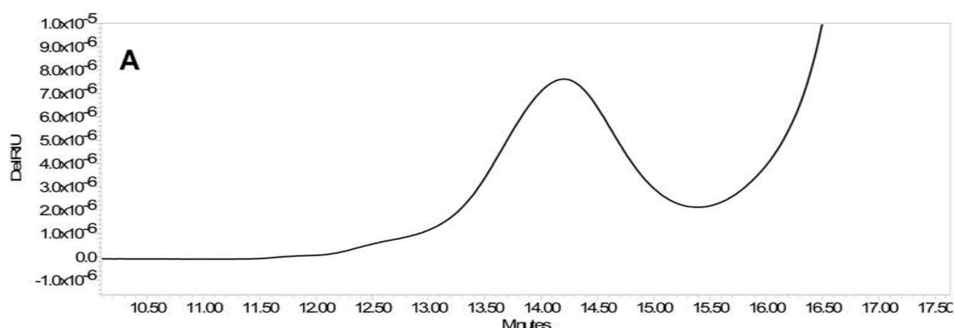

Figure 6: HPLC-SEC profile of representative S. Typhimurium isolate, Ke212 OAg (similar profiles obtained for all NTS strains)

4.1.3.2 O-antigen characterization by ^{1}H Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) and High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

^{1}H Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) analysis on a set of 118 samples (73 *S*. Typhimurium and 45 *S.* Enteritidis) showed that only 6.8% of *S.* Typhimurium OAg were not O-acetylated, compared with 35.6% and 42.2% of *S.* Enteritidis OAg with no or traces of O-acetyl groups, respectively. For *S.* Typhimurium, OAg, O-acetylation was detected on both rhamnose (Rha) and abequose (Abe) sugar residues in the majority of isolates (63%), but also on Rha only (9.6%) and Abe only (17.8%) (Table 3; Figure 7 and Figure 8; Appendix Table 5 a.i, Appendix Table 5 a.ii, Appendix Table 5 b.i and Table 5 b.ii).

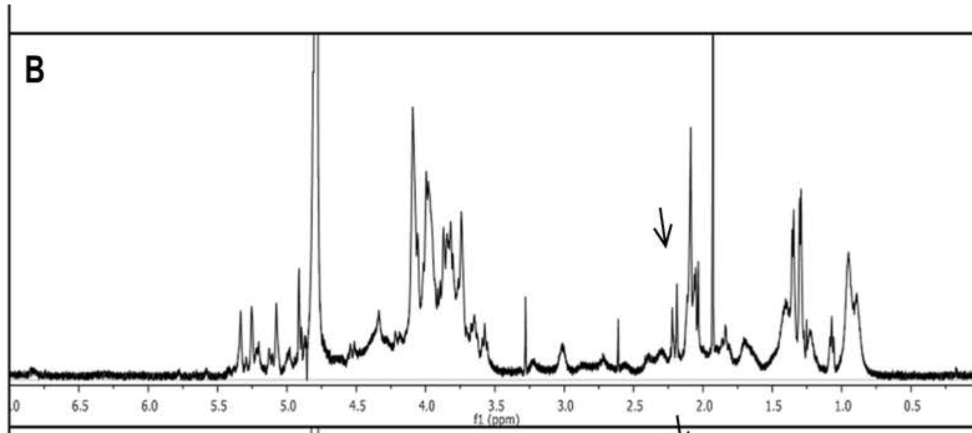

Figure 7: ^{1}H NMR profile of a representative S. Enteritidis isolate, Ke189 OAg

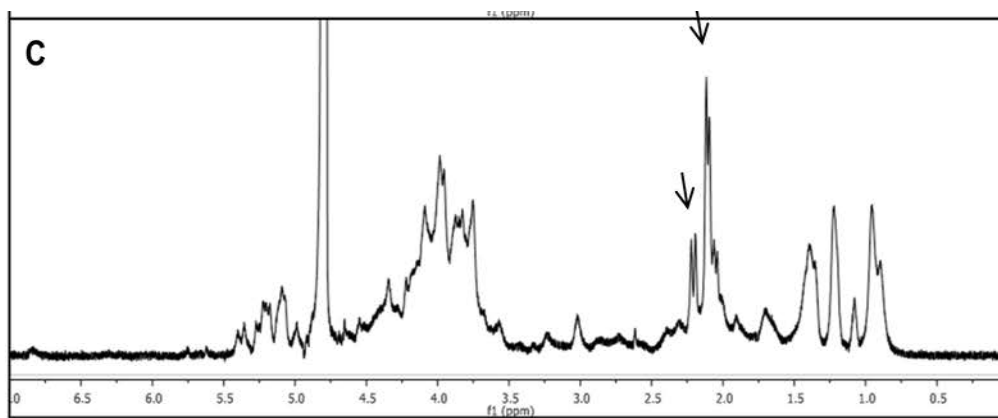

Figure 8: ^{1}H NMR of profile of a representative S. Typhimurium isolate, Ke004 OAg (arrows indicate O-acetyl groups)

Table 3: Proportion distribution in percentages of O-acetylation and glucosylation levels among NTS isolates

	O-Acetylation (n=73 *S.* Typhimurium, 44 *S.* Enteritidis)					**Glucosylation (n= 55 *S.* Typhimurium, 36 *S.* Enteritidis)**		
	% strains with O-acetyl groups			**% strains with traces or no O-acetyl groups**		**% strains with glucosylation level**		
	On both Rha & Abe	On Abe only	On Rha only	With traces of O-acetyl groups	No O-acetyl groups	<20%	20-50%	>50%
***S*. Typhimurium**	63.0	17.8	9.6	3.0	6.8	29.1	56.4	14.5
***S*. Enteritidis**	22.2			42.2	35.6	13.9	72.2	13.9

n= Total number; Rha = rhamnose; Abe =abequose

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was done on a subset of 91 isolates (55 S. Typhimurium and 36 *S*. Enteritidis) and showed that for both serovars, the majority of strains (72.2%) expressed OAg with 20-50% glucosylation levels (Figure 9, Figure 10 for *S*. Typhimurium and Figure 11 for *S*. Enteritidis, Appendix Table 5 b.i and Appendix Table 5 b.ii).

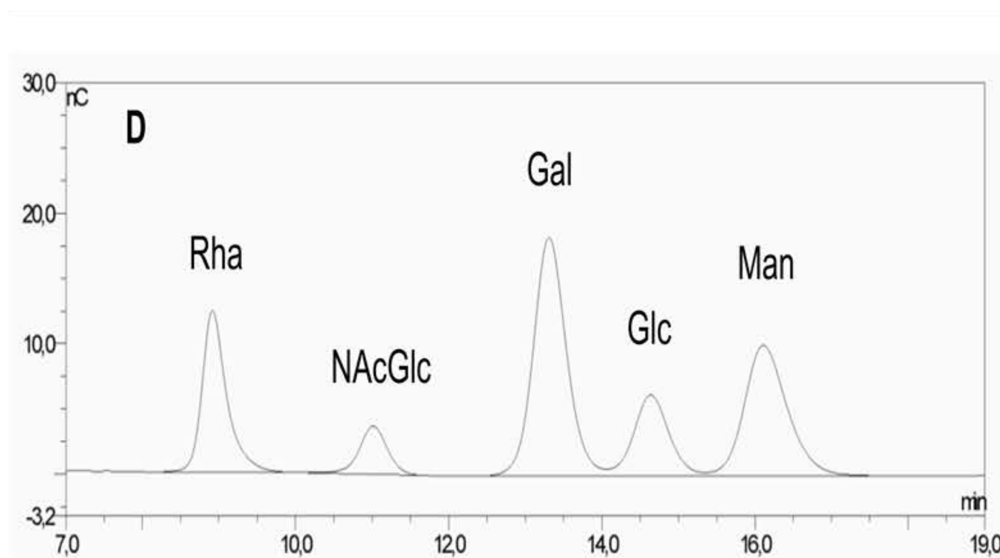

Figure 9: HPAEC-PAD profile of S. Typhimurium Ke012 OAg sample indicating typical sugars of the repeating unit (Rha, Gal, Glc and Man) and of the core region (Gal, Glc and NAcGlc) (similar profiles obtained for all NTS strains).

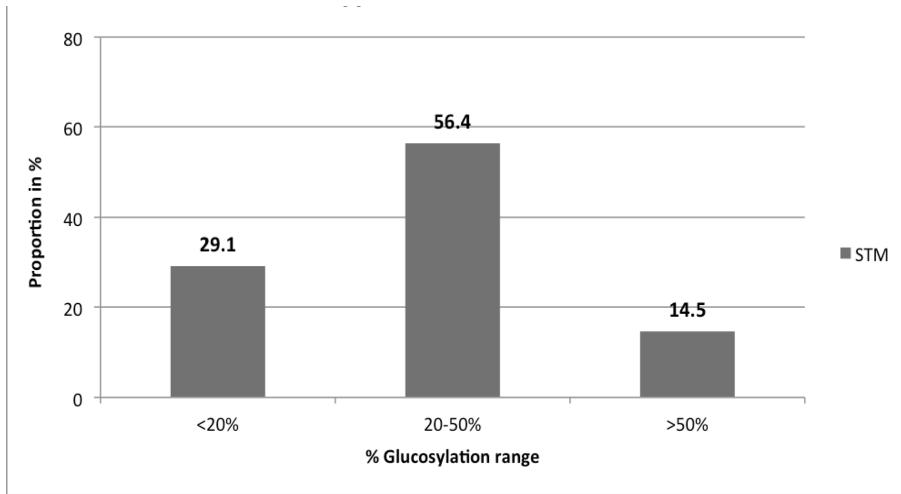

Figure 10: Proportions in percentage of glucosylation levels in Salmonella Typhimurium isolates.

Among the 55 S. Typhimurium isolates that were analysed, majority (56.4%)had a glucosylation range of between 20 - 50%. The data label on each bar of the graph represents proportion in percentage of the isolates with the given glucosylation range.

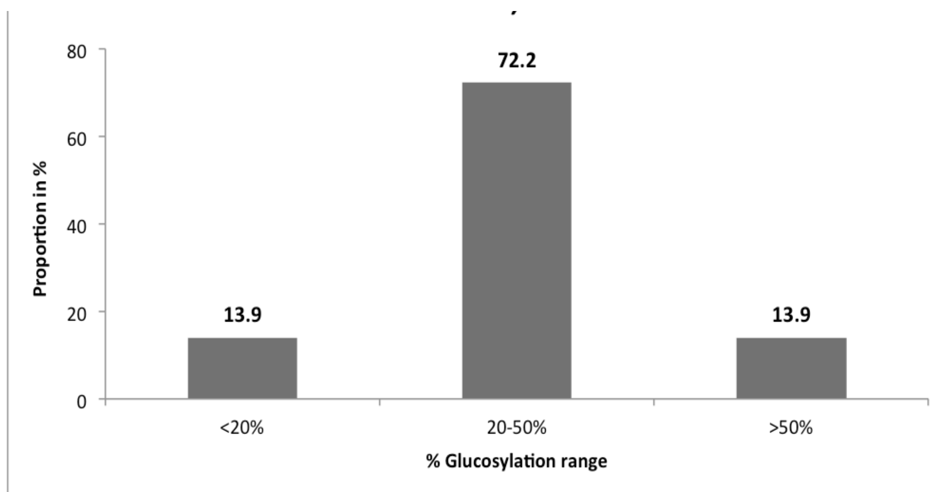

Figure 11: Proportions in percentage of glucosylation levels in Salmonella Enteritidis isolates.

Among the 36 S. Enteritidis isolates that were analysed, majority (72.2%)had a glucosylation range of between 20 - 50%. The data label on each bar of the graph represents proportion in percentage of the isolates with the given glucosylation range.

4.1.4 Analysis of associations between clinical presentation and bacterial characteristics

SBA results, OAg features and clinical presentation were compared. There was a clear inter-serovar difference, with *S.* Enteritidis isolates having more OAg than *S.* Typhimurium and being more resistant to antibody-mediated killing than *S.* Typhimurium. Among *S.* Typhimurium isolates, there was a correlation between amount of OAg and resistance to antibody-mediated killing (Spearman r = 0.29, 95% CI 0.10 to 0.45, p=0.002), Figure 12 (A), in accordance with the initial hypothesis of this study, but this was not present for *S.* Enteritidis, Figure 12 (B).

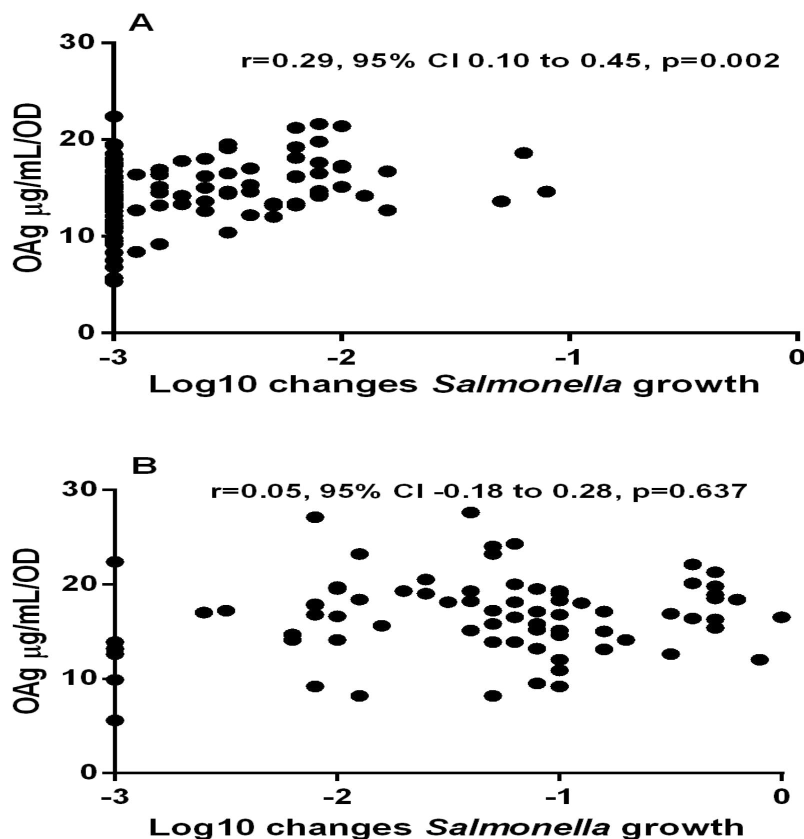

Figure 9: Correlation of SBA results and O-antigen production levels

Correlation of SBA results and OAg production levels in (A) S. Typhimurium isolates (p=0.002) and (B) S. Enteritidis isolates (p=0.637). r is the Spearman correlation coefficient. CI is confidence interval.

No correlation was found between NTS clinical presentation (invasive, gastrointestinal and carrier) and either antibody susceptibility (SBA) or polysaccharide (OAg) production, for either *S*. Typhimurium, Figure 13 (A, B) or *S*. Enteritidis isolates, Figure 13 (C, D). There was no clear correlation between O-acetylation levels/position and glucosylation levels with clinical presentation, antibody susceptibility (SBA) and OAg production in both *S*. Typhimurium and *S*. Enteritidis isolates. For *S*. Typhimurium, there was an association between lower glucosylation levels and higher OAg production (Spearman r = 0.51, 95% CI -0.69 to -0.27, p value<0.001) (Figure 14).

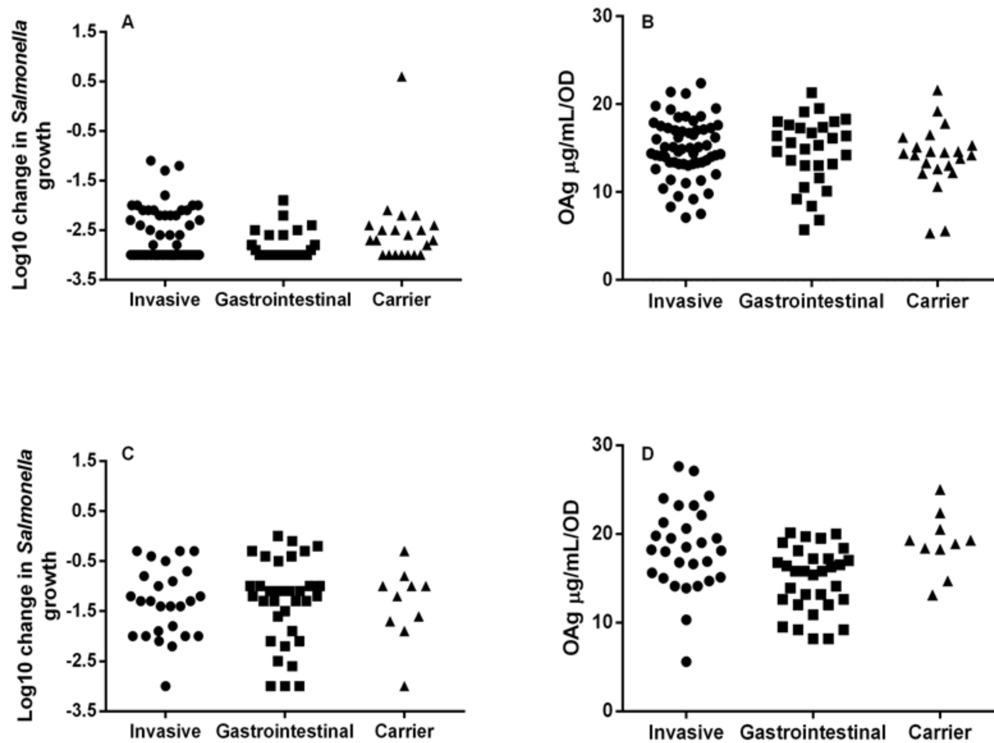

Figure 10: Correlation of SBA results and O-antigen production levels with clinical presentations in S. Typhimurium and S. Enteritidis

Correlation of SBA results (A, C) and O-antigen production levels (B, D) in S. Typhimurium (A, B) and S. Enteritidis (C, D) isolates with clinical presentations. Samples for which no clinical presentation was determined were excluded from analysis. Pooled adult human serum was used for SBA.

Invasive: Salmonella isolates from blood, urine, CSF.

Gastrointestinal: Salmonella isolates from stools.

Controls: Salmonella isolates from stools of healthy controls.

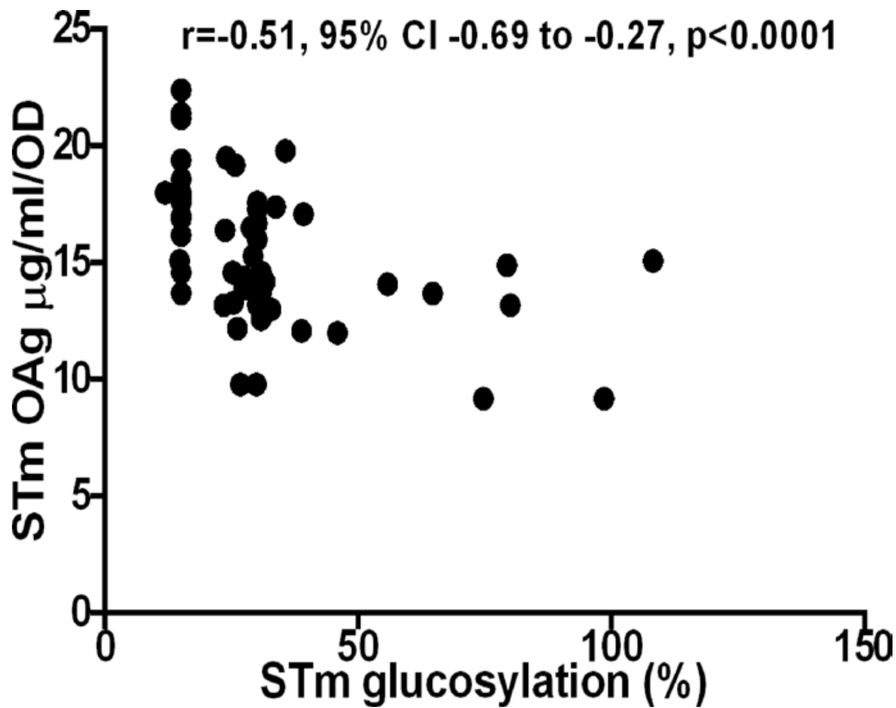

Figure 11: Correlation of S. Typhimurium (STm) OAg production levels and glucosylation (p<0.0001). r is the Spearman correlation coefficient. CI is confidence interval.

It was also observed that there was no clear correlation between O-acetylation levels/position and glucosylation levels with clinical presentation, antibody susceptibility and sugar production in both *S.* Typhimurium (Figures 15, 16 & 17) and *S*. Enteritidis (Figures 18, 19 & 20).

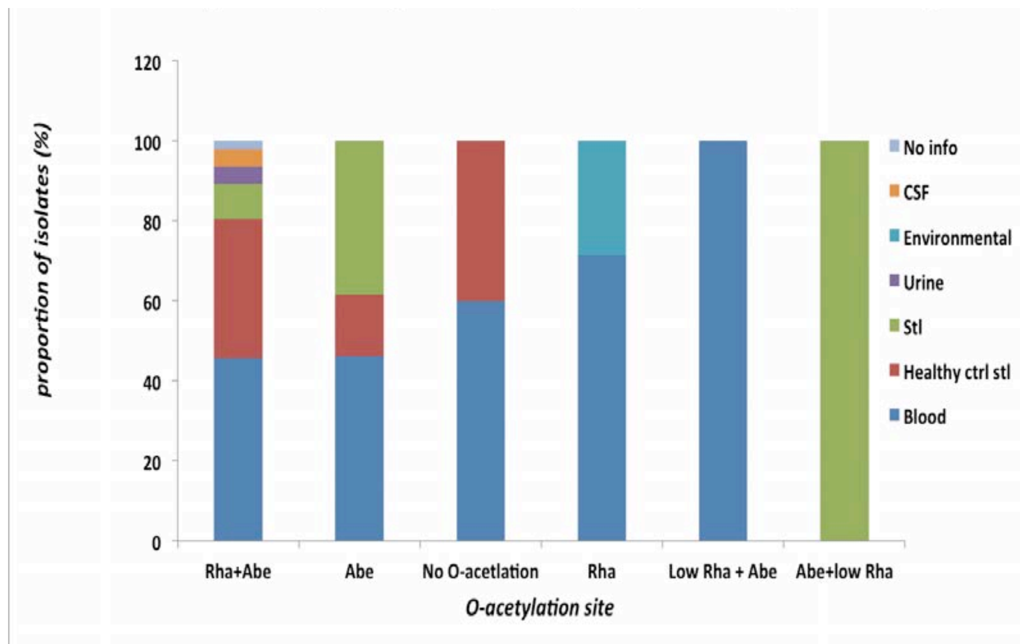

Figure 12: Comparison between O-acetylation and clinical forms in percentage proportions in Salmonella Typhimurium isolates.

A sub-set of 73 S. Typhimurium isolates were analysed. A Majority of S. Typhimurium isolates that expressed O-acetylation at various sites (Rha + Abe, Abe only and Rha only) were isolated from blood, i.e 45.7% of the 46 that expressed O-acetylation on both Rh + Abe, 46.2% of the 13 isolates that expressed O-acetylation on Abe only and 71.4% of the 7 isolates that expressed O-acetylation on Rha only. The two isolates that expressed O-acetylation on both Rha (low) + Abe and on both Abe + Rha (low) respectively were isolated from blood and stool respectively. 60% of the 5 isolates that expressed no O-acetylation were isolated from blood. There was no clear association between O-acetylation and the clinical forms of the S. Typhimurium isolates analysed.

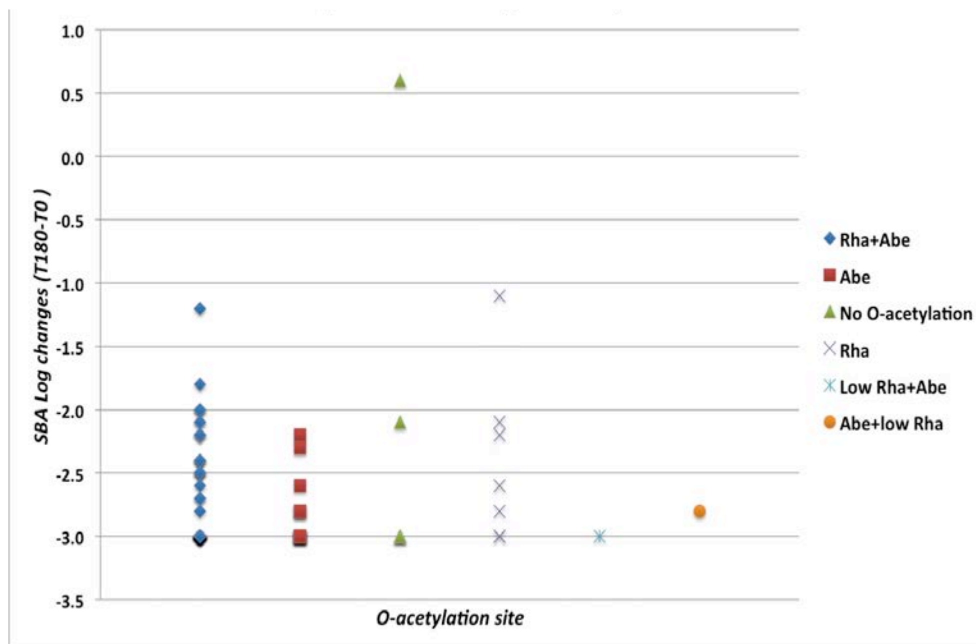

Figure 13: Correlation of O-acetylation with Serum bactericidal activity using adult human sera in Salmonella Typhimurium.

A sub-set of 73 S. Typhimurium isolates were analysed. The isolates that expressed O-acetylation on both Rha + Abe had colony forming units per millitre (CFU/ml) log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to -1.2, those isolates with O-acetylation on Abe had CFU/ml log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to -2.2, those isolates with O-acetylation on Rha had CFU/ml log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to -1.1. Two isolates with O-acetylation on both Rha (low) + Abe and on both Abe + Rha (low) respectively had CFU/ml log change (T_{180} min - T_0 min) SBA values of -3.0 and -2.8 respectively. Those isolates with no O-acetylation expression had CFU/ml log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to 0.6. There was no clear association between O-acetylation with Serum bactericidal activity using adult human sera among S. Typhimurium isolates studied.

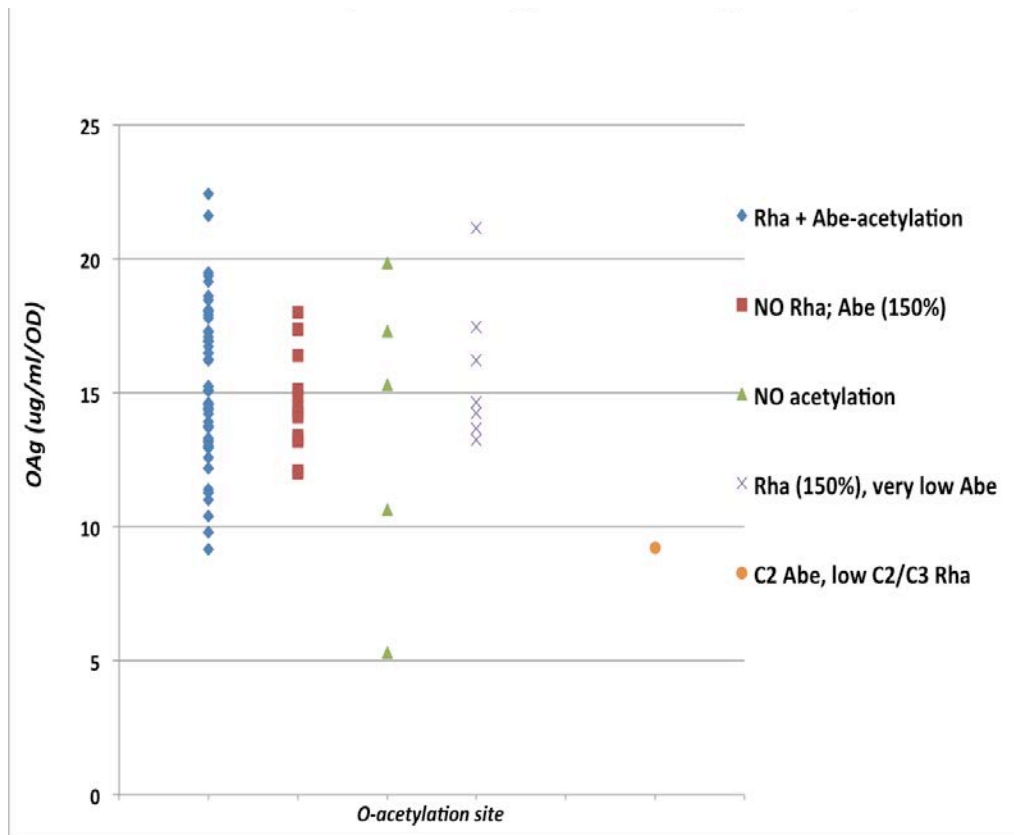

Figure 14: Correlation of O-acetylation with O-antigen production in Salmonella Typhimurium isolates.

A sub-set of 73 S. Typhimurium isolates were analysed. The isolates that expressed O-acetylation on both Rha + Abe produced O-antigen amount ranging between 17.9 µg/ml/OD - 22.4 µg/ml/OD, those with O-acetylation at Abe produced O-antigen amount ranging between 12.0 µg/ml/OD - 18.0 µg/ml/OD and those that expressed O-acetylation on Rha + Abe (low) produced O-antigen amount ranging between 13.2 µg/ml/OD - 21.2 µg/ml/OD. Those that had no O- acetylation produced O-antigen amount ranging between 5.3 µg/ml/OD - 19.8 µg/ml/OD while the single isolate that expressed O- acetylation on Abe and Rha (low) produced O-antigen amount of 9.2 µg/ml/OD. There was no clear association between O-acetylation and O-antigen production among S. Typhimurium isolates studied.

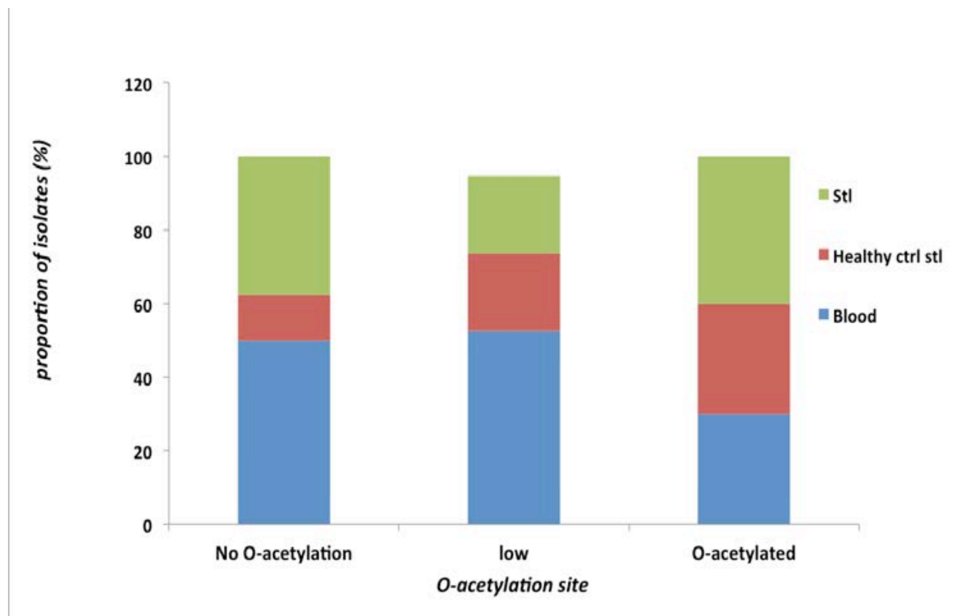

Figure 15: Comparison between O-acetylation and clinical forms in percentage proportions in Salmonella Enteritidis.

A sub-set of 45 S. Enteritidis isolates were analysed. A Majority of S. Enteritidis isolates had either low O-acetylation (42%) or no O-acetylation expression at all (36%). Only 22% of the isolates expressed O-acetylation, majority of them (40%) isolated from stool. Majority of those that expressed either low O-acetylation or no O-acetylation were isolated from blood i.e 52.6% of those that expressed low O-acetylation were isolated from blood and 50% of those that expressed no O-acetylation at all were isolated from blood. This contrasts with what was observed among S. Typhimurium isolates, where a majority of those that expressed O-acetylation were isolated from blood. There was no clear association between O-acetylation and the clinical forms of the S. Enteritidis isolates analysed, an observation that was similary made among S. Typhimurium isolates.

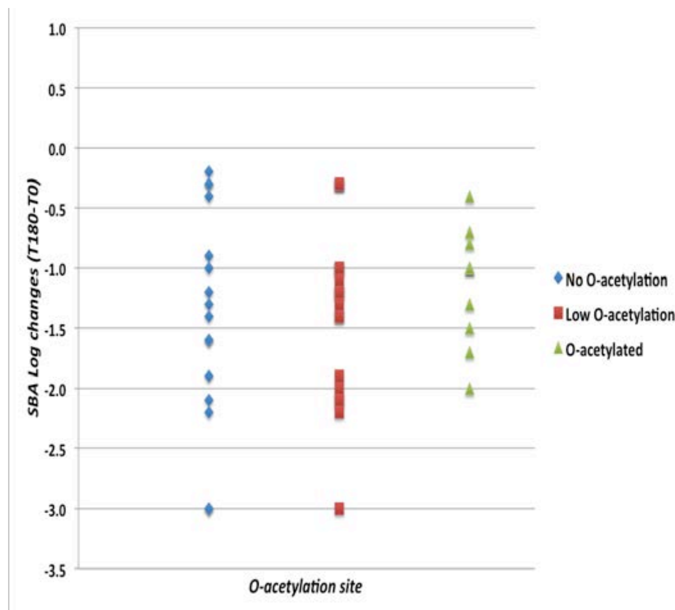

Figure 16: Correlation of O-acetylation with Serum bactericidal activity using pooled adult human sera in Salmonella Enteritidis isolates.

A sub-set of 45 S. Enteritidis isolates were analysed. The isolates that expressed no O-acetylation had colony forming units per millitre (CFU/ml) log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to -0.2, those isolates which expressed low O-acetylation had CFU/ml log change (T_{180} min - T_0 min) SBA values ranging from -3.0 to -032. Those isolates with O-acetylation had CFU/ml log change (T_{180} min - T_0 min) SBA values ranging from -2.0 to -0.4. There was no clear association between O-acetylation with Serum bactericidal activity using adult human sera among S. Enteritidis isolates studied, a similar observation that was made among S. Typhimurium isolates.

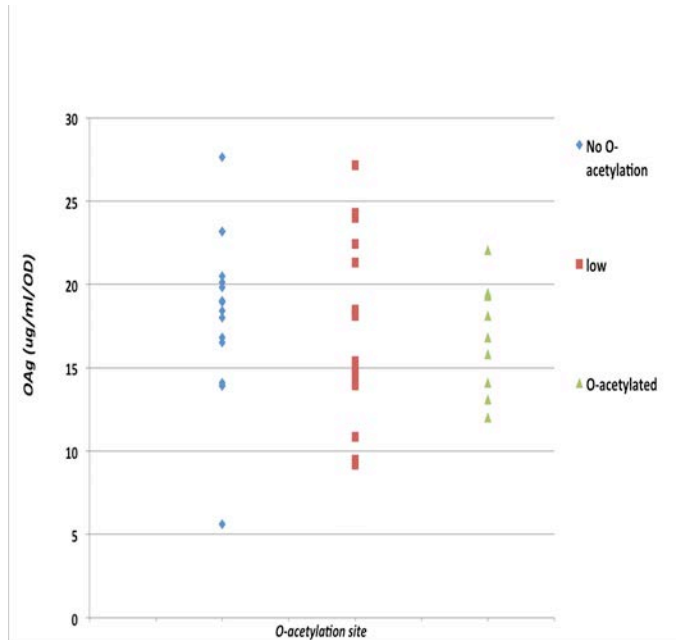

Figure 17: Correlation of O-acetylation with O-antigen production in Salmonella Enteritidis isolates.

A sub-set of 45 S. Enteritidis were analysed. The isolates hat expressed no O-acetylation produced O-antigen amount ranging between 5.6 µg/ml/OD - 27.6 µg/ml/OD, those with low O-acetylation produced O-antigen amount ranging between 9.2 µg/ml/OD - 27.1 µg/ml/OD and those that had O-acetylation produced O-antigen amount ranging between 12.0 µg/ml/OD - 22.1 µg/ml/OD. There was no clear association between O-acetylation and O-antigen production among S. Enteritidis isolates studied as was similary observed among S. Typhimurium isolates.

4.1.5 Antimicrobial Susceptibility testing: Multi-drug resistance (MDR) and resistance phenotypes

Antibiotic susceptibility test was first performed on all the 193 NTS isolates against a panel of 9 antimicrobials commonly prescribed for bacterial infections. These included ampicillin (AMP), ceftriaxone (CRO), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), tetracycline (TE), trimethoprim (W), co-trimoxazole (SXT), nitrofurantoin (F). Antibiograms were obtained for all the NTS isolates in this study (Appendix Table 6 A.i, Appendix Table 6 A.ii, Appendix Table 6 B.i and Appendix Table 6 B.ii).

The majority (77%) of the *S*. Typhimurium isolates tested were resistant to at least 3 antibiotics, and as a result were deemed multidrug resistant (MDR) (Appendix Table 4, Appendix Table 6 A.i and Appendix Table 6 A.ii). Only 3% were fully susceptible to all the 9 antibiotics used in the susceptibility testing panel (Table 4). Majority (34%) of the MDR *S*. Typhimurium isolates were resistant to 4 antibiotics (Table 4). The most common antimicrobial resistance phenotype among *S*. Typhimurium isolates was AMP-C-NA-W-SXT, with 20% (23) of *S*. Typhimurium isolates having this phenotype (Table 5).

On the other hand, of the 79 *S*. Enteritidis (Appendix Table 6 B.i and Appendix Table 6 B.ii) isolates in the study, 30% were MDR (resistant to at least 3 antibiotics) while only 8% were fully sensitive to all the 9 antibiotics tested (Table 4). A significant proportion (24%) of the MDR (resistant to at least 3 antibiotics) *S*. Enteritidis isolates were resistant to 5 antibiotics (Table 4) and the most common resistance phenotype was AMP-C-TE-W-SXT, with 24% of the *S*. Enteritidis isolates having this phenotype (Table 5). It is worthy to note that this resistance phenotype that was observed among the *S*. Enteritidis isolates (AMP-C-TE-W-SXT) differs from *S*. Typhimurium isolates whose most common resistance phenotype was AMP-C-NA-W-SXT, with resistance to TE and NA antibiotics separating the two serovars.

Overall, *S*. Typhimurium serovar had a higher proportion of isolates exhibiting antibiotic resistance, with 97% (111) of the isolates showing resistance to atleast one

antibiotic, compared to 92% (73) of the *S*. Enteritidis isolates that were resistant to atleast one antibiotic (Table 4).

Table 4: Multi-drug Resistance (MDR) distribution in percentage among NTS isolates

No. of antibiotics resisted by isolates	**Proportion in % (Frequency)**	
	***S.* Typhimurium, n=114**	***S.* Enteritidis, n=79**
0 (Fully sensitive to all 9 antibiotics tested)	3% (3)	8% (6)
1	13% (15)	32% (25)
2	7% (8)	30% (24)
3	12% (14)	1% (1)
4	30% (34)	1% (1)
5	27% (31)	24% (19)
6	5% (6)	4% (3)
7	2% (2)	0% (0)
8	0% (0)	0% (0)
9 (Resistant to all 9 antibiotics tested)	1% (1)	0% (0)

Table 5: Resistance phenotypes distribution in percentage among NTS isolates

Resistance phenotypes	**Proportion in % (Frequency)**	
	***S.* Typhimurium, n=114**	***S.* Enteritidis, n=79**
AMP-C-NA-W-SXT	20% (23)	0% (0)
AMP-C-TE-W-SXT	0% (0)	24% (19)
AMP-C-W-SXT	17% (19)	0% (0)
AMP-W-SXT	12% (14)	1% (1)
AMP-W-SXT-F	5% (6)	0% (0)
AMP-TE-W-SXT	5% (6)	0% (0)
AMP-CRO-C-TE-W-SXT	4% (5)	1% (1)
AMP-C-TE--W-SXT-F	1% (1)	3% (2)
AMP-TE-W-SXT	3% (3)	0% (0)
AMP-CRO-C-CIP-TE-W-SXT	2% (2)	0% (0)
AMP-NA-W-SXT	2% (2)	0% (0)
AMP-NA-W-SXT-F	2% (2)	0% (0)
AMP-C-W-SXT-F	1% (1)	0% (0)
AMP-CRO-W-SXT	1% (1)	0% (0)
AMP-CRO-W-SXT-F	1% (1)	0% (0)
AMP-NA-TE-W-SXT	1% (1)	0% (0)
CRO-C-NA-F	0% (0)	1% (1)
AMP-CRO-C-CIP-NA-TE-W-SXT-F	1% (1)	0% (0)
Legend: **AMP**-Ampicillin (10 µg), **CRO**-Ceftriaxone (30 µg), **C**-Chloramphenicol (30 µg), **CIP**-Ciprofloxacin (1 µg), **NA**-Nalidixic acid (30 µg), **TE** -Tetracycline (10 µg), **W**-Trimethoprim (2.5 µg), **SXT**-Trimethoprim /sulphamethoxazole 1:19 (25 µg), **F**-Nitrofurantoin (300 µg)		

4.1.6 Whole-genome based phylogenetic analyses

Sequence reads mapped to an average of 86.5% of the reference genome for *S.* Typhimurium samples, with a mean depth of 65.2-fold in mapped regions across all isolates and an average of 95.4 % of the reference genome for *S.* Enteritidis samples, with a mean depth of 89.5-fold in mapped regions across all isolates (Appendix Table 6A.i, Appendix Table 6A.ii, Appendix Table 6B.i, Appendix Table 6b.ii).

4.1.7 Accession numbers

The raw sequence data is available under the listed accession numbers at the Welcome Trust Sanger Institute (WTSI) Internal Nucleotide Archive (the full list of accession codes is given in Appendix Table 6A.i, Appendix Table 6A.ii, Appendix Table 6B.i, Appendix Table 6b.ii)

4.1.7.1 Salmonella Typhimurium: Whole genome single-nucleotide polymorphism (SNP)-based phylogenetic tree and Multi-locus sequence type (MLST) distribution

Whole genome analysis of the study isolates sequences showed that the majority (80%; 91/114) of the *S.* Typhimurium analysed belonged to the sequence type ST313 which also fell within the two lineages while 25 (22%) isolates belong to ST19 (Figure 21, Appendix Table 6 A.i and Appendix Table 6 A.ii).

A maximum-likelihood analysis of the core single nucleotide sequences (SNPs) was performed and revealed that the isolates clustered into four lineages which, for purposes of discussing findings from this study were designated as I, II, IIIA and IIIB (Figure 21). The isolates in lineage I (invasive-associated A130-like) & II (invasive-associated D23580-like) all belong to ST313 while isolates in lineage III A (gastroenteritis-associated SL1344-like) and IIIB all belong to ST19. An average of 297 SNPs separates ST313 lineages I from ST313 lineage II while an average 2,479 SNPs separates ST19 lineage IIIA from ST19 lineage IIIB.

Records from the patients clinical metadata (Appendix Table 3 a.i and Appendix Table 3 a.ii) indicates that the majority (61%) of the *S.* Typhimurium isolates within Lineage I and II were isolated from invasive-associated tissues (blood, CSF, urine).

Six isolates characterised in the study that were collected from blood belong to ST19 and map within linages IIIA and IIIB.

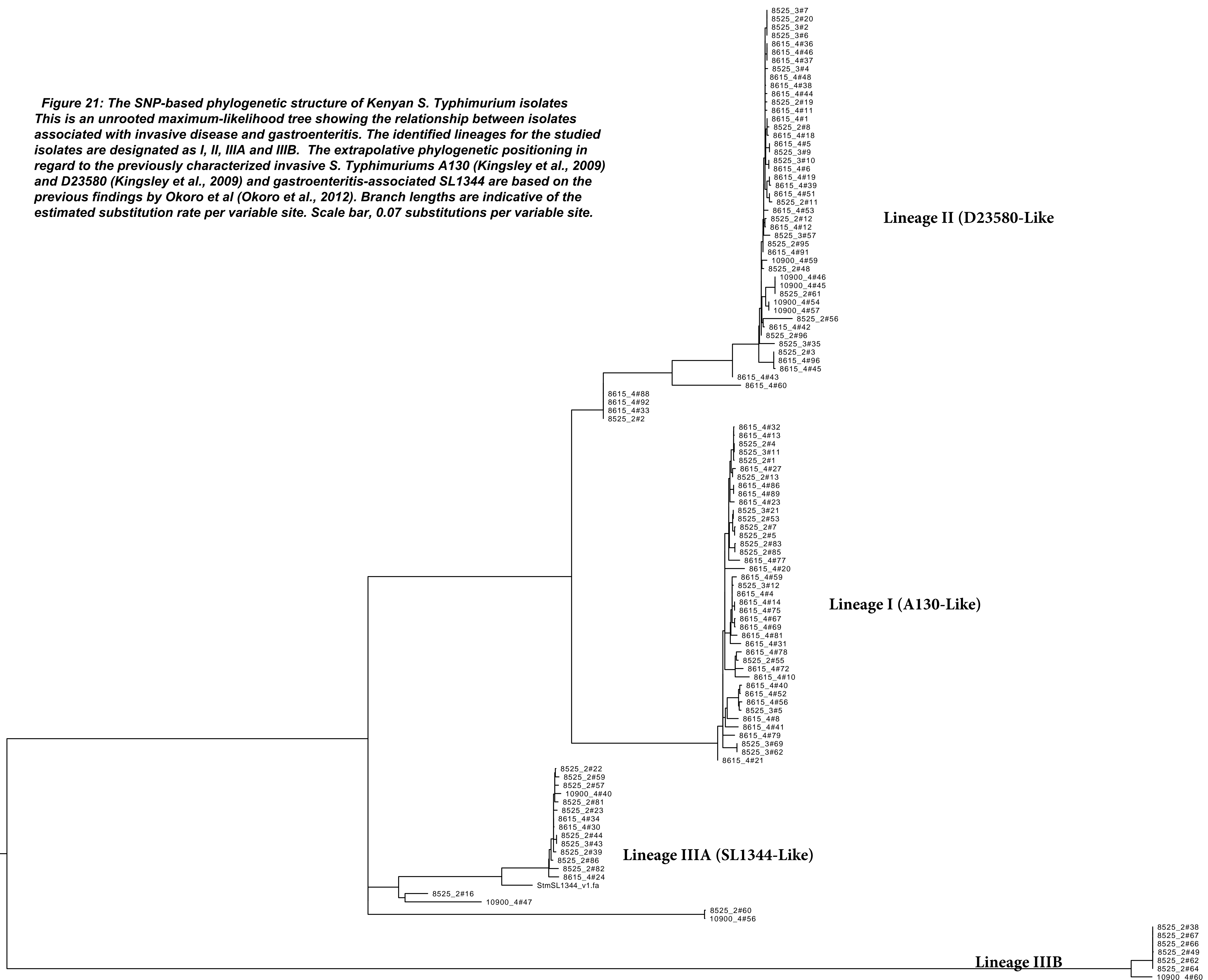

Figure 21: The SNP-based phylogenetic structure of Kenyan S. Typhimurium isolates
This is an unrooted maximum-likelihood tree showing the relationship between isolates associated with invasive disease and gastroenteritis. The identified lineages for the studied isolates are designated as I, II, IIIA and IIIB. The extrapolative phylogenetic positioning in regard to the previously characterized invasive S. Typhimuriums A130 (Kingsley et al., 2009) and D23580 (Kingsley et al., 2009) and gastroenteritis-associated SL1344 are based on the previous findings by Okoro et al (Okoro et al., 2012). Branch lengths are indicative of the estimated substitution rate per variable site. Scale bar, 0.07 substitutions per variable site.

4.1.7.2 Relationship between antimicrobial resistance and genotype: S. Typhimurium phylogeny in Kenya is defined by MDR phenotype and patient disease outcome.

The antibiotic profiles of the *S*. Typhimurium study isolates were found to be generally congruent with their phylogenetic positioning (Figure 22). Nearly all (99%, 91/92) of the ST313 isolates in lineages I and II were MDR (Figure 22, Appendix Table 6 A.i and Appendix Table 6 A.ii). Conversely, the majority (68%, 17/25) of ST19 isolates in Lineage IIIA & B are susceptible to most antibiotics in the testing panel (Figure 22, Appendix Table 6 A.i and Appendix Table 6 A.ii). Resistance against chloramphenicol was observed constrained only among *S*. Typhimurium isolates within ST313 lineages I and II (Figure 22, Appendix Table 6 A.i and Appendix Table 6 A.ii).

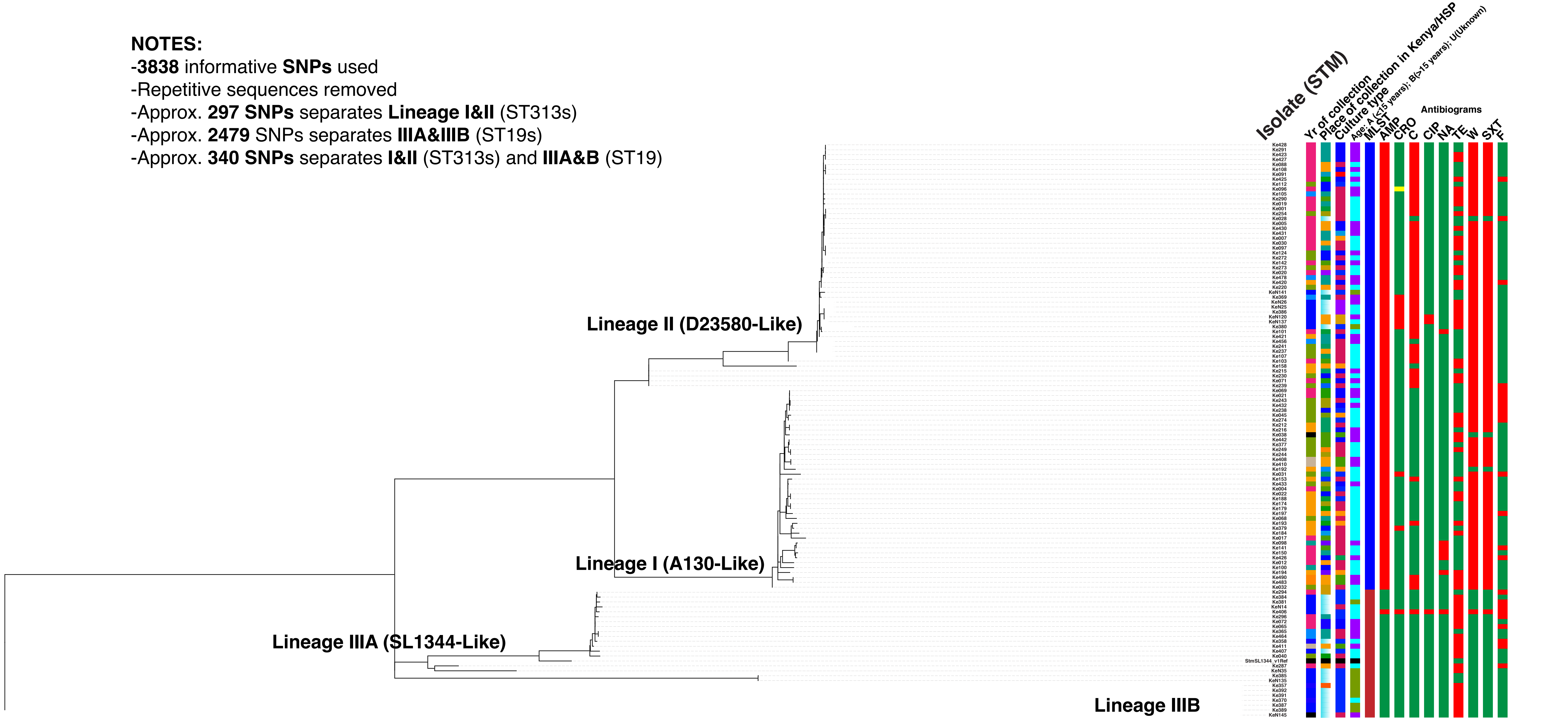

Figure 22: Associated strain metadata and antibiotic resustance profiles (red and green profiles) of S. Typhimurium isolates aligned to their phylogenetic framework Colour legends highlights isolate information (place, year and patient age), culture type, multilocus sequence type (MLST) and antibiotic resistance profiles.

4.1.7.3 Salmonella Enteritidis: Whole genome single-nucleotide polymorphism (SNP)-based phylogenetic tree and Multi-locus sequence type (MLST) distribution.

Similar MLST analysis extracted from the whole genome data showed that all the 79 *S*. Enteritidis belong to ST11 (Appendix Table 6 B.i and Appendix Table 6 B.ii). *Salmonella* Enteritidis isolates fell into two distinct and separate lineages that, for purposes of discussing findings from this study were designated as I and II (*S*. Enteritidis PT4 P125109-like) (Figure 23). The lineages are separated by approximately 2150 SNPs. Similar to what was observed in *S*. Typhimurium phylogeny, records from the patients clinical metadata (Appendix Table 3 b.i and Appendix Table 3 b.ii) indicate that 46% (11/24) of the *S*. Enteritidis strains within lineage I were isolated from blood compared to 29% (16/55) of lineage II. In addition, 46% (11/24) of patients of the samples in lineage I had invasive disease-related clinical complaints namely fever, vomiting and cough, compared to 15% (8/55) of the patients samples with similar complaints in lineage II in the available clinical records (Appendix Table 3 b.i and Appendix Table 3 b.ii).

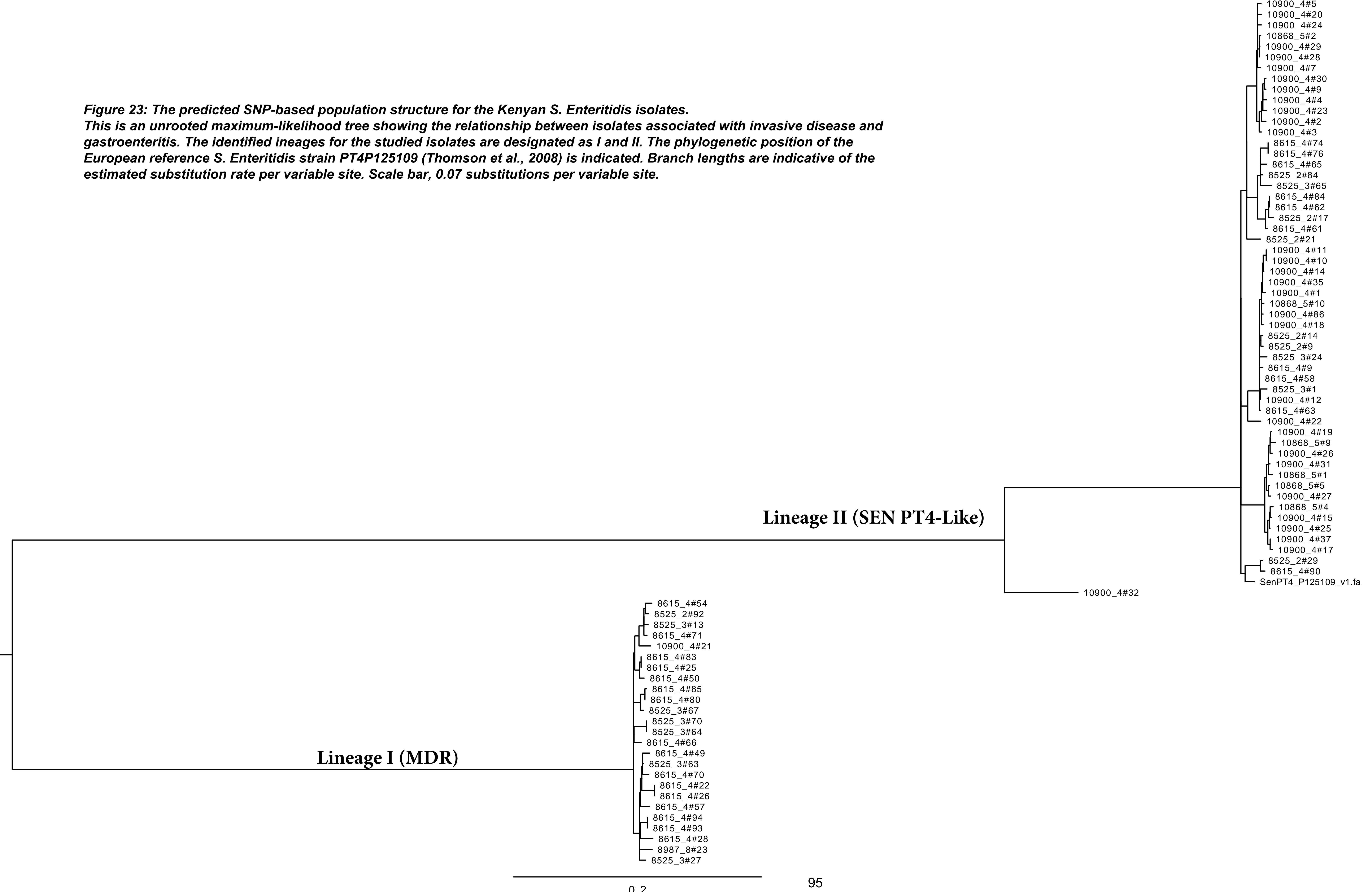

Figure 23: The predicted SNP-based population structure for the Kenyan S. Enteritidis isolates.
This is an unrooted maximum-likelihood tree showing the relationship between isolates associated with invasive disease and gastroenteritis. The identified ineages for the studied isolates are designated as I and II. The phylogenetic position of the European reference S. Enteritidis strain PT4P125109 (Thomson et al., 2008) is indicated. Branch lengths are indicative of the estimated substitution rate per variable site. Scale bar, 0.07 substitutions per variable site.

4.1.7.4 Relationship between antimicrobial resistance and genotype: S. Enteritidis phylogeny in Kenya is defined by MDR phenotype and patient disease outcome.

Similar to *S*. Typhimurium phylogeny, the majority (96%; 23/24) of *S*. Enteritidis isolates in lineage I were MDR, compared to 45% (25/55) in lineage II (*S*. Enteritidis PT4 P125109-like) which are MDR (Figure 24, Appendix Table 6B.i and Appendix Table 6B.ii). Conversely, the majority (55%, 30/55) of isolates in lineage II (*S*. Enteritidis PT4 P125109-like) were susceptible to most antibiotics in the testing panel (Figure 24, Appendix Table 6B.i and Appendix Table 6B.ii).

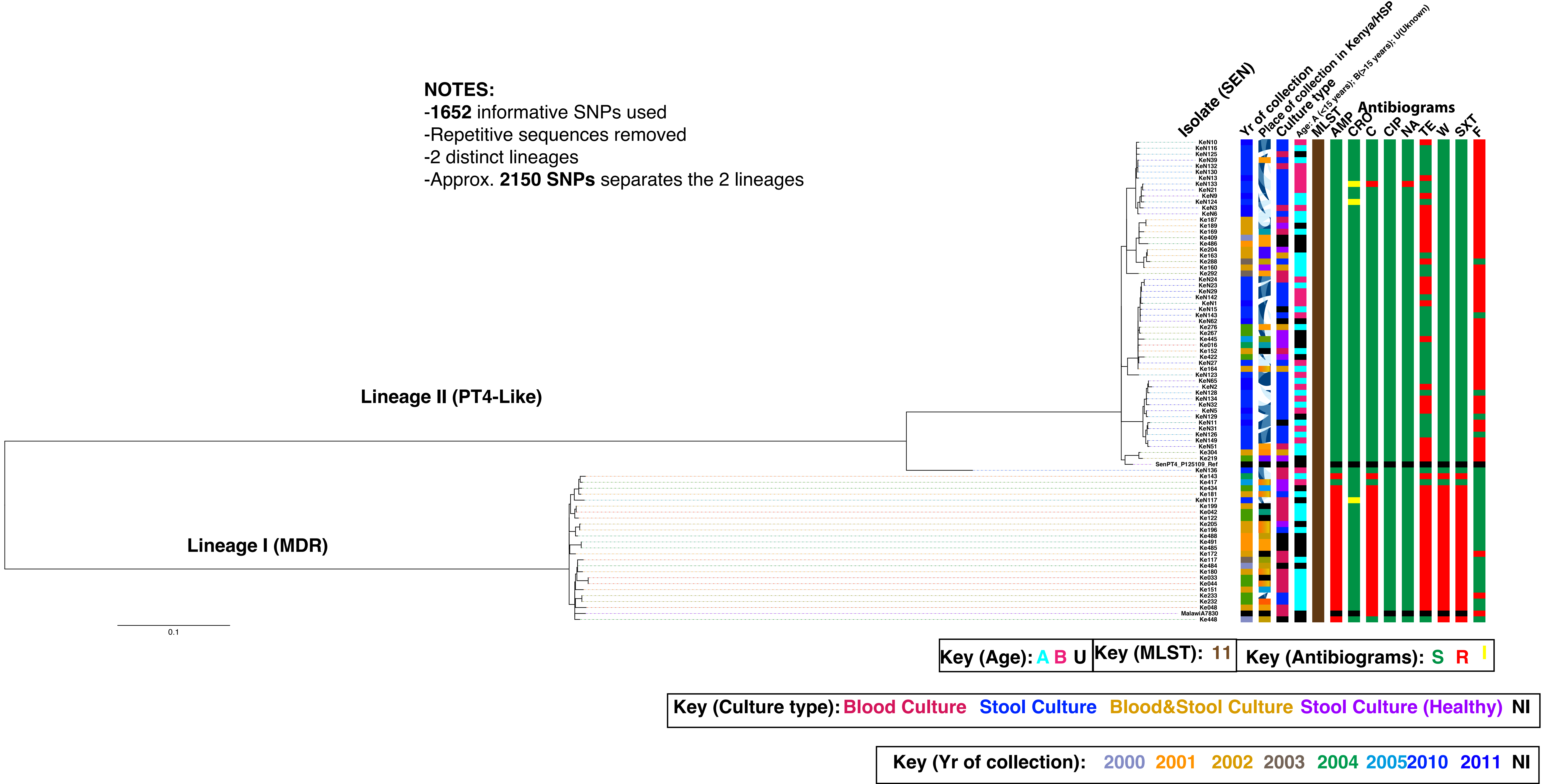

Figure 24: Assocaited strain metadata and antibiotic resistance profiles (red and green profiles) of S. Enteritidis isolates aligned to their phylogenetic framework. Colour legends highlights isolate information (place, year and patient age), culture type, MLST and antibiotic resistance profiles

4.2 Discussion

4.2.1 Introduction

Nontyphoidal *Salmonella* infections are a major problem in sub-Saharan Africa and can either present as bacteraemia, with symptoms of gastroenteritis in under half of patients, or as diarrheal disease without bloodstream infection (Feasey et al., 2012; Gordon et al., 2008; Kotloff et al., 2013). Both presentations can be life-threatening, with case fatality rates of 20-25% for iNTS bacteremia in children (Brent et al., 2006; Feasey et al., 2012; Graham et al., 2000a; MacLennan and Levine, 2013) and up to 50% in HIV-infected adults (Gordon et al., 2002), and a recognized association between NTS diarrhoea with mortality (O'Reilly et al., 2012). No vaccine is currently available.

4.2.2 Microbiological and immunological analyses

The main finding of the study is that all isolates studied, apart from one *S.* Typhimurium, were susceptible to antibody-dependent complement-mediated killing, using a serum pool from an endemic region. Previously it has been shown that the antibody response specific to NTS is acquired with age, corresponding with a decline in cases of NTS bacteraemia (MacLennan et al., 2008; Nyirenda et al., 2014). The serum pool used in the study contained high levels of both *S.* Typhimurium and *S.* Enteritidis anti-OAg antibodies as expected among healthy adults in a *Salmonella* endemic region of sub-Saharan Africa such as Kenya, Malawi or Tanzania (MacLennan et al., 2010). It has also previously been demonstrated that this killing is through the antibody-dependent complement-mediated mechanism and requires the presence of specific antibodies and intact complement function (MacLennan et al., 2008). The study therefore confirms the bactericidal activity of anti-*Salmonella* antibodies against the vast majority of endemic NTS isolates in the current study. These findings support an important role for antibodies in protective vaccines against iNTS disease (Goh and MacLennan, 2013; O'Shaughnessy et al., 2012; Rondini et al., 2013).

Another key finding is the difference in relationship between susceptibility to antibody-mediated killing and levels of OAg expression for the two *Salmonella* serovars studied. According to the study hypothesis, decreased susceptibility of *S.* Enteritidis compared with *S*. Typhimurium could be the result of higher levels of OAg, not excluding the contributions of other pathogen features, not investigated here. A previous report from Malaysia found that *S.* Enteritidis isolates were more often isolated from the blood than stool of patients compared with other *Salmonella* serovars (Dhanoa and Fatt, 2009), but the reasons for this were not explored. Considering that OAg length and density play a role in pathogen virulence (Joiner et al., 1986), increased levels of OAg expression may enhance the ability of *S.* Enteritidis isolates to evade host first line defence mechanisms and gain access to the bloodstream. It has also been reported that, when exposed to serum, *S*. Typhimurium upregulates genes for increased production of very long chain OAg to escape complement-dependent killing (Murray et al., 2006). The finding in this study of a correlation between OAg expression and resistance to antibody-mediated killing among *S*. Typhimurium isolates is consistent with the hypothesis that high OAg expression protects against such killing. It is also well known that strains lacking OAg are rapidly eliminated from the host (Moller et al., 2003; Nevola et al., 1987), and there were no such isolates in the study collection. However, the lack of correlation between OAg expressed by *S.* Enteritidis isolates and susceptibility to antibody-mediated killing does not support the study hypothesis for this serovar concerning OAg levels and resistance to antibody killing. This finding suggests that other inherent factors of *Salmonella,* particularly *S.* Enteritidis, have a role in determining loss of sensitivity. In other words, quantity of OAg is more important for resistance to antibody-killing of *S*. Typhimurium than *S.* Enteritidis. Further studies are required to better understand the factors contributing to the inherent resistance of *S.* Enteritidis.

In relation to OAg fine specificity characterization of the study isolate collection, O-acetylation analyses revealed that *S*. Typhimurium were more heterogeneous than *S.* Enteritidis. OAg O-acetylation occurred in almost all *S*. Typhimurium isolates, and

most often at two different sites: C-2 Abe, known to provide factor O:5 specificity and C-2/C-3 Rha. Rha O-acetylation was first reported by Wollin et al (Wollin et al., 1987) and this has previously been reported as part of the characterisation of an invasive Malawian *S*. Typhimurium isolate (Micoli et al., 2014). The extent to which this additional O-acetylation was widespread in the study collection suggests a possible role in pathogenesis that should be investigated further. Moreover, the fact that *S*. Typhimurium isolates with either Abe- or Rha-O-acetylation only could be identified in the study, shows that O-acetylation at both sites can occur independently. A *S*. Typhimurium OAg-based vaccine for Africa would need to induce antibodies capable of binding to both OAg specificities. In contrast, O-acetylation of *S.* Enteritidis OAg was not as common, with more than two-thirds of the isolates lacking or having very low levels of O-acetylation.

Glucosylation is another OAg modification for which variation has been described for *S*. Typhimurium and can occur on the O:12 antigen galactose (C4 position) generating the 12-2 variant, and on the O:1 antigen galactose (C6 position) (phage-determined) (Makela, 1973). It has been reported that O:12 glucosylation in Typhimurium is not constitutive, occurs during intracellular macrophage growth and is associated with enhanced intestinal colonization (Bogomolnaya et al., 2008). All *S*. Typhimurium isolates in the study collection were glucosylated, with less than 30% of them at levels < 20%, showing that glucosylation is a very common feature among endemic strains. Similarly, 86% *S.* Enteritidis isolates analysed in this study showed a glucosylation level of >20%. Glucosylation in *S.* Enteritidis is not associated with specific OAg factors and it has not been extensively studied. A report on *S.* Enteritidis food-borne salmonellosis strains showed glucosylated LPS in some mouse isolates, closely related to strains obtained from eggs (Parker et al., 2001), but not in archived *S.* Enteritidis strains.

In *Shigella*, LPS glucosylation promotes bacterial invasion by enhancing type III secretion system (T3SS) function and is associated with reduced LPS chain length (West et al., 2005). In *Salmonella*, T3SS is necessary for invasion of intestinal epithelium and survival within macrophages and further investigation into different

glucosylation levels and T3SS-mediated invasion is still to be performed. The inverse correlation between *S*. Typhimurium OAg glucosylation and OAg expression observed in the current study may be related to T3SS function, as previously reported for *Shigella* (West et al., 2005) and *S*. Typhimurium (Holzer et al., 2009). *Salmonella* isolates defective for the synthesis of long and very long OAg species have been shown to have an increased translocation of a *Salmonella* pathogenicity island 1 (SPI1)-T3SS effector protein promoting invasion. Therefore, also for *Salmonella,* there may be the need to balance T3SS activity, possibly enhanced by shorter and glucosylated LPS, and protection against innate immune effectors, which is favoured by a thicker LPS layer (Joiner et al., 1986; Murray et al., 2006).

The reasons for NTS disease presenting as invasive or enteric disease are not well understood and could relate to host immunity and/or pathogen virulence. Epidemiological data indicate a frequent presentation of the invasive syndrome, without diarrhoea symptoms, in younger age groups (Kariuki et al., 2006a), often associated with other infections such as malaria and HIV (MacLennan, 2012; Gordon, 2008; Biggs et al., 2014). However, specific investigations on the relationship between invasive and diarrheal disease are extremely limited, partially due to the limited microbiological resources in endemic African countries and few data are available on contemporaneous presence of NTS in stool and blood samples (Onsare and MacLennan, 2014). Another problem is that the concentration of *Salmonella* in the blood of bacteraemic patients can be as low as 1 CFU/ml (Tennant et al., 2011) and blood culture sensitivity is estimated to be about 50% (Tennant et al., 2011).

The lack of obvious difference between phenotype of NTS isolates from invasive and gastrointestinal disease episodes is consistent with the concept that invasive NTS isolates are able to cause diarrhoea and *vice versa*. If true, this would implicate host immunity as the major determinant of NTS disease presentation in endemic sub-Saharan African countries. Other previous studies (Kariuki et al., 2006a) have not managed to identify specific pathogen determinants for either invasive or

gastrointestinal syndromes, therefore the interplay between host and pathogen may be key.

4.2.3 Multi-drug resistance in Nontyphoidal *Salmonella*

Many studies have shown that multidrug resistance is a major health challenge in sub-Saharan Africa and globally (Bronowski et al., 2013; Lunguya et al., 2013). *Salmonella* Typhimurium and *S*. Enteritidis are increasingly multiply antimicrobial resistance to commonly prescribed antibiotics and such MDR isolates have been reported in various Sub-Saharan African countries including Kenya (Kariuki et al., 2006b), Malawi (Bronowski et al., 2013) and DR Congo (Lunguya et al., 2013). The finding in the present study that a vast majority (77%, 88/114) of the tested *S*. Typhimurium and 30% (24/79) of *S*. Enteritidis isolates were MDR confirms widespread MDR among NTS isolates from Kenya. In addition the finding in the present study that, overall, *S*. Typhimurium serovar had a higher proportion of isolates exhibiting antibiotic resistance to atleast one antibiotic (97%, 111/114) compared to *S*. Enteritidis isolates (92%, 73/79) is in agreement with other studies in sub-Saharan Africa that have reported the same trend (Lunguya et al., 2013). The observed difference in resistance between *S*. Typhimurium and *S.* Enteritidis confirms data from Malawi (Gordon et al., 2008). The antibiotic resistance profile observed among the NTS isolates from Kenya in this study is similar to the ones described previously in Togo (Dagnra et al., 2007), in Ghana, Kenya, Malawi and Mozambique (Gordon et al., 2008; Mandomando et al., 2009; Nielsen et al., 2012; Tabu et al., 2012), although no 3rd generation cephalosporin resistance was observed in the later four studies. In Nigeria on the other hand, resistance in *S*. Enteritidis appears to be more elevated (Akinyemi et al., 2007). As suggested by authors of these publications, acquisition of MDR and increased prevalence observed in the present study may suggest competition for an ecological transmission niche between these two serovars. In the present study, the most common resistance phenotype among *S.* Typhimurium isolates was AMP-C-NA-W-SXT (Ampicilin - chloramphenicol - nalidixic acid - trimethoprim - trimethoprim/sulphamethoxazole) while that for *S*. Enteritidis isolates was AMP-C-TE-W-SXT (Ampicilin -

chloramphenicol - tetracycline - trimethoprim - trimethoprim/sulphamethoxazole), with resistance to TE and NA antibiotics differentiating the two serovars. This demonstrates existence of co-resistance against first-line antibiotics ampicillin (AMP), chloramphenicol (C) and trimethoprim/sulphamethoxazole (SXT) thus explaining the observed emerging resistance among antibiotics such as fluoroquinolones or third generation cephalosporins used as alternative drugs of choice which, unfortunately are threatened by increasing resistance. Many factors may have selected for the observed higher resistance of the study NTS isolates against the antibiotics tested. Possible factors include increased use of first line antibiotics, both overuse and misuse/abuse of the antimicrobial agents in both human and veterinary practices, the presence of antimicrobials in the environment – in healthcare, agriculture and industrial settings which has been associated with the survival of bacterial strains with mutations which confer resistance among other factors. Antibacterial resistance–associated infections are known to increase morbidity, mortality, and cost of treatment, and to potentially put others in the community at higher risk of infections (Kariuki and Dougan, 2014).

4.2.4 Genomic analyses and antimicrobial susceptibility profiles

Data from whole genome analysis of the study isolates sequences are in line with previous observations from the sub-Saharan Africa region. The phylogenetic structure of *S.* Typhimurium isolates from Kenya collected over a decade was determined by whole genome sequence analysis and they were found to be similar to a previously published population structure of a geographically diverse invasive *S.* Typhimurium from across sub-Saharan African (Okoro et al., 2012). This implies that invasive NTS disease in Kenya may mirror what is reported in several different countries in the sub-Saharan African region.

A maximum-likelihood analysis of the core single nucleotide sequences (SNPs) performed exactly as previously described (Okoro et al., 2012) revealed that ST19 pathotype is a common cause of gastroenteritis in the sub-Saharan Africa region, an observation that has not previously been made in similar studies. The results from this study revealed that an average of 297 SNPs separates ST313 lineages I from

ST313 lineage II while an average 2,479 SNPs separates ST19 Lineage IIIA from ST19 Lineage IIIB. This implies that ST19 lineage isolates are more diverse compared to ST313 lineage isolates. Again, this analysis holds up well with previous analysis but defines two new ST19 lineages circulating within Kenya.

Records from the patients clinical metadata (Table 3 a.i and Table 3 a.ii of Appendix 3) indicates that the majority (61%) of the *S*. Typhimurium isolates within Lineage I and II were isolated from invasive-associated tissues (blood, CSF, urine). This confirms that the *S*. Typhimurium isolates in lineages I and II are associated with invasive NTS disease. This is consistent with the previous findings that the majority of *S*. Typhimurium isolates implicated in invasive disease in sub-Saharan Africa comprised mainly two closely related ST313 lineages, more closely related to each other than other *S*. Typhimurium. Interestingly, 6 isolates characterised in the study that were collected from blood belong to ST19 and map within linages IIIA and IIIB. While additional clinical presentations data for most of these 6 isolates is not available, from information available in the patients' records (Table 3 a.i and Table 3 a.ii of Appendix 3), these pateints from where the six diarrheal *S*. Typhimurium strains were isolated from had complaints associated with febrile illness namely, cough, fever, respiratory distress and fever, general body weakness complaints. While this presentation is relatively novel, this supports a premise that other *S*. Typhimurium lineages are potentially capable of establishing opportunistic invasive disease in immunocompetent individuals, as reported in previous studies conducted outside of the sub-Saharan region.

Compared to *S*. Typhimurium, less phylogenetic diversity was observed among *S*. Enteritidis isolates as demonstrated by MLST analysis extracted from the whole genome data that showed that all the 79 *S*. Enteritidis belongs to one sequence type, ST11 and clustered into two distinct and separate lineages that were designated as lineage I and II. Similar to what was observed in *S*. Typhimurium phylogeny, information from patients' clinical metadata records indicating that 46% of the *S*. Enteritidis strains within lineage I were isolated from blood compared to 29% of lineage II suggests that *S*. Enteritidis isolates in lineage I could be more associated

with invasive NTS disease. In addition, the patients' clinical metadata indicated that 46% of patients of the samples in lineage I had invasive disease-related clinical complaints namely, fever, vomiting and cough compared to 15% of the patients samples with similar complaints in lineage II in the available clinical records. There are ongoing studies investigating the population structure of a collection of *S*. Enteritidis isolates from various countries in sub-Saharan Africa (Feasey, 2013). However, based on the observation made in the phylogeny of *S*. Typhimurium isolates from Kenya, it is possible to speculate that the phylogenetic structure of *S*. Enteritidis isolates from Kenya may also mirror the *S*. Enteritidis disease condition in other parts of the sub-Saharan Africa region.

Findings from whole-genome analyses and antimicrobial susceptibility tests in this study demonstrate a strong association between antimicrobial susceptibility profiles and population structure of both *S*. Typhimurium and *S*. Enteritidis isolates.

The antibiotic profiles of the *S*. Typhimurium study isolates were found to be generally congruent with their phylogenetic positioning with nearly all (99%, 91/92) of the ST313 isolates in lineages I and II being MDR. Conversely, the majority (68%, 17/25) of ST19 isolates in lineage IIIA & B were found to be susceptible to most antibiotics in the testing panel.

Consistent with this, but in a contrasting manner, while in the previous study isolates within lineage II (invasive-associated D23580-like) were resistant to chloramphenicol, data from this study show that all the samples within both ST313 lineages I (invasive-associated A130-like) and II (invasive-associated D23580-like) were resistant to chloramphenicol. The acquisition of chloramphenicol resistance may have afforded *S*. Typhimurium isolates in the invasiveness-associated lineages I & II a greater opportunity to survive treatment and hence be able to be transmitted easily thus contributing to their observed clonal replacement. Strong evidence collected in previous studies indicates that, at least in the case of *S*. Typhimurium, antimicrobial resistance to chloramphenicol has been important in driving the spread of *Salmonella* pathovars across Africa.

The *S*. Typhimurium pathotype ST313 dominates among multi-drug resistant *S*. Typhimurium study isolates, a majority of them primarily associated with invasive disease and febrile illness in Kenya. Multilocus sequence type (ST) 313 is geographically confined to sub-Saharan Africa and represents the predominant ST-type among invasive *S*. Typhimurium strains in Malawi and Kenya (Kingsley et al., 2009). ST313 genetically differs from other STs within the serogroup by a degraded genome capacity – a feature which is also noted among *S*. Typhi and *S*. Paratyphi and which suggest adaptation of ST313 to the human host (Kingsley et al., 2009). It was previously hypothesized that ST313 lineage II that is circulating in East-Africa originated in DR Congo, it is interesting to note that *S*. Typhimurium isolates in lineage I and II belong to ST313 pathotype and were all resistant to chloramphenicol thus further illustaring the dominance of ST313 pathotype among multi-drug resistant *S*. Typhimurium isolates.

Similar to *S*. Typhimurium phylogeny, a strong association was observed between antimicrobial susceptibility and the population structure of *S*. Enteritidis isolates as demonstrated by the majority of isolates in lineage I being MDR, compared to those in lineage II. The high level of MDR among lineage I isolates typifies the reported high antimicrobial resistance against NTS isolates in sub-Saharan Africa (Kariuki and Dougan, 2014). Conversely, majority of *S*. Enteritidis isolates in lineage II were susceptible to most antibiotics in the testing panel.

Indeed this and other published studies (Okoro et al., 2012) implies that antimicrobial resistance is a key player influencing the persistence, disease outcome and pattern of transmission and spread of both *S*. Typhimurium and *S*. Enteritidis isolates.

Previously, even with very high resolution molecular approaches, up to and including MLST, it was not possible to establish whether two isolates are epidemiologically related without an appreciation of the population diversity and structure of the bacterium in question (van Belkum et al., 2001). However, the results in this study shows that it is now possible, through the application of whole-genome phylogenetic

methods to investigate the population structure of the isolates in order to gain deeper insight into the epidemiology of NTS in Kenya. The SNP -based phylogenetic analyses applied in this study has made it possible to investigate the activities of the genotypes and prepare the ground for further studies involving functional genomics. These insights contribute to the ongoing efforts of clearer understanding of NTS pathogenicity and epidemiology, important in disease surveillance and vaccine design efforts.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSIONS, RECOMMENDATIONS AND OUTPUT OF THE STUDY

5.1 Summary

In this study, a bacterial collection of 193 NTS isolates (114 *S*. Typhimurium and 79 *S*. Enteritidis) derived from patients presenting to three main Kenyan hospitals in Nairobi and from 31 healthy carriers related to patients were analysed. The primary aim of the study was to characterise NTS isolates circulating in endemic areas by both immunological and whole-genome methods, with the goal of identifying bacterial features that may be related to either invasive or gastrointestinal pathology.

It was hypothesised that invasive isolates would, by nature, be more resistant to survival in the blood than gastrointestinal isolates, and focused the analysis on the susceptibility of isolates to antibody-mediated killing and characterization of the O-antigen (OAg) expressed by the various isolates.

The total amount of sugar expressed by the various isolates and OAg specific characteristics such as molecular weight (MW), O-acetylation and glucosylation levels were investigated. Additionally, the sensitivity of the isolates to antibody killing was tested, using pooled human serum from healthy addults from Malawi, an African country where iNTS disease is endemic. The results obtained were compared with clinical presentations to identify possible associations.
To complement immunological investigations and in order to provide insights into the genomic variation of NTS in Kenya, whole-genome based phylogenetic methods were employed to deduce the population structure of the study isolates to identify any unique genomic features that characterize the NTS isolate. Antimicrobial susceptibility testing was carried on the NTS isolates and their antibiograms compared with the genomic data.

5.2 Conclusions

Kenyan NTS isolates from patients with bacteraemia and gastroenteritis were susceptible to antibody-mediated killing. This supports the development of an antibody-inducing vaccine against NTS for Africa. *S.* Enteritidis were generally less susceptible to killing than *S.* Typhimurium isolates and expressed higher levels of OAg. However, while OAg expression correlated with antibody resistance for *S.* Typhimurium isolates, no correlation could be found for *S.* Enteritidis, supporting a role for other inherent bacterial factors in conferring resistance to antibody killing for this *Salmonella* serogroup. Serovar- and strain-specific differences in OAg expression and fine specificity were found. Those features could not be correlated to clinical presentation, so the same strains may be able to cause both invasive disease and diarrhoea.

Whole genome SNP-based phylogenetic trees of both *S.* Typhimurium and *S.* Enteritidis in Kenya are defined by an MDR phenotype thus implying that antimicrobial resistance is a key player influencing the persistence, disease outcome and pattern of transmission and spread of NTS. A vast majority (77%, 88/114) of *S.* Typhimurium and a substantial proportion of *S.* Enteritidis (30%, 24/79) isolates tested were MDR, with *S.* Typhimurium isolates having a higher proportion of isolates exhibiting antibiotic resistance to atleast one antibiotic (97%, 111/114) compared to *S.* Enteritidis isolates (92%, 73/79). This confirms widespread MDR among NTS isolates from Kenya.

Whole genome SNP-based phylogenetic trees of both *S.* Typhimurium and *S.* Enteritidis isolates collected over a decade in Kenya was determined and was found to be similar to a previously published population structure of a geographically diverse invasive *S.* Typhimurium from across sub-Saharan African thus implying that invasive NTS disease in Kenya may mirror what is reported in several different countries in the sub-Saharan African region. The finding that *S.* Typhimurium isolates from diarrheal samples can be associated with febrile invasive illness implies that it is possible that other *S.* Typhimurium lineages (ST19 lineages), previously associated mainly with gastroenteritis are potentially capable of establishing

opportunistic invasive disease in immunocompromised individuals, as reported in studies conducted outside of the sub-Saharan region. The SNP-based phylogenetic analyses applied in this study has made it possible to investigate the activities of the genotypes and prepare the ground for further studies involving functional genomics. These insights contribute to the ongoing efforts of clearer understanding of NTS pathogenicity and epidemiology, important in disease surveillance and vaccine design efforts.

5.3 Recommendations

From the study findings, Kenyan NTS isolates from patients with bacteraemia and gastroenteritis were found to be susceptible to antibody-mediated killing. Efforts should therefore be made to pursue development of an antibody-inducing vaccine against NTS for Africa.

More in-depth whole genome analysis of invasive and gastrointestinal NTS isolates from Africa are needed to provide additional insights regarding whether these strains are the same or different (Onsare and MacLennan, 2014). This will require a prospective study that recruit patients with bacteremia and gastroenteritis, investigate the presence of NTS in blood and stool of all participants, and use whole-genome based methods to characterize the isolates. This will provide further understanding of the relationship between African invasive and diarrhogenic NTS disease.

Close surveillance of NTS and their microbial resistance patterns is recommended to follow-up on emerging resistance, for which this study provides important baseline insights so as to prevent outbreaks, and to further rationalize therapy.

5.4 Limitations of the study

There were limitations to the current study: categorization of isolates as “invasive” or “gastroenteric” was based on clinical presentation, as confirmed by microbiological analysis. Blood and stool samples belonging to the same patient were rarely collected, making it impossible to exclude that bacteria from bacteremic patients were not also present in stool and vice versa (Onsare and MacLennan, 2014).

Another limitation was the lack of patients' outcome data, which could give further insights into possible differences in serovar- and isolate pathogenicity.

5.5 Output, application and dissemination of the study findings

1. PhD thesis: The key findings from the study have been developed in the form a dissertation for the award of a PhD degree of JKUAT for the consumption of educators, clinicians, research scientists and any other readership audience that may find it relevant.
2. Abstracts and presentations at conference/workshops: As a way of disseminating information from the study abstracts and presentations to the scientific community, some of the findings were presented at the following conferences:
   a) Title of meeting: GENDRIVAX Meningococcus/Salmonella 2013 Conference
      Venue: Accra, Ghana
      Date: 9-10th December 2013
      Role: Attendance and a PowerPoint oral presentation.
   b) Title of meeting: 6th Annual Novartis Vaccines PhD Students Workshop
      Venue: Siena, Italy
      Date: 20th - 21th November 2013.
      Role: Attendance and a PowerPoint oral presentation.
   c) GENDRIVAX Salmonella/Meningococcus 2012 Conference
      Venue: Jacaranda Hotel in Nairobi, Kenya
      Date: 8th - 10th December 2012.
      Role: Attendance and a PowerPoint oral presentation.
3. Journal publications: The key findings from the study have also been published in the following peer-reviewed international journals:

a) **ONSARE, R. S**., MICOLI, F., LANZILAO, L., ALFINI, R., OKORO, C. K., MUIGAI, A. W., REVATHI, G., SAUL, A., KARIUKI, S., MACLENNAN, C. A. & RONDINI, S. 2015. Relationship between Antibody Susceptibility and Lipopolysaccharide O-Antigen Characteristics of Invasive and Gastrointestinal

Nontyphoidal Salmonellae Isolates from Kenya. *PLoS Negl Trop Dis,* 9**,** e0003573.

b) **ONSARE, R. S.** & MACLENNAN, C. A. 2014. New genomics studies to understand the link between nontyphoidal Salmonella bacteremia and gastroenteritis in Africa. *J Infect Dev Ctries,* 8**,** 252-3.

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Appendix 1: Scientific steering committee (SSC, KEMRI) approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
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ESACIPAC/SSC/9635 **11th August , 2011**

Robert Sanaya

Thro'
Director, CMR
NAIROBI

Forwarded 15/8/11 Dr C Bii

REF: SSC No. 2115 (Revised) – Genomic and immunologiacal characterization of clinical isolates of non-typhoidal salmonella (NTS) from Kenya

Thank you for your letter dated 10th August, 2011 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

FOR: Sammy Njenga, PhD
SECRETARY, SSC

Appendix 2: Ethical Review Committee (KEMRI) approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
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KEMRI/RES/7/3/1 **September 23, 2011**

TO: **ROBERT ONSARE SANAYA (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. SAMUEL KARIUKI**
THE DIRECTOR, CMR,
NAIROBI

Forwarded 27/9/11 Dr C. Bii

Dear Sir,

RE: SSC PROTOCOL No. 2115- REVISED (*RE-SUBMISSION*): GENOMIC AND IMMUNOLOGICAL CHARACTERIZATION OF CLINICAL ISOLATES OF NON TYPHOIDAL SALMONELLA (NTS) FROM KENYA

Reference is made to your letter dated September 22, 2011. We acknowledge receipt of the Revised Study Protocol and Revised Informed Consent Documents on September 22, 2011.

This is to inform you that the Ethics Review Committee (ERC) finds that the issues raised at the 193rd meeting of September 13, 2011 have been adequately addressed. Consequently, the study is granted approval for implementation effective this **23rd day of September 2011**.

Please note that authorization to conduct this study will automatically expire on **September 21, 2012**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **August 10, 2012**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

Appendix 3: List of STm & SEn study isolates & their available basic metadata:

Appendix 3 (a.i): List of S. Typhimurium study isolates and their available basic metadata								
Lab. isolate No.	Hospital Name	Date of Collect	Age (Yrs)	Gender	Source of isolation	Major complaint	Temperature of patient on admission	Location in Kenya of Patient
Ke001	KNH	5/28/04	0.5	M	Case blood	Anemia	NI	Kawangware
Ke004	KNH	7/2/04	7	M	Case stool	NI	NI	KNH
Ke005	GGCH	6/21/04	NI	NI	Healthy control stool	NI	NI	GGCH
Ke007	KNH	6/27/04	12	M	Case blood+stool	Diarrhoea,Swollen stomack	39	Kibera
Ke012	KNH	6/27/04	4.5	M	Case blood	Diarrhoea,Vomiting, Fever	39.5	Eastleigh
Ke017	KNH	1/23/04	3	M	Case blood	Swollen	NI	KNH
Ke019	KNH	3/14/04	3	M	Case blood	NI	NI	Kibera
Ke020	KNH	4/25/04	0.6	F	Case stool	Fever, Diarrhoea, Vomiting	39	Ngumo
Ke021	AKH	21/11/2004	NI	NI	Healthy control stool	NI	NI	Kangemi
Ke022	KNH	10/14/02	1.8	M	Case blood	Fever, Diarrhoea, Cough	NI	Lungalunga
Ke030	KNH	2/24/04	1.5	M	Case blood	Diarrhoea, Vomiting	37	Dandora
Ke031	KNH	3/22/03	3	M	Case stool	Body weakness	NI	Kayole
Ke032	KNH	6/16/03	0.8	M	Case blood	Pneumonia	37	Githurai
Ke038	KNH	NI	NI	NI	NI	NI	NI	KNH
Ke040	KNH	7/18/03	1.4	M	Case blood	Cough, Fever, Respiratory distress	40	Kariobangi
Ke045	KNH	6/6/03	1.3	F	Case blood+stool	Pneumonia	37.5	Juja
Ke065	GGCH	10/23/04	NI	NI	Healthy control stool	NI	NI	Muthangari
Ke068	KNH	11/1/03	9M	F	Case blood	Vomiting, Fever	NI	Kibera
Ke069	AKH	11/21/04	NI	NI	Healthy control stool	NI	NI	Kangemi
Ke071	AKH	11/21/04	NI	NI	Healthy control stool	NI	NI	Kangemi
Ke072	GGCH	10/23/04	NI	NI	Healthy control stool	NI	NI	Muthangari
Ke088	KNH	9/12/04	7.5	M	Case blood	Vomiting, Diarrhoea, Headache	36.8	Dandora
Ke091	KNH	9/10/04	3	M	Case blood	Fever	NI	Kikuyu
Ke096	KNH	10/8/04	NI	M	Case blood	Fever	38.5	Mathare
Ke097	KNH	8/1/04	0.9	F	Case blood	Diarrhoea, Vomiting	37.8	Kibera
Ke098	KNH	2/3/05	NI	M	Case blood	NI	39.5	Ngong
Ke100	KNH	2/2/05	5	M	Case blood	Fever, Cough	37.8	Mathare
Ke101	KNH	9/22/04	2	M	Case blood	Vomiting, Anemia	36.5	Kawangware
Ke103	KNH	8/21/04	0.6	F	Case blood	Cough, Mouth ulcers	35.6	KNH
Ke105	CDC	8/1/08	NI	NI	Case blood	NI	NI	Kibera
Ke107	KNH	3/3/03	0.1	F	Case blood	Respiratory distress	37.8	Kayole
Ke108	KNH	9/9/04	NI	NI	Healthy control stool	NI	NI	Eastleigh
Ke112	KNH	6/6/03	0.4	M	Case stool	Diarrhoea, Vomiting	NI	Mathare
Ke124	KNH	9/25/03	NI	NI	Healthy control stool	NI	NI	Lungalunga
Ke141	KNH	12/12/04	NI	M	Case blood	Jaundice	37	KNH
Ke142	KNH	9/24/04	NI	NI	Healthy control stool	NI	NI	KNH
Ke150	KNH	8/16/04	0.3	F	Case blood	Diarrhoea, Vomiting	36.3	Kibera
Ke153	KNH	5/23/02	3	F	Case blood	Diarrhoea, Fever, Vomiting	39	Lucky Summer
Ke158	GGCH	5/22/02	6.5	M	Case blood+stool	Fever, Diarrhoea	39	Dandora
Ke174	AKH	9/5/02	6	M	Case blood	Fever, Vomiting, headache	NI	Huruma
Ke179	KNH	9/12/02	1	F	Case blood	NI	NI	Kariobangi
Ke184	KNH	9/12/02	1	F	Case blood	Fever	NI	Kiserian
Ke188	KNH	9/23/02	0.2	M	Case stool	Anemia	NI	Kawangware
Ke192	KNH	10/28/02	5	F	Case blood+stool	NI	NI	Komarock
Ke193	KNH	10/31/02	3	F	Case blood+stool	NI	NI	Kariobangi
Ke194	KNH	10/31/02	1.8	M	Case blood+stool	Fever, Diarrhoea, Vomiting	NI	Ngong
Ke197	GGCH	10/12/02	2	F	Case blood+stool	Diarrhoea, Bloody stool	NI	GGCH
Ke212	KNH	2/17/02	1.2	F	Case blood	Fever, Cough, Anemia	NI	Kayole
Ke215	KNH	2/17/02	NI	NI	Healthy control stool	NI	NI	Kayole
Ke216	KNH	2/17/02	NI	NI	Healthy control stool	NI	NI	Kayole
Ke220	KNH	4/4/03	0.6	F	Case blood	Cough, Diarrhoea, Oral sores	37.1	Eastleigh
Ke230	KNH	5/20/03	0.8	M	Case blood	Fever, Convulsions	NI	Majengo
Ke237	KNH	7/21/03	1.2	F	Case blood	MP, Pneumonia	39	Dandora
Ke238	KNH	6/24/03	0.9	F	Case stool	Diarrhoea, Vomiting, Fever	NI	Lungalunga
Ke239	KNH	7/7/03	6	F	Case blood	Fever	39	Korogocho
Ke241	KNH	7/21/03	1.1	F	Case blood	Marasmus	36	Kayole
Ke243	KNH	7/12/03	0.8	M	Case blood	Fever, Vomiting, Diarrhoea	40	Huruma
Ke244	KNH	7/14/03	7	F	Case blood	Fever	37.5	Huruma
Ke249	KNH	7/31/03	1	M	Case blood	NI	NI	Dagoretti
Ke254	KNH	9/1/03	1.3	M	Case blood	Diarrhoea, Abdominal pains	38.5	Huruma
Ke272	KNH	9/25/03	1	NI	Case blood	Fever, Vomiting, Cough ,Diarrhoea	NI	Lungalunga
Ke273	KNH	11/6/03	1.5	M	Case blood	Diarrhoea, Vomiting, Fever	NI	Githurai
Ke274	KNH	11/24/03	4	M	Case stool	SDD, Vomiting	NI	Kayole
Ke287	KNH	2/18/04	4	M	Case blood	Fever, Weak	38.5	Eastleigh

Appendix 3 (a.ii): List of S. Typhimurium study isolates and their available basic metadata								
Lab. isolate No.	**Hospital Name**	**Date of Collect**	**Age (Yrs)**	**Gender**	**Source of isolation**	**Major complaint**	**Temperature of patient on admission**	**Location in Kenya of Patient**
Ke290	KNH	4/19/04	4	F	Case blood	Fever, Cough	37	KNH
Ke291	KNH	5/10/04	NI	NI	Healthy control stool	NI	NI	Kibera
Ke294	KNH	5/28/04	3	F	Case stool	Fever, Diarrhoea, Vomiting	NI	Githurai
Ke296	KNH	5/31/04	1.5	F	Case stool	Diarrhoea, Vomiting	37.2	Kibera
Ke357	AKH	3/19/11	39	M	Case stool	NI	NI	Buru buru clinic
Ke358	AKH	3/19/11	1	M	Case stool	NI	NI	AKH
Ke365	CDC	8/1/08	NI	NI	Case blood	NI	NI	Kibera
Ke369	CDC	8/1/08	NI	NI	Case blood	NI	NI	Kibera
Ke370	AKH	1/30/11	8	M	Case stool	NI	NI	AKH
Ke377	KNH	1/23/03	1.2	F	Case blood	Fever, Diarhoea, Fever	NI	KNH
Ke379	KNH	11/13/02	0.3	F	Case blood	Fever, Diarrhoea	NI	Mathare
Ke380	AKH	5/5/10	43	M	Case Stool	ISS patient-Septic screen	NI	AKH
Ke381	AKH	6/24/10	37	F	Case Stool	NI	NI	AKH
Ke384	AKH	7/11/10	4	F	Case Stool	Abdominal pain	NI	AKH
Ke385	AKH	7/19/10	34	F	Case Stool	NI	NI	AKH
Ke386	AKH	5/1/10	1Mt	M	Case urine	NI	NI	AKH
Ke387	AKH	10/22/10	45	F	Case Stool	NI	NI	AKH
Ke389	AKH	10/23/10	44	F	Case Stool	Acute GE and dehydration	NI	AKH
Ke391	AKH	10/24/10	59	M	Case Stool	NI	NI	AKH
Ke392	AKH	10/22/10	40	F	Case stool	NI	NI	AKH
Ke406	AKH	5/11/10	13	F	Case Stool	NI	NI	AKH
Ke407	AKH	4/3/10	2	M	Case Stool	NI	NI	AKH
Ke408	GGCH	4/11/00	NI	NI	NI	NI	NI	GGCH
Ke410	GGCH	4/11/00	NI	NI	NI	NI	NI	GGCH
Ke411	GGCH	4/11/00	NI	NI	NI	NI	NI	GGCH
Ke420	KNH	12/20/02	NI	NI	Healthy control stool	NI	NI	Kibera
Ke421	KNH	12/20/02	NI	NI	Healthy control stool	NI	NI	Kibera
Ke423	KNH	5/10/04	NI	NI	Healthy control stool	NI	NI	Kibera
Ke426	KNH	5/28/04	NI	NI	Sewer	NI	NI	Mathare
Ke427	KNH	5/10/04	NI	NI	Healthy control stool	NI	NI	Kibera
Ke428	KNH	5/10/04	NI	NI	Healthy control stool	NI	NI	Kibera
Ke430	GGCH	6/21/04	NI	NI	Healthy control stool	NI	NI	GGCH
Ke431	KNH	6/27/04	NI	NI	Soil	NI	NI	Kibera
Ke432	KNH	7/12/03	NI	NI	Healthy control stool	NI	NI	Huruma
Ke433	KNH	4/23/03	NI	NI	Healthy control stool	NI	NI	Huruma
Ke442	KNH	1/23/03	NI	NI	Healthy control stool	NI	NI	KNH
Ke456	CDC	7/29/08	NI	NI	Case blood	NI	NI	Kibera
Ke464	CDC	8/1/08	NI	NI	Case blood	NI	NI	Kibera
Ke478	CDC	7/29/08	NI	NI	Case blood	NI	NI	Kibera
Ke483	GGCH	1/4/01	NI	NI	NI	NI	NI	GGCH
Ke490	GGCH	1/4/01	NI	NI	NI	NI	NI	GGCH
KeN120	GGCH	11/02/2010	NI	NI	Case CSF	NI	NI	GGCH
KeN135	AKH	19/07/2010	43	F	Case stool	NI	NI	AKH
KeN137	GGCH	04/07/2010	0.3	F	Case CSF	NI	NI	GGCH
KeN14	AKH	09/04/2010	0.5	F	Case blood	NI	NI	AKH
KeN141	AKH	25/01/2010	29	F	Case stool	NI	NI	AKH
KeN145	AKH	NI	NI	M	Case blood	NI	NI	AKH
KeN25	AKH	01/05/2010	0.1	M	Case Urine	NI	NI	AKH
KeN26	AKH	05/05/2010	NI	NI	Case Urine	NI	NI	AKH
KeN35	AKH	06/04/2010	29	M	Case stool	NI	NI	AKH

Key: NI-No information available; **KNH-**Kenyatta National Hospital; **AKH-**Aga Khan University Hospital, Nairobi; **GGCH**-Gertrude's Children Hospital; **CDC**-Centers for Disease Control and Preventions clinic, Nairobi

Appendix 3 (b.i): List of *S*. Enteritidis study isolates and their available basic metadata								
Lab. isolate No.	Hospital N	Date of Collectio	Age (Yrs)	Gender	Source of isolation	Major complaint	Temperature of patient on admission	Location in Kenya of Patient
Ke016	KNH	28/05/2004	NI	NI	Healthy control sto	NI	NI	Komarock
Ke033	KNH	17/07/2003	2.5	F	Case blood	NI	38.5	Pipeline
Ke042	KNH	10/02/2003	6	F	Case blood	Anemia, Diarrhoea, Malaria	NI	Kiserian
Ke044	KNH	28/07/2003	2.5	F	Case blood	Fever,Vomiting,	38	Kayole
Ke048	KNH	03/12/2002	2	F	Case blood	Diarrhoea, Fever	NI	Huruma
Ke117	KNH	23/02/2004	1.4	F	Case blood	Cough, Fever	36	Kaloleni
Ke122	KNH	24/07/2003	0.6	M	Case blood	Fever	NI	Outer ring
Ke143	KNH	08/10/2004	1.5	F	Case blood	NI	39	Baba Dogo
Ke151	GGCH	03/05/2002	9.5	F	Case blood	Fever, Cough	NI	Lucky Summer
Ke152	AKH	13/05/2002	3	F	Case blood	Fever	40	Ongata
Ke160	AKH	20/04/2002	3	M	Case blood+stool	NI	38.5	Upper hill
Ke163	AKH	11/06/2002	5	M	Case blood+stool	Fever,Diarrhoea,Cough	38.5	Saika
Ke164	GGCH	18/06/2002	2	F	Case blood+stool	Abdominal pains,Fever, DY	39.5	Karen
Ke169	KNH	01/08/2002	0.4	M	Case blood	Diarrhoea, Fever	39.5	Komarock
Ke172	AKH	26/08/2002	NI	F	Case blood	NI	NI	Mathare North
Ke180	KNH	12/09/2002	0.8	M	Case blood	NI	NI	Kibera
Ke181	KNH	12/09/2002	0.8	F	Case stool	NI	NI	Kiserian
Ke187	AKH	17/09/2002	1.3	M	Case blood	NI	NI	AKH
Ke189	AKH	17/09/2002	NI	NI	Healthy control sto	NI	NI	AKH
Ke196	KNH	13/11/2002	1.5	F	Case stool	Diarrhoea, Dehydration	NI	Kayole
Ke199	KNH	23/11/2002	0.2	M	Case blood	Fever, Cough, Pneumonia	NI	Njiru
Ke204	AKH	11/06/2002	NI	NI	Healthy control sto	NI	NI	Saika
Ke205	KNH	13/11/2002	NI	NI	Healthy control sto	NI	NI	Kayole
Ke219	GGCH	11/03/2003	NI	NI	Healthy control sto	NI	NI	Shauri Moyo
Ke232	KNH	13/06/2003	0.8	M	Case stool	Vomiting,Fever	NI	Bahati
Ke233	KNH	17/06/2003	0.9	M	Case stool	Vomiting, Diarrhoea	37.8	Baba Dogo
Ke267	AKH	29/09/2003	NI	NI	Healthy control sto	NI	NI	AKH
Ke276	GGCH	24/11/2003	6	F	Case blood+stool	Abdominal pains on & off	NI	GGCH
Ke288	KNH	08/03/2004	1.6	M	Case stool	Diarrhoea,Vomiting,Fever, Stomack pains	37	KNH
Ke292	KNH	27/05/2004	2.1	F	Case blood	Fever,Diarrhoea	38.5	Githurai
Ke304	MRT	15/06/2002	3.5	F	Case blood+stool	Fever, Cough, Diarrhoea	40	Buru buru
Ke409	GGCH	11/04/2000	NI	NI	NI	NI	NI	GGCH
Ke417	KDH	10/12/2005	NI	F	Healthy control sto	NI	NI	Kilifi
Ke422	AKH	08/10/2003	NI	NI	Healthy control sto	NI	NI	AKH
Ke434	KNH	21/03/2003	NI	NI	Healthy control sto	NI	NI	Mathare
Ke445	KNH	04/03/2005	NI	NI	Healthy control sto	NI	NI	Kampala
Ke448	KNH	15/04/2000	NI	NI	NI	NI	NI	KNH
Ke484	KNH	11/04/2000	NI	NI	NI	NI	NI	KNH
Ke485	GGCH	04/01/2001	NI	NI	NI	NI	NI	GGCH
Ke486	GGCH	04/01/2001	NI	NI	NI	NI	NI	GGCH
Ke488	KNH	04/01/2001	NI	NI	NI	NI	NI	KNH
Ke491	GGCH	04/01/2001	NI	NI	NI	NI	NI	GGCH
KeN1	AKH	18/03/2011	36	M	Case stool	NI	NI	AKH
KeN10	AKH	04/02/2011	20	M	Case stool	NI	NI	AKH
KeN11	AKH	15/03/2011	0	F	NI	NI	NI	AKH
KeN116	AKH	01/08/2010	2	M	Case stool	NI	NI	AKH
KeN117	AKH	15/11/2010	NI	M	Case blood	NI	NI	AKH
KeN123	AKH	21/11/2010	25	F	Case stool	NI	NI	AKH
KeN124	AKH	19/11/2010	1	M	Case stool	NI	NI	AKH
KeN125	AKH	27/10/2010	NI	NI	Case blood	NI	NI	AKH
KeN126	AKH	27/10/2010	0.8	F	Case stool	NI	NI	AKH

Appendix 3 (b.ii): List of *S*. Enteritidis study isolates and their available basic metadata								
Lab. isolate No.	Hospital N	Date of Collectio	Age (Yrs)	Gender	Source of isolation	Major complaint	Temperature of patient on admission	Location in Kenya of Patient
KeN128	AKH	23/10/2010	3	F	Case stool	NI	NI	AKH
KeN129	AKH	08/08/2010	NI	F	Case stool	NI	NI	AKH
KeN13	AKH	14/01/2011	25	F	Case stool	NI	NI	AKH
KeN130	AKH	08/08/2010	42	M	Case stool	NI	NI	AKH
KeN132	AKH	21/08/2010	23	F	Case blood	NI	NI	AKH
KeN133	AKH	23/07/2010	20	F	Case stool	NI	NI	AKH
KeN134	AKH	06/07/2010	18	M	Case stool	NI	NI	AKH
KeN136	AKH	07/08/2010	47	M	Case blood	NI	NI	AKH
KeN142	AKH	14/03/2010	23	F	Case stool	NI	NI	AKH
KeN143	AKH	29/06/2010	41	M	Case stool	NI	NI	AKH
KeN149	AKH	30/03/2010	36	M	Case stool	NI	NI	AKH
KeN15	AKH	28/02/2010	2.5	F	NI	NI	NI	AKH
KeN2	AKH	18/03/2011	33	F	Case stool	NI	NI	AKH
KeN21	AKH	22/12/2010	25	F	Case stool	NI	NI	AKH
KeN23	AKH	17/04/2010	8	F	Case stool	NI	NI	AKH
KeN24	AKH	30/04/2010	32	M	Case blood	NI	NI	AKH
KeN27	AKH	20/04/2010	64	M	Case stool	NI	NI	AKH
KeN29	AKH	25/01/2010	46	F	Case stool	NI	NI	AKH
KeN3	AKH	25/03/2011	48	M	Case blood	NI	NI	AKH
KeN31	AKH	25/02/2010	17	M	Case stool	NI	NI	AKH
KeN32	AKH	06/01/2010	2	M	Case stool	NI	NI	AKH
KeN39	GGCH	19/02/2010	3.6	M	Case stool	NI	NI	GGCH
KeN5	AKH	14/03/2011	29	M	Case stool	NI	NI	AKH
KeN51	GGCH	11/02/2010	1.3	M	Case blood	NI	NI	GGCH
KeN6	AKH	29/04/2011	1	F	Case stool	NI	NI	AKH
KeN62	AKH	21/01/2011	NI	NI	NI	NI	NI	AKH
KeN65	AKH	02/01/2011	5	M	Case stool	NI	NI	AKH
KeN9	AKH	25/02/2011	7	M	Case stool	NI	NI	AKH

Key: NI-No information available; **KNH**-Kenyatta National Hospital; **AKH**-Aga Khan University Hospital, Nairobi; **GGCH**-Gertrude's Children Hospital; **CDC**-Centers for Disease Control and Preventions clinic, Nairobi

Appendix 4: SBA (using pooled adult human sera) results of STm & SEn isolates:

Appendix 4 (a.i): Serum bactericidal assay (SBA) results for *S*. Typhimurium (STm) isolates against pooled Malawian sera

Serovar	Lab. isolate No.	Log changes (T180-T0)	
		Fresh serum	HI Serum (Negative control)
STm	Ke001	-3.0	0.6
STm	Ke007	-3.0	0.7
STm	Ke012	-2.2	0.6
STm	Ke017	-1.8	0.1
STm	Ke019	-3.0	0.8
STm	Ke022	-2.4	0.8
STm	Ke030	-2.8	0.4
STm	Ke032	-1.2	0.4
STm	Ke040	-3.0	0.4
STm	Ke045	-2.5	0.8
STm	Ke068	-2.0	0.8
STm	Ke088	-3.0	0.7
STm	Ke091	-3.0	0.9
STm	Ke096	-3.0	1.0
STm	Ke097	-3.0	1.0
STm	Ke098	-3.0	0.3
STm	Ke100	-3.0	0.8
STm	Ke101	-3.0	0.8
STm	Ke103	-3.0	0.8
STm	Ke105	-3.0	0.3
STm	Ke107	-2.1	0.7
STm	Ke141	-2.6	0.7
STm	Ke150	-3.0	0.7
STm	Ke153	-2.2	0.5
STm	Ke158	-2.1	0.8
STm	Ke174	-2.4	0.2
STm	Ke179	-2.6	0.4
STm	Ke184	-2.1	0.7
STm	Ke192	-3.0	0.7
STm	Ke193	-2.0	0.5
STm	Ke194	-2.0	0.2
STm	Ke197	-2.1	0.7
STm	Ke212	-2.3	0.7
STm	Ke220	-3.0	0.6
STm	Ke230	-3.0	0.4
STm	Ke237	-3.0	0.8
STm	Ke238	-2.1	0.5
STm	Ke239	-3.0	0.9
STm	Ke241	-3.0	0.8
STm	Ke243	-2.0	0.7
STm	Ke244	-2.2	0.5
STm	Ke249	-3.0	0.8
STm	Ke254	-3.0	0.9
STm	Ke272	-3.0	0.4
STm	Ke273	-3.0	0.8
STm	Ke287	-1.1	0.6
STm	Ke290	-3.0	0.8
STm	Ke365	-2.8	0.2
STm	Ke369	-2.6	0.8
STm	Ke377	-2.2	0.5
STm	Ke379	-1.3	0.3
STm	Ke456	-3.0	0.0
STm	Ke464	-3.0	0.1
STm	Ke478	-3.0	1.0
STm	KeN120	-3.0	1.0
STm	KeN137	-3.0	0.8
STm	KeN14	-3.0	0.2
STm	KeN145	-2.3	0.8
STm	Ke004	-3.0	0.6
STm	Ke020	-3.0	0.3

Appendix 4 (a.ii): Serum bactericidal assay (SBA) results for *S.* Typhimurium (STm) isolates against pooled Malawian sera			
Serovar	**Lab. isolate No.**	**Log changes (T180-T0)**	
		Fresh serum	**HI Serum (Negative control)**
STm	Ke188	-2.2	0.7
STm	Ke274	-3.0	0.5
STm	Ke294	-3.0	0.0
STm	Ke296	-2.5	0.1
STm	Ke357	-3.0	0.6
STm	Ke358	-3.0	-0.2
STm	Ke370	-3.0	0.6
STm	Ke380	-3.0	0.6
STm	Ke381	-1.9	0.0
STm	Ke384	-2.9	-0.1
STm	Ke385	-3.0	-0.1
STm	Ke387	-2.6	0.8
STm	Ke389	-2.8	0.6
STm	Ke391	-3.0	0.7
STm	Ke392	-3.0	0.4
STm	Ke406	-2.9	0.7
STm	Ke407	-3.0	0.2
STm	KeN135	-2.6	0.2
STm	KeN141	-2.5	0.3
STm	KeN35	-2.8	0.6
STm	Ke215	-2.7	0.6
STm	Ke216	-2.7	0.8
STm	Ke005	-3.0	0.0
STm	Ke021	-2.2	0.6
STm	Ke065	-3.0	0.4
STm	Ke069	-2.1	0.6
STm	Ke071	-3.0	1.1
STm	Ke072	-2.8	0.5
STm	Ke108	-3.0	0.8
STm	Ke124	-2.7	0.9
STm	Ke142	-3.0	0.7
STm	Ke291	-3.0	0.5
STm	Ke420	0.6	0.5
STm	Ke421	-3.0	0.8
STm	Ke423	-2.5	0.9
STm	Ke427	-2.5	0.8
STm	Ke428	-2.5	0.8
STm	Ke430	-2.4	0.7
STm	Ke432	-2.2	0.6
STm	Ke433	-2.6	0.3
STm	Ke442	-2.4	0.3
STm	Ke386	-3.0	0.5
STm	KeN25	-3.0	0.7
STm	KeN26	-3.0	1.1
STm	Ke426	-2.8	0.8
STm	Ke431	-3.0	0.9
STm	Ke038	-3.0	0.8
STm	Ke408	-2.9	0.6
STm	Ke410	-3.0	0.8
STm	Ke411	-2.1	0.4
STm	Ke483	-2.3	0.1
STm	Ke490	-1.8	0.5
Key: HI-Heat - inactivated			

Appendix 4 (B.i): Serum bactericidal assay (SBA) results for *S.* Enteritidis (SEn) isolates against pooled adult human sera			
Serovar	Lab. isolate No.	Log changes (T180-T0)	
		Fresh serum	HI Serum (Negative control)
SEn	Ke048	-3.0	0.6
SEn	Ke117	-1.3	0.4
SEn	Ke143	-1.2	0.4
SEn	Ke044	-1.4	0.0
SEn	Ke152	-0.4	0.3
SEn	Ke122	-1.4	0.4
SEn	Ke042	-1.3	0.8
SEn	Ke033	-1.9	0.4
SEn	Ke199	-0.3	0.6
SEn	Ke169	-1.2	0.0
SEn	Ke172	-1.3	0.2
SEn	Ke164	-0.8	0.3
SEn	Ke160	-2.2	0.0
SEn	Ke180	-2.0	0.1
SEn	Ke163	-2.0	-0.2
SEn	Ke187	-1.8	0.2
SEn	Ke151	-2.1	0.0
SEn	Ke276	-0.3	0.8
SEn	Ke292	-0.7	0.2
SEn	KeN117	-1.0	0.5
SEn	KeN125	-2.0	0.4
SEn	KeN132	-1.4	0.7
SEn	KeN136	-2.0	0.8
SEn	KeN24	-0.9	0.4
SEn	KeN3	-0.5	0.9
SEn	KeN51	-0.3	0.6
SEn	Ke288	-3.0	1.1
SEn	Ke233	-1.1	0.5
SEn	Ke196	-0.4	0.5
SEn	Ke181	-1.2	0.1
SEn	Ke232	-1.0	0.7
SEn	Ke304	-2.1	0.8
SEn	KeN1	-1.0	0.0
SEn	KeN10	-1.5	0.0

Appendix 4 (B.ii): Serum bactericidal assay (SBA) results for *S.* Enteritidis (SEn) isolates against pooled adult human sera

Serovar	Lab. isolate No.	Log changes (T180-T0)	
		Fresh serum	HI Serum (Negative control)
SEn	KeN124	-1.6	0.4
SEn	KeN126	-1.0	0.5
SEn	KeN128	-0.2	0.5
SEn	KeN129	-1.0	0.4
SEn	KeN13	-2.1	0.2
SEn	KeN130	-2.2	0.0
SEn	KeN133	-1.3	0.7
SEn	KeN134	-3.0	0.6
SEn	KeN142	-0.5	0.6
SEn	KeN143	-1.3	0.5
SEn	KeN149	-0.4	0.0
SEn	KeN2	-0.3	-0.1
SEn	KeN21	-1.9	0.2
SEn	KeN23	-1.1	-0.7
SEn	KeN27	-1.1	0.7
SEn	KeN29	-3.0	0.6
SEn	KeN31	-2.5	0.0
SEn	KeN32	-0.1	0.6
SEn	KeN39	-1.1	0.6
SEn	KeN5	-1.2	0.0
SEn	KeN6	-2.6	0.2
SEn	KeN65	0.0	0.4
SEn	KeN9	-1.3	0.5
SEn	Ke267	-1.2	0.2
SEn	Ke219	-0.3	0.5
SEn	Ke205	-1.6	0.1
SEn	Ke204	-1.9	0.2
SEn	Ke189	-0.8	0.8
SEn	Ke016	-3.0	0.1
SEn	Ke422	-1.7	0.2
SEn	Ke434	-1.0	0.4
SEn	Ke445	-1.0	0.7
SEn	Ke409	-1.0	0.8
SEn	Ke448	-2.1	0.6
SEn	Ke484	-2.1	0.4
SEn	Ke485	-1.0	0.5
SEn	Ke486	-3.0	0.1
SEn	Ke488	-1.4	0.6
SEn	Ke491	-1.1	0.2
SEn	KeN11	-0.8	0.1
SEn	KeN62	-1.1	0.5
SEn	KeN15	-1.0	0.4

Key: HI-Heat - inactivated

Appendix 5: O-Acetylation and glucosylation results (STm & SEn):

Appendix 5 (a.i): O-acetylation results for S. Typhimurium (STm) study isolates

Serovar	Lab. isolate No.	O-acetylation results by NMR
STm	Ke001	Rha O-acetylation
STm	Ke004	Rha + Abe-O-acetylation
STm	Ke005	Rha (<150%); Abe (<150%) O-acetylation
STm	Ke007	Rha + Abe-O-acetylation
STm	Ke017	Rha + Abe-O-acetylation
STm	Ke019	Rha and Abe (150%) O-acetylation
STm	Ke020	Rha and Abe (150%) O-acetylation
STm	Ke021	Rha and Abe (150%) O-acetylation
STm	Ke022	Rha and Abe (150%) O-acetylation
STm	Ke030	Rha and Abe (150%) O-acetylation
STm	Ke031	Rha and Abe (150%) O-acetylation
STm	Ke032	Rha and Abe (150%) O-acetylation
STm	Ke038	Rha and Abe (150%) O-acetylation
STm	Ke045	Rha and Abe (150%) O-acetylation
STm	Ke068	Rha + Abe-O-acetylation
STm	Ke069	Rha + Abe-O-acetylation
STm	Ke088	Rha + Abe-O-acetylation
STm	Ke098	Rha (slightly less 150%); Abe (<150%) O-acetylation
STm	Ke101	Rha and Abe (150%) O-acetylation
STm	Ke103	Rha (<150%); Abe (<150%) O-acetylation
STm	Ke105	Rha and Tyv (150%) O-acetylation
STm	Ke107	Rha and Abe (150%) O-acetylation
STm	Ke108	Rha and Abe (150%) O-acetylation
STm	Ke112	Rha and Abe (150%) O-acetylation
STm	Ke124	Rha and Abe (150%) O-acetylation
STm	Ke142	Rha and Abe (150%) O-acetylation
STm	Ke192	Abe and Rha O-acetylation
STm	Ke193	Abe and Rha O-acetylation
STm	Ke194	Abe and Rha O-acetylation
STm	Ke216	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke237	Rha + Abe O-acetylation
STm	Ke244	Rha + Abe O-acetylation
STm	Ke249	Rha + Abe O-acetylation
STm	Ke291	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke386	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke421	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke423	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke427	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke428	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke430	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke432	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke433	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke442	C2/C3 Rha + C2 Abe O-acetylation
STm	KeN120	C2/C3 Rha + C2 Abe O-acetylation
STm	KeN137	C2/C3 Rha + C2 Abe O-acetylation
STm	KeN25	C2/C3 Rha + C2 Abe O-acetylation
STm	Ke012	No O-acetylation on Rha; Abe (150%) O-acetylation
STm	Ke040	No O-acetylation on Rha; Abe (>150%) O-acetylation
STm	Ke065	NO-acetylation on Rha; Abe O-acetylation
STm	Ke072	NO-acetylation on Rha; Abe O-acetylation
STm	Ke100	No O-acetylation on Rha; Rha; Abe (150%) O-acetylation
STm	Ke294	No O-acetylation Rha; Abe O-acetylation
STm	Ke357	No O-acetylation Rha; Abe O-acetylation
STm	Ke365	No O-acetylation Rha; Abe O-acetylation
STm	Ke370	No O-acetylation Rha; Abe O-acetylation
STm	KeN135	No O-acetylation Rha; Abe O-acetylation
STm	KeN14	No O-acetylation Rha; Abe O-acetylation
STm	KeN145	No O-acetylation Rha; Abe O-acetylation
STm	KeN35	No O-acetylation Rha; Abe O-acetylation
STm	Ke071	No O-acetylation
STm	Ke096	No O-acetylation
STm	Ke097	No O-acetylation
STm	Ke158	No O-acetylation
STm	Ke420	No O-acetylation
STm	Ke141	O-acetylation on Rha; very low or no O-acetylation on Abe
STm	Ke150	O-acetylation on Rha; very low or no O-acetylation on Abe
STm	Ke153	Rha (150%) O-acetylation, very low O-acetylation on Abe
STm	Ke197	Rha (150%) O-acetylation, very low O-acetylation on Abe
STm	Ke287	low C2/C3 O-acetylation on Rha
STm	Ke426	C2/C3 O-acetylation on Rha
STm	Ke431	C2/C3 Rha O-acetylation + no/very low O-acetylation on Abe
STm	Ke091	Very low O-acetylation on Rha; very low O-acetylation on Abe
STm	Ke389	C2 O-acetylation on Abe, low C2/C3 O-acetylation on Rha

Key: Rha-Rhamnose, **Abe**-Abequose, **C**-Carbon

Appendix 5 (a.ii): O-acetylation results for S. Enteritidis (SEn) study isolates		
Serovar	**Lab. isolate No.**	**O-acetylation results by NMR**
SEn	Ke033	No O-acetylation
SEn	Ke048	No O-acetylation, or vey low on Abe
SEn	Ke117	No O-acetylation
SEn	Ke122	No O-acetylation
SEn	Ke181	No O-acetylation
SEn	Ke196	No O-acetylation
SEn	Ke199	No O-acetylation
SEn	Ke205	No O-acetylation
SEn	Ke219	No O-acetylation
SEn	Ke288	No O-acetylation
SEn	Ke304	No O-acetylation
SEn	KeN117	No O-acetylation
SEn	KeN124	No O-acetylation
SEn	KeN128	No O-acetylation
SEn	KeN130	No O-acetylation
SEn	KeN24	No O-acetylation
SEn	Ke016	Very low O-acetylation on Rha, very low or no O-acetylation on Abe
SEn	Ke042	No O-acetylation on Rha, very low or no O-acetylation Abe
SEn	Ke044	Very low/no Rha only
SEn	Ke143	very low on Rha, no or very low on Abe
SEn	Ke151	Very low on Rha
SEn	Ke160	Very low Rha
SEn	Ke163	No/very low
SEn	Ke169	Very low
SEn	Ke204	low
SEn	Ke232	Very low
SEn	Ke233	No/very low
SEn	Ke267	No/very low
SEn	Ke276	low
SEn	Ke445	Very low
SEn	KeN123	No/low OAc
SEn	KeN13	Very low
SEn	KeN132	No/very low
SEn	KeN51	Very low
SEn	Ke152	Rha (<150%), low
SEn	Ke189	O-acetylated
SEn	Ke292	O-acetylated
SEn	Ke422	O-acetylated
SEn	Ke434	O-acetylated
SEn	KeN1	O-acetylated
SEn	KeN10	O-acetylated
SEn	KeN116	O-acetylated
SEn	KeN126	O-acetylated
SEn	KeN136	O-acetylated
Key: Rha-Rhamnose, **Abe**-Abequose, **C**-Carbon		

Appendix 5 (b.i): Glucosylation results for S. Typhimurium (STm) study isolates

Serovar	Lab. isolate No.	% Glucosylation	% Glucosylation range
STm	Ke019	15	< 20
STm	Ke021	15	< 20
STm	Ke022	15	< 20
STm	Ke030	15	< 20
STm	Ke031	15	< 20
STm	Ke032	15	< 20
STm	Ke038	15	< 20
STm	Ke112	15	< 20
STm	Ke124	15	< 20
STm	Ke141	15	< 20
STm	Ke153	15	< 20
STm	Ke194	14.72	<20
STm	Ke238	15	< 20
STm	Ke243	15	< 20
STm	Ke249	15	< 20
STm	KeN135	11.88	<20
STm	Ke012	30	20-50
STm	Ke017	30	20-50
STm	Ke020	30	20-50
STm	Ke065	38.77	20-50
STm	Ke158	35.59	20-50
STm	Ke193	39.21	20-50
STm	Ke197	30.09	20-50
STm	Ke216	25.39	20-50
STm	Ke239	30	20-50
STm	Ke241	30	20-50
STm	Ke254	30	20-50
STm	Ke287	25.23	20-50
STm	Ke291	30.94	20-50
STm	Ke357	33.74	20-50
STm	Ke370	30.92	20-50
STm	Ke386	32.75	20-50
STm	Ke421	31.63	20-50
STm	Ke423	28.8	20-50
STm	Ke426	23.49	20-50
STm	Ke427	30.87	20-50
STm	Ke428	27.37	20-50
STm	Ke430	29.22	20-50
STm	Ke432	25.73	20-50
STm	Ke433	30.77	20-50
STm	Ke442	26.14	20-50
STm	KeN120	27.28	20-50
STm	KeN137	26.71	20-50
STm	KeN145	45.91	20-50
STm	KeN25	23.97	20-50
STm	KeN26	29.85	20-50
STm	KeN35	23.7	20-50
STm	Ke007	80	> 80
STm	Ke192	98.56	>50
STm	Ke294	79.35	>50
STm	Ke365	108.2	>50
STm	Ke389	74.7	>50
STm	Ke420	861.13	NI
STm	Ke431	64.69	>50
STm	KeN14	55.83	>50

Key: **N**I-No information

Appendix 5 (b.ii): Glucosylation results for S. Enteritidis (SEn) study isolates			
Serovar	**Lab. isolate No.**	**% Glucosylation**	**% Glucosylation range**
SEn	Ke196	18.12	<20
SEn	Ke205	15.68	<20
SEn	Ke233	18.64	<20
SEn	KeN123	19.36	<20
SEn	KeN51	18.98	<20
SEn	Ke016	30	20-50
SEn	Ke163	27.76	20-50
SEn	Ke164	20.21	20-50
SEn	Ke169	19.8	20-50
SEn	Ke181	29.15	20-50
SEn	Ke189	22.2	20-50
SEn	Ke199	23.13	20-50
SEn	Ke204	30.52	20-50
SEn	Ke219	22.62	20-50
SEn	Ke232	27.35	20-50
SEn	Ke267	22.18	20-50
SEn	Ke276	45.69	20-50
SEn	Ke288	24.44	20-50
SEn	Ke292	20.67	20-50
SEn	Ke304	21.98	20-50
SEn	Ke422	39.23	20-50
SEn	Ke434	23.27	20-50
SEn	Ke445	32.03	20-50
SEn	KeN1	21.33	20-50
SEn	KeN10	28.15	20-50
SEn	KeN116	24.08	20-50
SEn	KeN124	24.77	20-50
SEn	KeN126	19.85	20-50
SEn	KeN130	24.23	20-50
SEn	KeN24	19.99	20-50
SEn	KeN117	63.42	>50
SEn	KeN128	59.94	>50
SEn	KeN13	61.35	>50
SEn	KeN132	66.33	>50
SEn	KeN136	52.64	>50
Key: NI-No information			

Appendix 6: AST, MLST, Resistance phenotypes & Accession Numbers for STm & SEn:

Appendix 6 A.i: Antimcrobial Susceptibility Test (AST) results, Resistance phenotypes & Genomic metadata (MLST & Phylogenetic lineages) for S. Typhimurium

Sample	Antibiograms									No.of antibiotics (Resistant)	Antimicrobial resistance phenotypes	MLST	Phylogenetic lineage	Accession Number
	AMP	CRO	C	CIP	NA	TE	W	SXT	F					
Ke001	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158353
Ke004	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158356
Ke005	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158357
Ke007	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158358
Ke012	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158360
Ke017	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158362
Ke019	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158363
Ke020	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158364
Ke021	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158365
Ke022	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	I	ERS158366
Ke030	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158371
Ke031	R	R	S	S	S	S	R	R	R	5	AMP-CRO-W-SXT-F	313	I	ERS158372
Ke032	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	I	ERS158373
Ke038	R	S	S	S	S	R	S	S	S	2	AMP-TE	313	I	ERS158375
Ke040	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS158376
Ke045	R	S	S	S	S	R	R	R	R	5	AMP-TE-W-SXT-F	313	I	ERS158379
Ke065	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158382
Ke068	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158383
Ke069	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158384
Ke071	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158385
Ke072	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS158386
Ke088	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158388
Ke091	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158389
Ke096	R	I	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	313	II	ERS158390
Ke097	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158391
Ke098	R	S	S	S	R	S	R	R	S	4	AMP-NA-W-SXT	313	I	ERS158392
Ke100	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158393
Ke101	R	S	R	S	R	S	R	R	S	5	AMP-C-NA-W-SXT	313	II	ERS158394
Ke103	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158395
Ke105	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158396
Ke107	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158397
Ke108	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158398
Ke112	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158400
Ke124	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158403
Ke141	R	S	S	S	R	S	R	R	R	5	AMP-NA-W-SXT-F	313	I	ERS158404
Ke142	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158405
Ke150	R	S	S	S	R	S	R	R	S	4	AMP-NA-W-SXT	313	I	ERS158408
Ke153	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	I	ERS158411
Ke158	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	II	ERS158412
Ke174	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158419
Ke179	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158421
Ke184	R	S	S	S	S	R	R	R	S	5	AMP-TE-W-SXT-F	313	I	ERS158424
Ke188	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	I	ERS158427
Ke192	R	S	S	S	S	S	S	S	S	1	AMP	313	I	ERS158429
Ke193	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	I	ERS158430
Ke194	R	S	S	S	R	R	R	R	S	5	AMP-NA-TE-W-SXT	313	I	ERS158431
Ke197	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158433
Ke212	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	I	ERS158438
Ke215	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158440
Ke216	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158441
Ke220	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158443
Ke230	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158444
Ke237	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158448
Ke238	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158449
Ke239	R	S	R	S	S	S	R	R	R	5	AMP-C-W-SXT-F	313	II	ERS158450
Ke241	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158451
Ke243	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158452
Ke244	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158453
Ke249	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	I	ERS158455
Ke254	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158456

Legend: AMP-Ampicillin (10 µg), CRO-Ceftriaxone (30 µg), C-Chloramphenicol (30 µg), CIP-Ciprofloxacin (1 µg), NA-Nalidixic acid (30 µg), TE - Tetracycline (10 µg), W-Trimethoprim (2.5 µg), SXT-Trimethoprim /sulphamethoxazole 1:19 (25 µg), F-Nitrofurantoin (300 µg)

Appendix 6 A.ii: Antimcrobial Susceptibility Test (AST) results, Resistance phenotypes & Genomic metadata (MLST & Phylogenetic lineages) for S. Typhimurium

Sample	Antibiograms									No.of antibiotics (Resistant)	Antimicrobial resistance phenotypes	MLST	Phylogenetic lineage	Accession Number
	AMP	CRO	C	CIP	NA	TE	W	SXT	F					
Ke272	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158459
Ke273	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158460
Ke274	R	S	S	S	S	R	R	R	R	5	AMP-TE-W-SXT-F	313	I	ERS158461
Ke287	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158464
Ke290	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158467
Ke291	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158468
Ke294	S	S	S	S	S	S	S	S	R	1	F	19	IIIA	ERS158470
Ke296	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158471
Ke357	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158486
Ke358	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158487
Ke365	S	S	S	S	S	S	S	S	S	0	Sensitive	19	IIIA	ERS158492
Ke369	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158496
Ke370	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158497
Ke377	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158501
Ke379	R	R	S	S	S	S	R	R	S	4	AMP-CRO-W-SXT	313	I	ERS158503
Ke380	R	R	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	313	II	ERS158504
Ke381	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158505
Ke384	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS158507
Ke385	S	S	S	S	S	S	S	S	S	0	Sensitive	19	IIIA	ERS158508
Ke386	R	R	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	313	II	ERS158509
Ke387	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158510
Ke389	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158512
Ke391	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158514
Ke392	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS158515
Ke406	R	R	R	R	R	R	R	R	R	9	AMP-CRO-C-CIP-NA-TE-W-SXT-F	19	IIIA	ERS158529
Ke407	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS158530
Ke408	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158531
Ke410	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158533
Ke411	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS158534
Ke420	R	S	R	S	S	R	R	R	R	6	AMP-C-TE-W-SXT-F	313	II	ERS158543
Ke421	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158544
Ke423	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158546
Ke426	R	S	S	S	R	S	R	R	R	5	AMP-NA-W-SXT-F	313	I	ERS158549
Ke427	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158550
Ke428	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158551
Ke430	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	II	ERS158553
Ke431	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158554
Ke432	R	S	S	S	S	S	R	R	R	4	AMP-W-SXT-F	313	I	ERS158555
Ke433	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	I	ERS158556
Ke442	R	S	S	S	S	R	R	R	S	4	AMP-TE-W-SXT	313	I	ERS158565
Ke456	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	313	II	ERS158579
Ke464	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS158587
Ke478	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS158601
Ke483	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	I	ERS158606
Ke490	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	313	I	ERS158613
KeN120	R	R	R	R	S	R	R	R	S	7	AMP-CRO-C-CIP-TE-W-SXT	313	II	ERS330080
KeN135	S	S	S	S	S	S	S	S	S	0	Sensitive	19	IIIA	ERS330082
KeN137	R	R	R	R	S	R	R	R	S	7	AMP-CRO-C-CIP-TE-W-SXT	313	II	ERS330083
KeN14	S	S	S	S	S	R	S	S	R	2	TE-F	19	IIIA	ERS330066
KeN141	R	S	R	S	S	S	R	R	S	4	AMP-C-W-SXT	313	II	ERS330085
KeN145	S	S	S	S	S	R	S	S	S	1	TE	19	IIIB	ERS330086
KeN25	R	R	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	313	II	ERS330071
KeN26	R	R	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	313	II	ERS330072
KeN35	S	S	S	S	S	R	S	S	S	1	TE	19	IIIA	ERS330073

Legend: AMP-Ampicillin (10 µg), CRO-Ceftriaxone (30 µg), C-Chloramphenicol (30 µg), CIP-Ciprofloxacin (1 µg), NA-Nalidixic acid (30 µg), TE -Tetracycline (10 µg), W-Trimethoprim (2.5 µg), SXT-Trimethoprim /sulphamethoxazole 1:19 (25 µg), F-Nitrofurantoin (300 µg)

Appendix 6 B.i: Antimcrobial Susceptibility Test (AST) results, Resistance phenotypes & Genomic metadata (MLST & Phylogenetic lineages) for S. Enteritidis

Sample	Antibiograms									No.of antibiotics (Resistant)	Antimicrobial resistance phenotypes	MLST	Phylogenetic lineage	Accession Number
	AMP	CRO	C	CIP	NA	TE	W	SXT	F					
Ke016	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158361
Ke033	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158374
Ke042	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158377
Ke044	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158378
Ke048	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158380
Ke117	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158401
Ke122	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158402
Ke143	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158406
Ke151	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158409
Ke152	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158410
Ke160	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158413
Ke163	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158414
Ke164	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158415
Ke169	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158417
Ke172	R	S	R	S	S	R	R	R	R	6	AMP-C-TE-W-SXT-F	11	I	ERS158418
Ke180	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158422
Ke181	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158423
Ke187	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158426
Ke189	S	S	S	S	S	R	S	S	R	1	TE	11	II	ERS158428
Ke196	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158432
Ke199	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158435
Ke204	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158436
Ke205	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158437
Ke219	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158442
Ke232	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158445
Ke233	R	S	R	S	S	R	R	R	R	6	AMP-C-TE-W-SXT-F	11	I	ERS158446
Ke267	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158457
Ke276	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158462
Ke288	S	S	S	S	S	R	S	S	S	1	TE	11	II	ERS158465
Ke292	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158469
Ke304	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158477
Ke409	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158532
Ke417	S	S	S	S	S	S	S	S	S	0	Sensitive	11	I	ERS158540
Ke422	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS158545
Ke434	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158557
Ke445	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158568
Ke448	R	S	S	S	S	S	R	R	S	3	AMP-W-SXT	11	I	ERS158571
Ke484	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158607
Ke485	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158608
Ke486	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS158609

Legend: AMP-Ampicillin (10 µg), CRO-Ceftriaxone (30 µg), C-Chloramphenicol (30 µg), CIP-Ciprofloxacin (1 µg), NA-Nalidixic acid (30 µg), TE - Tetracycline (10 µg), W-Trimethoprim (2.5 µg), SXT-Trimethoprim /sulphamethoxazole 1:19 (25 µg), F-Nitrofurantoin (300 µg)

Appendix 6 B.ii: Antimcrobial Susceptibility Test (AST) results, Resistance phenotypes & Genomic metadata (MLST & Phylogenetic lineages) for S. Enteritidis

Sample	Antibiograms									No.of antibiotics (Resistant)	Antimicrobial resistance phenotypes	MLST	Phylogenetic lineage	Accession Number
	AMP	CRO	C	CIP	NA	TE	W	SXT	F					
Ke488	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158611
Ke491	R	S	R	S	S	R	R	R	S	5	AMP-C-TE-W-SXT	11	I	ERS158614
KeN1	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330021
KeN10	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330027
KeN11	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330028
KeN116	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330046
KeN117	R	I	R	S	S	R	R	R	S	6	AMP-CRO-C-TE-W-SXT	11	I	ERS330047
KeN123	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330048
KeN124	S	I	S	S	S	S	S	S	R	2	CRO-F	11	II	ERS330049
KeN125	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330050
KeN126	S	S	S	S	S	S	S	S	S	0	Sensitive	11	II	ERS330051
KeN128	S	S	S	S	S	S	S	S	S	0	Sensitive	11	II	ERS330052
KeN129	S	S	S	S	S	S	S	S	S	0	Sensitive	11	II	ERS330053
KeN13	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330030
KeN130	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330054
KeN132	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330055
KeN133	S	I	R	S	R	S	S	S	R	4	CRO-C-NA-F	11	II	ERS330056
KeN134	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330057
KeN136	S	S	S	S	S	S	S	S	S	0	Sensitive	11	II	ERS330058
KeN142	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330061
KeN143	S	S	S	S	S	S	S	S	S	0	Sensitive	11	II	ERS330062
KeN149	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330063
KeN15	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330031
KeN2	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330022
KeN21	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330033
KeN23	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330034
KeN24	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330035
KeN27	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330036
KeN29	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330038
KeN3	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330023
KeN31	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330039
KeN32	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330040
KeN39	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330042
KeN5	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330024
KeN51	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330043
KeN6	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330025
KeN62	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330044
KeN65	S	S	S	S	S	S	S	S	R	1	F	11	II	ERS330045
KeN9	S	S	S	S	S	R	S	S	R	2	TE-F	11	II	ERS330026

Legend: AMP-Ampicillin (10 µg), CRO-Ceftriaxone (30 µg), C-Chloramphenicol (30 µg), CIP-Ciprofloxacin (1 µg), NA-Nalidixic acid (30 µg), TE - Tetracycline (10 µg), W-Trimethoprim (2.5 µg), SXT-Trimethoprim /sulphamethoxazole 1:19 (25 µg), F-Nitrofurantoin (300 µg)

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Relationship between Antibody Susceptibility and Lipopolysaccharide O-Antigen Characteristics of Invasive and Gastrointestinal Nontyphoidal *Salmonellae* Isolates from Kenya

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OPEN ACCESS

Citation: Onsare RS, Micoli F, Lanzilao L, Alfini R, Okoro C, Muigai AW, et al. (2015) Relationship between Antibody Susceptibility and Lipopolysaccharide O-Antigen Characteristics of Invasive and Gastrointestinal Nontyphoidal *Salmonellae* Isolates from Kenya. PLoS Negl Trop Dis 9(3): e0003573. doi:10.1371/journal.pntd.0003573

Editor: Stephen Baker, Oxford University Clinical Research Unit, VIETNAM

Received: August 21, 2014

Accepted: January 28, 2015

Published: March 4, 2015

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Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported through a PhD scholarship grant from the European GENDRIVAX project (Grant Agreement no. PIAP-GA-2009-251522). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Nontyphoidal *Salmonellae* (NTS) cause a large burden of invasive and gastrointestinal disease among young children in sub-Saharan Africa. No vaccine is currently available. Previous reports indicate the importance of the O-antigen of *Salmonella* lipopolysaccharide for virulence and resistance to antibody-mediated killing. We hypothesised that isolates with more O-antigen have increased resistance to antibody-mediated killing and are more likely to be invasive than gastrointestinal.

Methodology/Principal Findings

We studied 192 NTS isolates (114 Typhimurium, 78 Enteritidis) from blood and stools, mostly from paediatric admissions in Kenya 2000–2011. Isolates were tested for susceptibility to antibody-mediated killing, using whole adult serum. O-antigen structural characteristics, including O-acetylation and glucosylation, were investigated. Overall, isolates were susceptible to antibody-mediated killing, but *S.* Enteritidis were less susceptible and expressed more O-antigen than Typhimurium ($p < 0.0001$ for both comparisons). For *S.* Typhimurium, but not Enteritidis, O-antigen expression correlated with reduced sensitivity to killing ($r = 0.29$, 95% CI = 0.10-0.45, $p = 0.002$). Both serovars expressed O-antigen populations ranging 21–33 kDa average molecular weight. O-antigen from most Typhimurium were O-acetylated on rhamnose and abequose residues, while Enteritidis O-antigen had low or no O-acetylation. Both Typhimurium and Enteritidis O-antigen were approximately

Competing Interests: FM, LL, RA, AS, CAM, SR are employees of Novartis. AS owns Novartis shares. This does not alter our adherence to all PLOS policies on sharing data and materials.

20%–50% glucosylated. Amount of *S.* Typhimurium O-antigen and O-antigen glucosylation level were inversely related. There was no clear association between clinical presentation and antibody susceptibility, O-antigen level or other O-antigen features.

Conclusion/Significance

Kenyan *S.* Typhimurium and Enteritidis clinical isolates are susceptible to antibody-mediated killing, with degree of susceptibility varying with level of O-antigen for *S.* Typhimurium. This supports the development of an antibody-inducing vaccine against NTS for Africa. No clear differences were found in the phenotype of isolates from blood and stool, suggesting that the same isolates can cause invasive disease and gastroenteritis. Genome studies are required to understand whether invasive and gastrointestinal isolates differ at the genotypic level.

Author Summary

Nontyphoidal *Salmonellae* (NTS) are an emerging major cause of invasive bacterial disease in African children aged less than 5 years and immunocompromised adults, with an estimated case fatality rate of 20–25%. NTS also cause diarrhoea, a killer of about 1.5 million young children annually, mainly in low- and middle-income countries. No vaccine against NTS is available, but improved understanding of the *Salmonella* bacteria that cause disease in Africa would help the development of new vaccines. The authors characterized a collection of 192 Kenyan NTS strains (114 *S.* Typhimurium and 78 *S.* Enteritidis) from blood and stool specimens. All strains could be killed to differing extents by antibodies present in the blood of healthy HIV-uninfected African adults, supporting the development of a vaccine that will induce protective antibodies when given to African children. Differences in killing by antibody were partly related to the amount of O-antigen on the bacterial surface. There were no clear distinction between stains causing invasive disease and diarrhoea, suggesting that the same strains may be capable of causing both forms of disease. Clarification of this will require genomic analysis.

Introduction

NTS are a major but neglected cause of invasive disease (hence iNTS disease) in Africa [1–3]. *Salmonella enterica* serovars Typhimurium and Enteritidis account for nearly 80% of all human isolates reported globally [4]. While in developed countries, these predominantly cause a mild self-limiting gastroenteritis [5–7], in Africa they are responsible for bacteraemia, often associated with meningitis in young children, with incidence rates comparable to invasive *S. pneumoniae* disease [3]. The true burden of iNTS disease is uncertain due to the absence of a characteristic clinical presentation. Patients often present with nonspecific fever [8–10] and blood culture is necessary for diagnosis. Even where blood culture facilities are available, rapid clinical progression of NTS bacteraemia results in many patients dying before a microbiological diagnosis can be made [10]. No vaccine is available, and clinical management is made difficult by widespread multi-drug resistance and the need for late-generation expensive antibiotics [11–13].

In Kenya, iNTS disease is particularly frequent in rural areas [14], with incidence rates as high as 568/100,000 person-years [15]. A recent study from Western Kenya found an association between NTS diarrhoea and mortality in hospitalized children [16], indicating that NTS isolates in the region can cause fatal invasive and gastrointestinal disease, but it is currently unknown whether specific microbial phenotypic or genotypic characteristics are associated with each clinical presentations. Whole genome sequencing studies demonstrate that invasive African *S*. Typhimurium are genetically distinct from those in the rest of the world, and are characterized by a new sequence type, ST313, that has spread throughout the continent [17]. The ST313 pathovar is associated with genome degradation and pseudogene accumulation [13]. Equivalent studies are ongoing for *S*. Enteritidis which is more prevalent than *S*. Typhimurium in some parts of Africa [18,19], and globally [20]. Investigation of the relationship between genotypic and phenotypic features of Typhimurium isolates is ongoing and a systematic characterization of endemic Typhimurium and Enteritidis isolates from one endemic region is awaited.

Lipopolysaccharide (LPS) forms the outer layer of *Salmonella* and other Gram-negative bacteria, and is key to the interaction between *Salmonella* and its environment. The O-antigen chain (including core sugars, hereafter referred to as 'OAg') constitutes the outermost part of LPS [21]. In pathogenic bacteria such as *Salmonella*, LPS plays an important role in the interaction between the bacterium and its host and is a virulence factor required for colonization and resistance to antibody-mediated killing [22]. While the lipid A moiety is the predominant cause of the endotoxic effects of LPS, OAg is the most immunodominant portion of the molecule, and is responsible for serovar-specificity [23]. OAg protects bacteria from the environment and from serum complement [24–26], which can lead to bacterial killing by membrane attack complex formation. Rough strains of *Salmonella* that lack OAg are avirulent and succumb readily to complement-mediated killing [26]. The OAg structure plays a role in bacterial virulence, with longer OAg chains associated with increased complement and antibody resistance [27–29] and protection against other host antimicrobial factors [30].

In this study we analysed a bacterial collection of 114 *S*. Typhimurium and 78 *S*. Enteritidis isolates derived from patients, mostly children, presenting to three main Kenyan hospitals in Nairobi and from 31 healthy carriers related to these patients. Our primary objective was to characterise NTS isolates circulating in endemic areas, with the goal of identifying bacterial features associated with either invasive or gastrointestinal pathology. We hypothesised that invasive isolates would, by nature, be more resistant to survival in the blood than gastrointestinal isolates, and focused our analysis on the susceptibility of isolates to antibody-mediated killing and characterization of the OAg expressed by the various isolates. We investigated the total amount of OAg expressed by the various isolates and OAg specific characteristics such as molecular weight (MW), O-acetylation and glucosylation levels, parameters which can influence OAg immunogenicity. Additionally, we tested the sensitivity of the isolates to antibody killing, using whole human serum from HIV-uninfected adults [31]. The results obtained were compared with clinical presentation in order to identify possible associations.

Materials and Methods

Origin of bacterial isolates and serum samples

The study culture collection contained 192 archived NTS isolates (114 *S*. Typhimurium and 78 *S*. Enteritidis) collected between 2000 and 2011 at the Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya. Study isolates were selected from the archived bacterial culture collection based on availability of unequivocal serovar identification and availability of clinical records and metadata. The isolates were stored in

Tryptic Soy Broth with 15% glycerol at-80°C. The isolates were from blood and stools with a few additional samples from urine (3 samples), cerebrospinal fluid (CSF, 2 samples) and environmental sources (2 samples). The isolates were mostly from children admitted to three main hospitals in Nairobi county, namely: Kenyatta National Referral hospital, Gertrude's Children's Hospital and Aga Khan University Hospital. This collection also included isolates from healthy carriers (31 samples). Before use in the present study, the archived isolates were re-cultured and the serotype was confirmed by antibody-based agglutination (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Based on the clinical sources and sample type, the NTS isolates were divided into four main groups. Those isolates from body fluids that are normally sterile such as blood and CSF were grouped as 'invasive', those from stool samples of patients were grouped as 'gastroenteric', those from stool samples of healthy individuals were grouped as 'healthy carriers' while those from soil and sewer water sources were grouped as 'environmental'. The three isolates from urine samples were considered clinically significant invasive sterile site isolates since *Salmonellae* are not part of the normal perineal skin flora and the isolates were from patients with symptoms of urinary tract infection.

Undiluted sera from ten healthy HIV-uninfected Malawian adults were used to generate a pooled serum to assess sensitivity to antibody-mediated killing of the *Salmonella* isolates. Each serum was tested prior to pooling to ensure that killing of the index *S*. Typhimurium ST313 strain, D23580, was within the 0.9 to 3.0 Log_{10} range previously described for such sera [32]. Sera were handled at 4°C during the pooling process to ensure preservation of endogenous complement activity and frozen in aliquots at-80°C. Ethical approval for preparation of this serum was granted by the College of Medical Research and Ethics Committee, College of Medicine, University of Malawi [31].

Serum Bactericidal Activity (SBA) assay

The study isolates were tested for their susceptibility/resistance to killing against the serum pool described above, using a protocol involving whole serum and endogenous complement activity as previously described [26]. Briefly, 5 μl washed bacteria at 2 h log-growth phase at an OD of approximately 0.2 (with shaking at 180 rpm) was added to 45 μl undiluted serum at a final bacterial concentration of 1×10^6 colony forming units, CFU/ml and incubated at 37°C with the number of viable bacteria count determined by serial dilution on Luria Bertani (LB) agar after 0 and 180 min. As a negative control, reference sera were heat-inactivated at 56°C for 45 min to inactivate endogenous complement and included in each SBA experiment. Additional internal controls included testing the representative endemic African *S*. Typhimurium isolate D23580, previously shown to be susceptible to serum killing [26] and hyperimmune mouse serum containing high levels of anti-OAg antibodies [33]. Susceptibility to serum killing was determined as any reduction in viable bacterial count compared with the initial *Salmonella* concentration.

O-antigen extraction and quantification

OAg extraction was performed by acid hydrolysis [34]. Bacterial isolates were grown overnight in LB medium. As OAg expression can be influenced by growth conditions, identical conditions were used for the growth of all strains [29]. The bacterial OD was measured and the bacterial cultures were concentrated in PBS to OD: 35. Acetic acid (2% v/v) was then added to the concentrated growth bacterial culture (pH 3), which were incubated for 3h at 100°C. The reaction was stopped by the addition of 14% ammonium hydroxide, increasing the pH to around 6. Bacterial debris was removed by centrifugation and OAg in the supernatant was 0.22 μm

microfiltered and desalted by HiTrap Desalting 5 ml columns (GE Healthcare, UK).
Phenol sulphuric acid assay, using glucose as standard, was used for quantification of OAg content [34,35].

^{1}H Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

^{1}H NMR analysis was performed as a confirmation of the identity of the OAg samples and to verify the presence of O-acetyl groups along the OAg chain as previously described [34].

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Glucosylation level of OAg samples was estimated by HPAEC—PAD after acid hydrolysis of the OAg to release the monosaccharides constituting the sugar chain as described previously. Commercial glucose was used for building the calibration curve (0.5–10 μg/ml). Glucose (Glc) level is calculated as molar ratio of Glc to rhamnose (Rha), sugar present in each OAg repeating unit. The analysis was also used as confirmation of the OAg samples identity verifying the correct monosaccharides ratios in the repeating unit [34,36].

Size-exclusion High-Performance Liquid Chromatography (HPLC-SEC)

HPLC—SEC analysis was used to estimate the molecular size distribution of OAg populations [34,36]. Samples were run, without pre-treatment, on a TSK gel G3000 PWXL column (30 cm x_7.8 mm; particle size 7 um; cod. 808021) with a TSK gel PWXL guard column (4.0 cm _x 6.0 mm; particle size 12 um; cod. 808033) (Tosoh Bioscience, Tokyo, Japan). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2, at the flow rate of 0.5 ml/min (isocratic method for 30 min). OAg peaks were detected by differential refractive index (dRI). Void and bed volume calibration was performed with λ-DNA (λ-DNA molecular weight (MW) Marker III 0.12–21.2 kb; Roche) and sodium azide (Merck, New Jersey, USA), respectively. OAg average MW was estimated on standard dextrans (Sigma) calibration curve.

Statistical analyses

Serum susceptibility and OAg amounts of the two *Salmonella* serovars examined (*S*. Typhimurium and *S*. Enteritidis) was compared using the Mann-Whitney U test. Possible correlations (i.e. OAg amount and serum susceptibility, glucosylation levels and OAg production) were analysed by Spearman rank.

Results

NTS bacterial collection

Of the 192 NTS isolates in the study, 114 (59%) were *S*. Typhimurium and 78 (41%) were *S*. Enteritidis. This study collection included isolates from blood, stools (patients and healthy contacts), CSF, urine and environmental/animal sources as described in Table 1 (see S1a-b Table for supporting information). The isolates were obtained from hospitals based in Nairobi, an urban setting. Clinical information on patients' age was available for 67% of cases (73 with *S*. Typhimurium, 57 with *S*. Enteritidis). The age ranged from 1 month to 59 years for *S*. Typhimurium isolates and 0 months to 64 years for *S*. Enteritidis isolates, the majority (65%, 85 isolates) of these patients being below 5 years (53 *S*. Typhimurium, 32 *S*. Enteritidis). Overall, the median age of subjects from whom *S*. Typhimurium and *S*. Enteritidis strains were isolated was 2 and 3.5 years, respectively. Ages were not available for the healthy carriers.

Table 1. Number of study NTS isolates by serovar and origin.

	S. Typhimurium (% of total *S.* Typhimurium, n = 114)								*S.* Enteritidis (% of total *S.* Enteritidis, n = 78)				Total (% of total NTS)
	blood	**Stool**	**blood & stool**	**urine**	**CSF**	**Soil**	**Sewer water**	**ND**	**blood**	**Stool**	**blood & stool**	**ND**	
Invasive	48 (42%)	–	7 (6%)	3 (3%)	2 (2%)	–	–		22 (28%)	–	5 (6%)	–	87 (45%)
Gastrointestinal	–	24 (21%)	–	–	–	–	–	–	–	32 (41%)	–	–	56 (29%)
Healthy carriers	–	21 (18%)	–	–	–	–	–	–	–	10 (13%)	–	–	31 (16%)
Environmental	–	–	–	–	–	1 (1%)	1 (1%)	–	–	–	–	–	2 (1%)
ND	–	–	–	–	–	–	–	7 (6%)	–	–	–	9 (12%)	16 (8%)
Total	**114**								**78**				**192**

ND = Isolates for which no documentation on clinical form was available; n = Total number
Gastrointestinal and healthy carrier isolates were derived from stool samples.

doi:10.1371/journal.pntd.0003573.t001

SBA results

SBA results using the serum pool demonstrated a marked difference in susceptibility to antibody-mediated killing among individual isolates and a clear difference in susceptibility between *S.* Typhimurium and *S.* Enteritidis isolates (Fig. 1, and see S2a and S2b Table for supporting information).). We have previously demonstrated that this killing is through the antibody-dependent complement-mediated mechanism and requires the presence of specific antibodies and intact complement function [26]. We have also demonstrated that the control sera used in this assay from healthy Malawian adults contains abundant antibody levels to NTS [32]. All except one *S.* Typhimurium isolate (0.9%, 1/114) underwent a reduction in viable bacterial counts over the 180 minute time course of the assay, and so were designated 'sensitive' to antibody-mediated killing. Overall median Log_{10} reduction in viable bacteria after 180 minutes was 2.9 for *S.* Typhimurium. Indeed 50% (57/114) *S.* Typhimurium were highly susceptible to killing, undergoing a 3.0 Log_{10} reduction. In contrast *S.* Enteritidis as a group were less susceptible to antibody-mediated killing than *S.* Typhimurium, being killed by a median of 1.2 Log_{10} ($p<0.0001$, Mann Whitney test). However, all *S.* Enteritidis were sensitive to killing. A small proportion (10.3%, 6/78) was killed by a full 3.0 Log_{10}.

OAg characterization

OAg was extracted from all 192 NTS study isolates normalized to the same final OD and total amount was quantified by phenol sulphuric acid assay [34]. NTS isolates produced a range of OAg amounts, with low (<10 μg/ml/OD) and high producers (>25 μg/ml/OD). On average, *S.* Enteritidis isolates expressed more OAg than *S.* Typhimurium, with 70.9% of the isolates producing more than 15 μg/ml/OD OAg compared to only 42.1% *S.* Typhimurium. Median OAg production for *S.* Enteritidis isolates was 16.8 μg/ml/OD compared to 14.4 μg/ml/OD of *S.* Typhimurium ($p<0.0001$ by Mann Whitney test) (Fig. 2). All study isolates contained one single main OAg population with average MW of 21–33 kDa (Fig. 3a); <5% isolates contained OAg population with average MW < 6 kDa.

NMR analysis on a set of 118 samples (73 *S.* Typhimurium and 45 *S.* Enteritidis) showed that only 6.8% of *S.* Typhimurium OAg were not O-acetylated, compared with 35.6% and

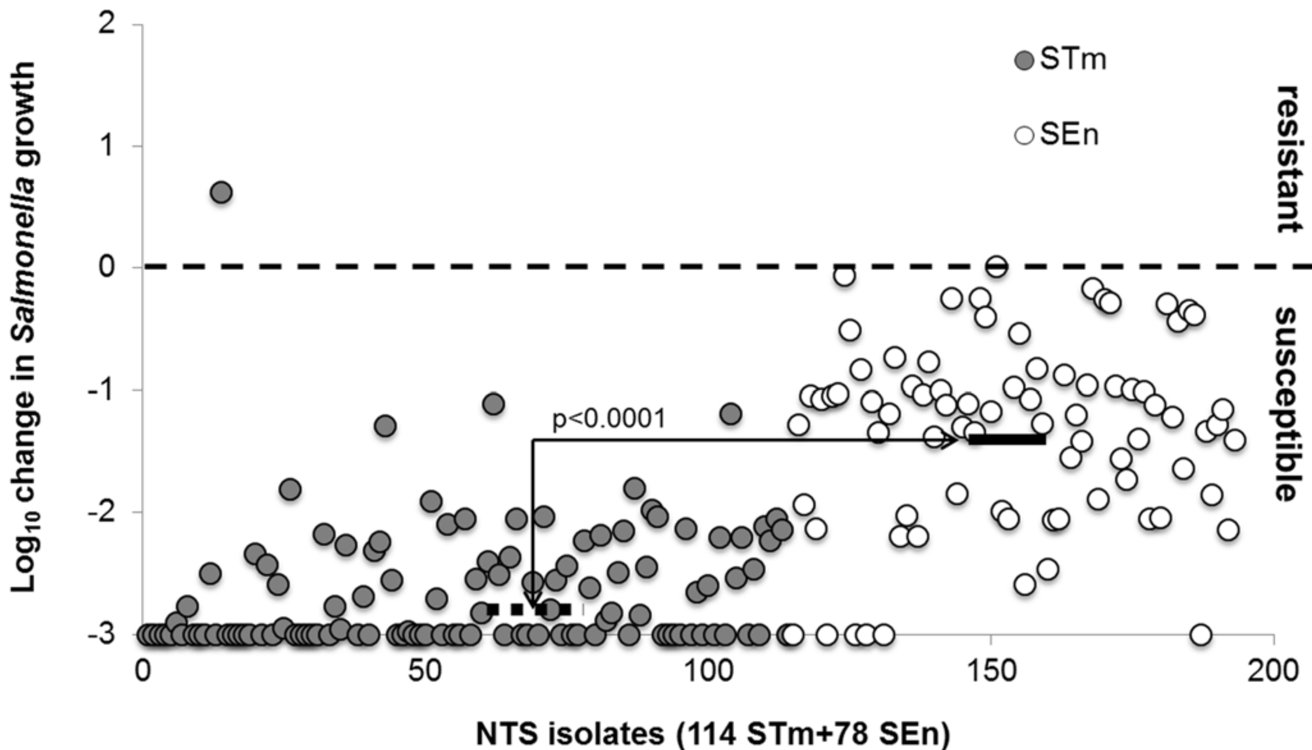

Fig 1. SBA results of STm and SEn isolates with Malawi serum pool. Bacterial colony forming units (CFU) were counted at time 0 (T0) and after 3 h (T180) incubation of bacteria in 45 μL undiluted serum (final bacterial concentration 1×10^6 CFU/ml). Killing/growth was determined by Log_{10} (CFU at T180)-Log_{10} (CFU at T0). Strains that could grow (Log_{10} change >0) were considered "resistant", strains that were killed (Log_{10} change <0) were considered susceptible. Dashed/continuous line indicates median Log_{10} change of STm (-2.9) and SEn (-1.2) isolates, respectively.

doi:10.1371/journal.pntd.0003573.g001

42.2% of *S*. Enteritidis OAg with no or traces of O-acetyl groups, respectively. For *S*. Typhimurium, OAg, O-acetylation was detected on both rhamnose (Rha) and abequose (Abe) sugar residues in the majority of isolates (63%), but also on Rha only (9.6%) and Abe only (17.8%) (Table 2; Fig. 3b and c; see S3a Table for supporting information). HPAEC-PAD was done on a subset of 91 isolates (55 S. Typhimurium and 36 SEn) and showed that for both serovars, the majority of strains (72.2%) expressed OAg with 20–50% glucosylation levels (Fig. 3d, see S3b Table for supporting information).

Analysis of associations between clinical presentation and bacterial characteristics

SBA results, OAg features and clinical presentation were compared. As mentioned above, there was a clear inter-serovar difference, with *S*. Enteritidis isolates having more OAg than *S*. Typhimurium and being more resistant to antibody-mediated killing than *S*. Typhimurium. Among *S*. Typhimurium isolates, there was a correlation between amount of OAg and resistance to antibody-mediated killing (Spearman r = 0.29, 95% CI 0.10 to 0.45, p = 0.002) in accordance with our initial hypothesis, but this was not present for *S*. Enteritidis (Fig. 4).

No correlation was found between NTS clinical presentation (invasive, gastrointestinal and carrier) and either antibody susceptibility or polysaccharide production, for either *S*. Typhimurium or *S*. Enteritidis isolates (Fig. 5). There was no clear correlation between O-acetylation levels/position and glucosylation levels with clinical presentation, antibody susceptibility and OAg production. For *S*. Typhimurium, we found an association between

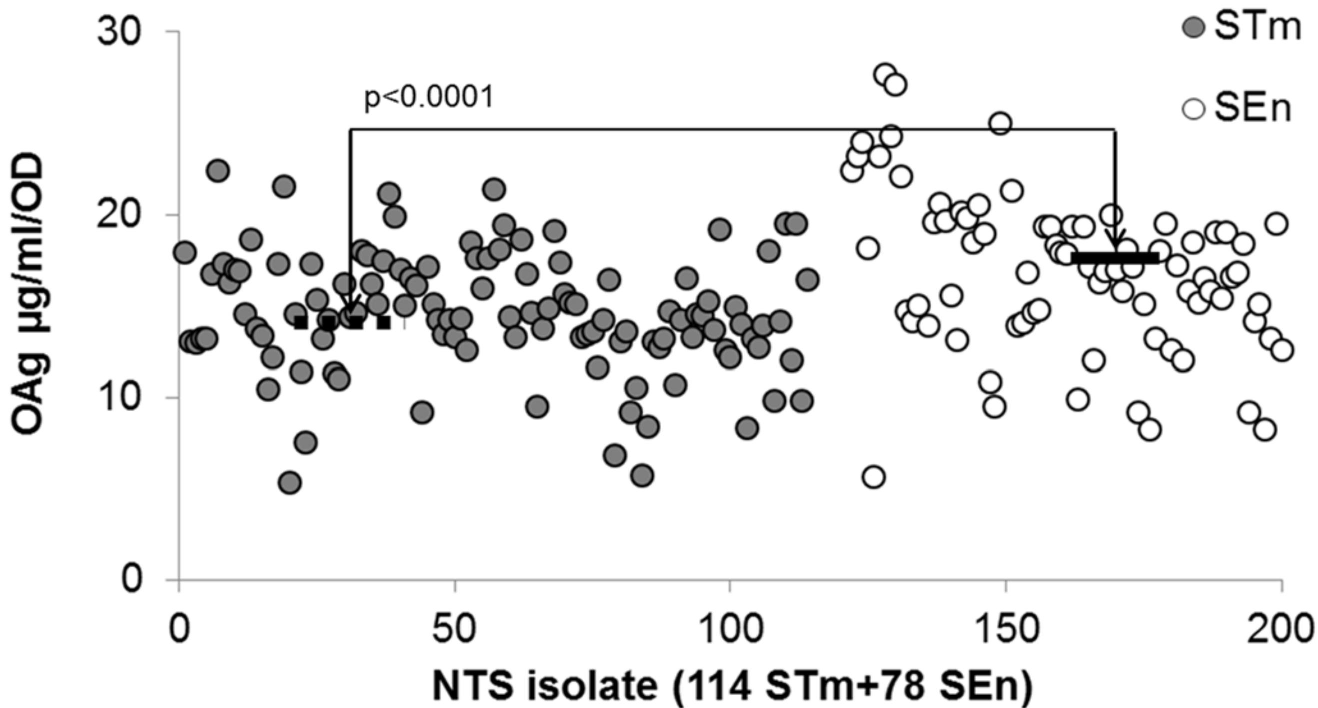

Fig 2. OAg production of *S.* Typhimurium (STm) and *S.* Enteritidis (SEn) isolates after growth and normalization to the same final OD: 35. Dashed/continuous line indicates median OAg production of *S*. Typhimurium (STm) (14.4 µg/ml/OD) and *S*. Enteritidis (SEn) (16.8 µg/ml/OD) isolates, respectively.

doi:10.1371/journal.pntd.0003573.g002

lower glucosylation levels and higher OAg production (Spearman r = 0.51, 95% CI-0.69 to-0.27, p value<0.001) (Fig. 6).

Discussion

NTS infections are a major problem in sub-Saharan Africa and can either present as bacteraemia, with symptoms of gastroenteritis in under half of patients, or as diarrheal disease without bloodstream infection [1,11,37]. Both presentations can be life-threatening, with case fatality rates of 20–25% for iNTS bacteremia in children [1,2,8,10] and up to 50% in HIV-infected adults [9], and a recognized association between NTS diarrhoea with mortality [16]. No vaccine is currently available.

The main finding of the study is that all isolates studied, apart from one *S*. Typhimurium, were susceptible to antibody-dependent complement-mediated killing, using a serum pool from an endemic region. Previously it has been shown that the antibody response specific to NTS is acquired with age, corresponding with a decline in cases of NTS bacteraemia [26,38]. The serum pool we used contained high levels of anti-OAg (both *S*. Typhimurium and *S*. Enteritidis) antibodies as expected among healthy adults in a *Salmonella* endemic region of sub-Saharan Africa such as Kenya, Malawi or Tanzania [32]. The study therefore confirms the bactericidal activity of anti-*Salmonella* antibodies against the vast majority of endemic NTS isolates in the current study. These findings support an important role for antibodies in protective vaccines against iNTS disease [31,33,39].

Another key finding is the difference in relationship between susceptibility to antibody-mediated killing and levels of OAg expression for the two *Salmonella* serovars studied. According to our study hypothesis, decreased susceptibility of *S*. Enteritidis compared with *S*. Typhimurium could be the result of higher levels of OAg, not excluding the contributions of other

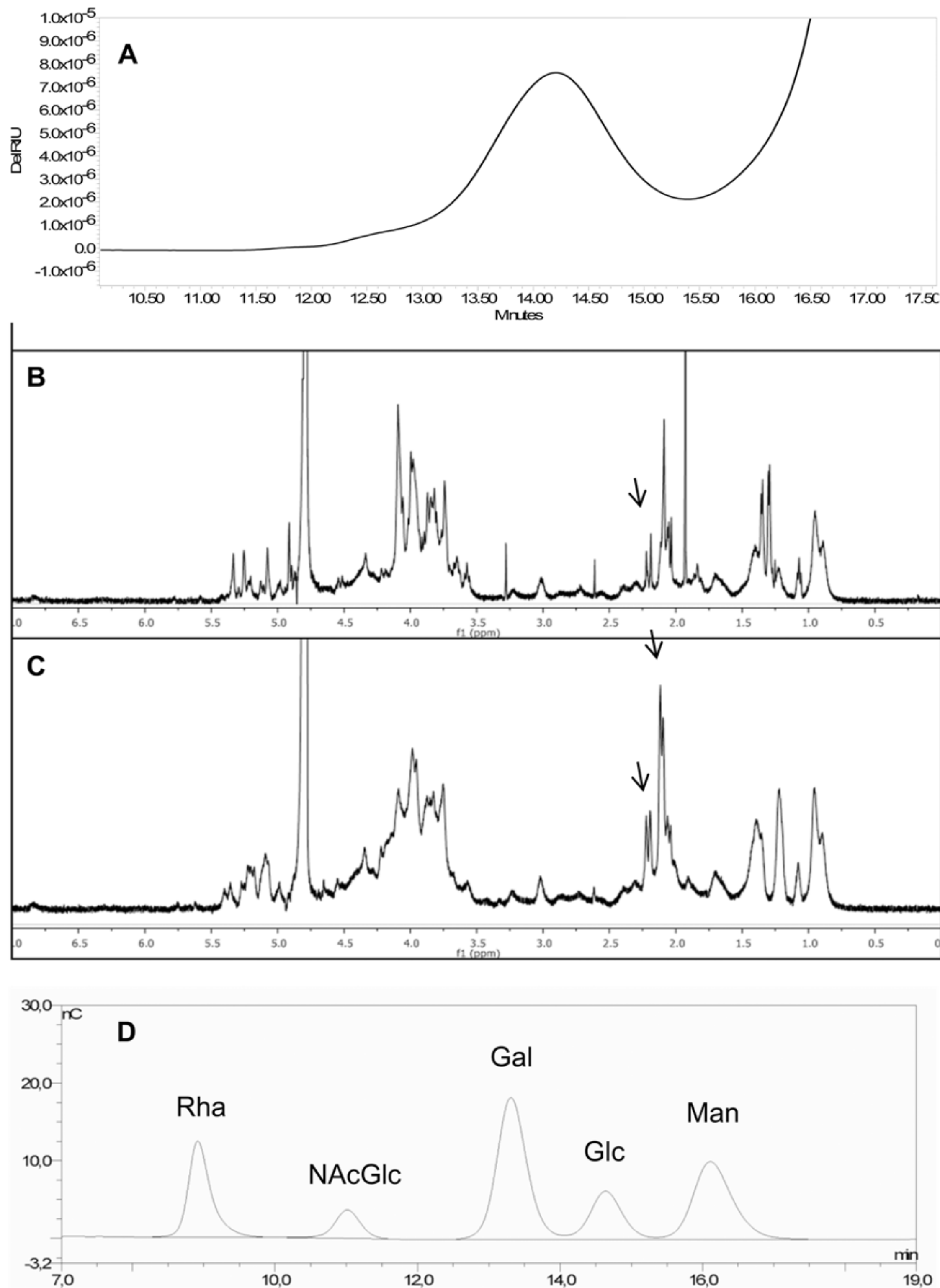

Fig 3. a) HPLC-SEC profile of *S*. Typhimurium Ke212 OAg (similar profiles obtained for all NTS strains; b) ^{1}H NMR of *S*. Enteritidis Ke189 OAg and c) *S*. Typhimurium Ke004 OAg (arrows indicate O-acetyl groups); d) HPAEC-PAD profile of *S*. Typhimurium Ke012 OAg sample indicating typical sugars of the repeating unit (Rha, Gal, Glc and Man) and of the core region (Gal, Glc and NAcGlc) (similar profiles obtained for all NTS strains).

doi:10.1371/journal.pntd.0003573.g003

Table 2. Proportion distribution in percentages of O-acetylation and glucosylation levels among NTS isolates.

	O-Acetylation (n = 73 *S*. Typhimurium, 44 *S*. Enteritidis)					Glucosylation (n = 55 *S*. Typhimurium, 36 *S*. Enteritidis)		
	% strains with O-acetyl groups			% strains with traces or no O-acetyl groups		% strains with glucosylation level		
	On both Rha & Abe	On Abe only	On Rha only	With traces of O-acetyl groups	No O-acetyl groups	<20%	20–50%	>50%
***S*. Typhimurium**	63.0	17.8	9.6	3.0	6.8	29.1	56.4	14.5
***S*. Enteritidis**	22.2			42.2	35.6	13.9	72.2	13.9

n = Total number; Rha = rhamnose; Abe = abequose

doi:10.1371/journal.pntd.0003573.t002

pathogen features, not investigated here. A previous report from Malaysia found that *S*. Enteritidis isolates were more often isolated from the blood than stool of patients compared with other *Salmonella* serovars [40], but the reasons for this were not explored. Considering that OAg length and density play a role in pathogen virulence [27], increased levels of OAg expression may enhance the ability of *S*. Enteritidis isolates to evade host first line defence mechanisms and gain access to the bloodstream. It has also been reported that, when exposed to serum, *S*. Typhimurium upregulates genes for increased production of very long chain OAg to escape complement-dependent killing [28]. Our finding of a correlation between OAg expression and resistance to antibody-mediated killing among *S*. Typhimurium isolates is consistent with the hypothesis that high OAg expression protects against such killing. It is also well know that strains lacking OAg are rapidly eliminated from the host [41,42], and there were no such isolates in our collection. However, the lack of correlation between OAg expressed by *S*. Enteritidis isolates and susceptibility to antibody-mediated killing does not support our hypothesis for this serovar concerning OAg levels and resistance to antibody killing. This finding suggests that other inherent factors of *Salmonella*, particularly *S*. Enteritidis, have a role in determining loss of sensitivity. In other words, quantity of OAg is more important for resistance to antibody-killing of *S*. Typhimurium than *S*. Enteritidis. Further studies are required to better understand the factors contributing to the inherent resistance of *S*. Enteritidis.

In relation to OAg fine specificity (O-acetylation) of the isolates in our collection, *S*. Typhimurium were more heterogeneous than *S*. Enteritidis. OAg O-acetylation occurred in almost all *S*. Typhimurium isolates, and most often at two different sites: C-2 Abe (known to provide factor O:5 specificity) and C-2/C-3 Rha. Rha O-acetylation was first reported by Wollin et al [43] and we have previously reported this as part of the characterisation of an invasive Malawian *S*. Typhimurium isolate [44]. The extent to which this additional O-acetylation was widespread in our collection suggests a possible role in pathogenesis that should be investigated further. Moreover, the fact that we could also identify *S*. Typhimurium isolates with either Abe- or Rha-O-acetylation only, shows that O-acetylation at both sites can occur independently. A *S*. Typhimurium OAg-based vaccine for Africa would need to induce antibodies capable of binding to both OAg specificities. In contrast, O-acetylation of *S*. Enteritidis OAg was not as common, with more than two-thirds of the isolates lacking or having very low levels of O-acetylation.

Glucosylation is another OAg modification for which variation has been described for *S*. Typhimurium and can occur on the O:12 antigen galactose (C4 position) generating the 12–2 variant, and on the O:1 antigen galactose (C6 position) (phage-determined) [45]. It has been

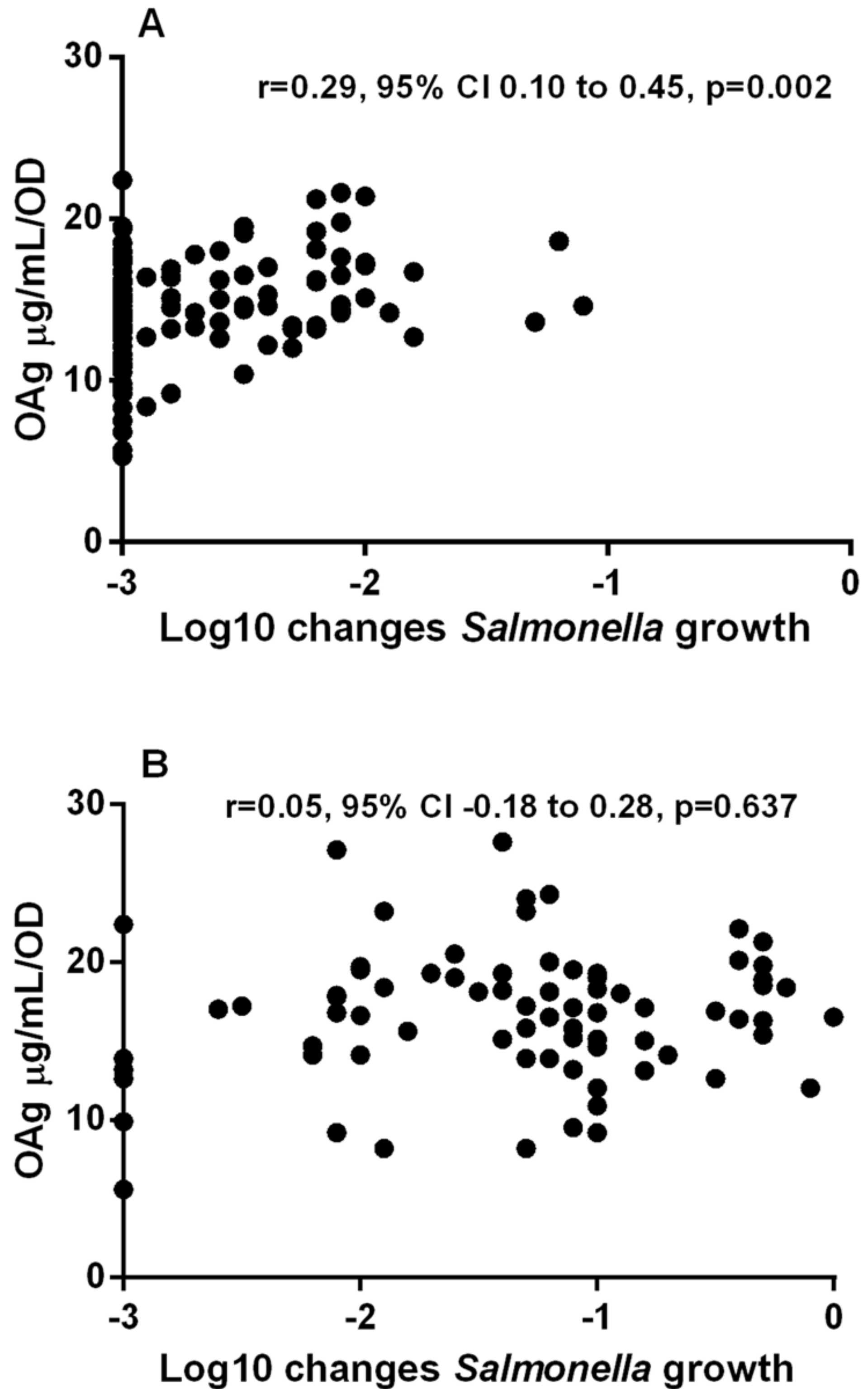

Fig 4. Correlation of SBA results and OAg production levels in (a) *S*. Typhimurium isolates (p = 0.002) and (b) *S*. Enteritidis isolates (p = 0.637). r is the Spearman correlation coefficient. CI is confidence interval.

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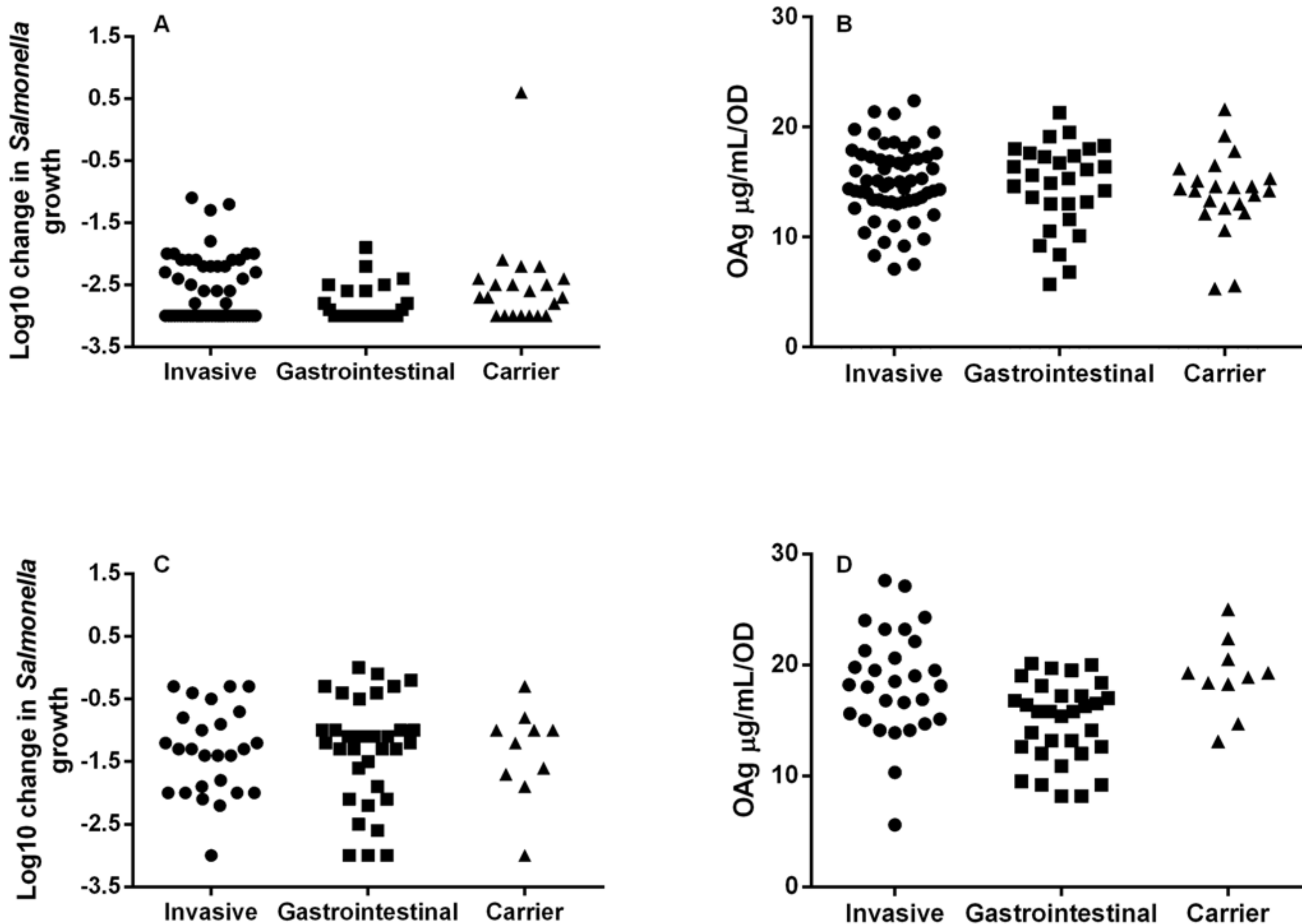

Fig 5. Correlation of SBA results (a, c) and OAg production levels (b, d) in *S*. Typhimurium (a, b) and *S*. Enteritidis (c, d) isolates with clinical presentations. Samples for which no clinical presentation was determined were excluded from analysis. Malawi serum pool was used for SBA. Invasive: *Salmonella* isolates from blood, urine, CSF. Gastrointestinal: *Salmonella* isolates from stools. Controls: *Salmonella* isolates from stools of healthy controls.

doi:10.1371/journal.pntd.0003573.g005

reported that O:12 glucosylation in Typhimurium is not constitutive, occurs during intracellular macrophage growth and is associated with enhanced intestinal colonization [46]. All *S*. Typhimurium isolates in our collection were glucosylated, with less than 30% of them at levels < 20%, showing that glucosylation is a very common feature among endemic strains. Similarly, 86% *S*. Enteritidis isolates investigated showed a glucosylation level >20%. Glucosylation in *S*. Enteritidis is not associated with specific OAg factors and it has not been extensively studied. A report on *S*. Enteritidis food-borne salmonellosis strains showed glucosylated LPS in some mouse isolates, closely related to strains obtained from eggs [47], but not in archived *S*. Enteritidis strains.

In *Shigella*, LPS glucosylation promotes bacterial invasion by enhancing type III secretion system (T3SS) function and is associated with reduced LPS chain length [48]. In *Salmonella*, T3SS is necessary for invasion of intestinal epithelium and survival within macrophages and further investigation into different glucosylation levels and T3SS-mediated invasion is still to be performed. The inverse correlation between *S*. Typhimurium OAg glucosylation and OAg expression observed in the current study may be related to T3SS function, as previously reported for *Shigella* [48] and *S*. Typhimurium [30]. *Salmonella* isolates defective for the synthesis of long and very long OAg species have been shown to have increased translocation of a

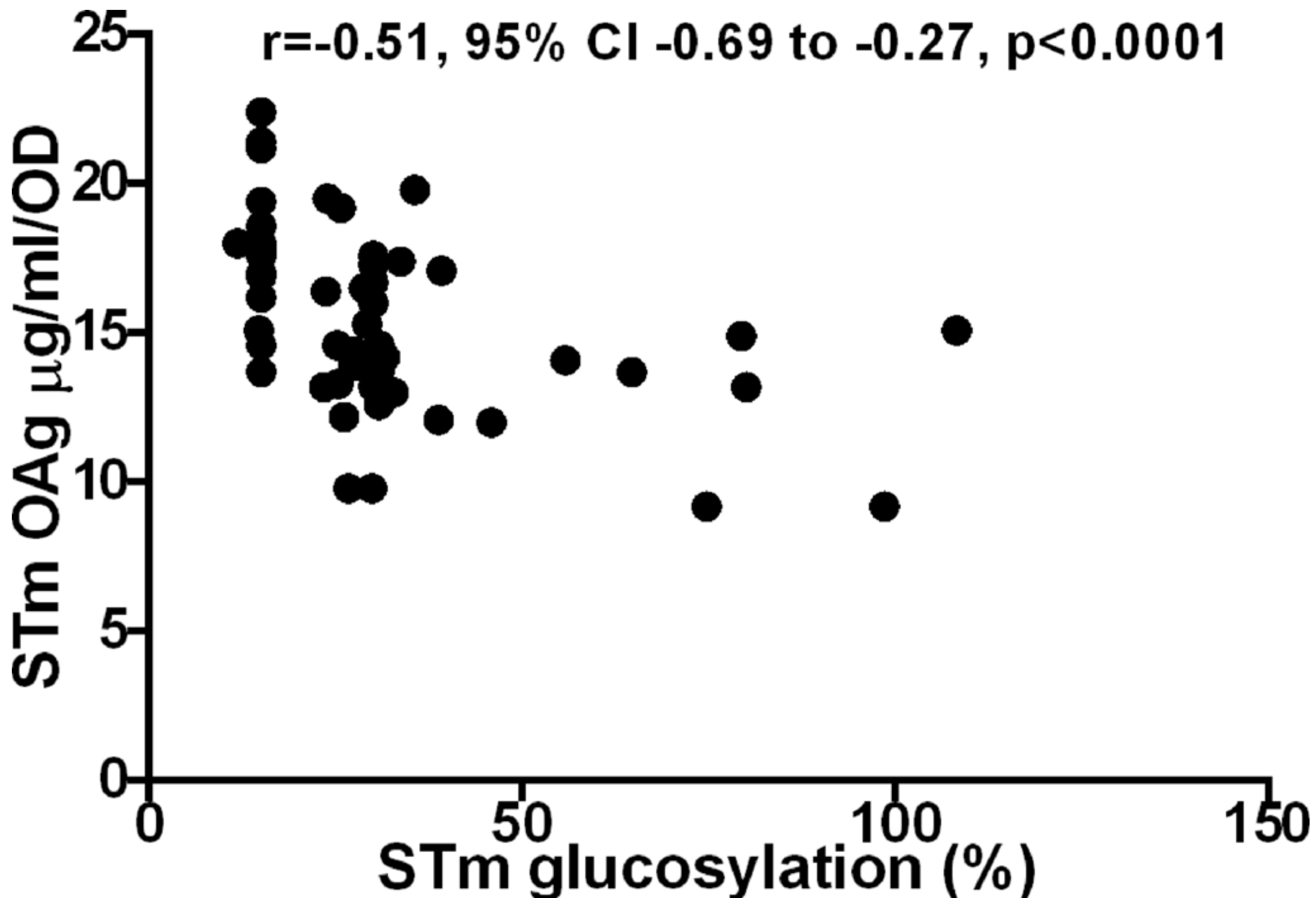

Fig 6. Correlation of *S*. Typhimurium (STm) OAg production levels and glucosylation (p<0.0001). r is the Spearman correlation coefficient. CI is confidence interval.

doi:10.1371/journal.pntd.0003573.g006

Salmonella pathogenicity island 1 (SPI1)-T3SS effector protein promoting invasion. Therefore, also for *Salmonella*, there may be the need to balance T3SS activity, possibly enhanced by shorter and glucosylated LPS, and protection against innate immune effectors, which is favoured by a thicker LPS layer [27,28].

The reasons for NTS disease presenting as invasive or enteric disease are not well understood and could relate to host immunity and/or pathogen virulence. Epidemiological data indicate a frequent presentation of the invasive syndrome, without diarrhoea symptoms, in younger age groups [49], often associated with other infections such as malaria and HIV [50–52]. However, specific investigations on the relationship between invasive and diarrheal disease are extremely limited, partially due to the limited microbiological resources in endemic African countries and few data are available on contemporaneous presence of NTS in stool and blood samples [53]. Another problem is that the concentration of *Salmonella* in the blood of bacteraemic patients can be as low as 1 CFU/ml [54] and blood culture sensitivity is estimated to be about 50% [54].

The lack of obvious difference between phenotype of NTS isolates from invasive and gastrointestinal disease episodes is consistent with the concept that invasive NTS isolates are able to cause diarrhoea and vice versa. If true, this would implicate host immunity as the major determinant of NTS disease presentation in endemic sub-Saharan African countries. Other previous

studies [49] have not managed to identify specific pathogen determinants for either invasive or gastrointestinal syndromes, therefore the interplay between host and pathogen may be key.

There are several limitations to the current study: categorization of isolates as "invasive" or "gastroenteric" was based on clinical presentation, as confirmed by microbiological analysis. Blood and stool samples belonging to the same patient were rarely collected, making it impossible to exclude that bacteria from bacteremic patients were not also present in stool and vice versa [53]. Another limitation was the lack of patients' outcome data, which could give further insights into possible differences in serovar- and isolate pathogenicity,

In summary, Kenyan NTS isolates from patients with bacteraemia and gastroenteritis were susceptible to antibody-mediated killing, supporting the development of an antibody-inducing vaccine against NTS for Africa. *S*. Enteritidis were generally less susceptible to killing than *S*. Typhimurium isolates and expressed higher levels of OAg, but while OAg expression correlated with antibody resistance for *S*. Typhimurium isolates, no correlation could be found for *S*. Enteritidis, supporting a role for other inherent bacterial factors in conferring resistance to antibody killing for this *Salmonella* serogroup. Serovar- and strain-specific differences in OAg expression and fine specificity were found. Those features could not be correlated to clinical presentation, so the same strains may be able to cause both invasive disease and diarrhoea. Whole genome analysis of invasive and gastrointestinal NTS isolates from Africa will provide additional insights regarding whether these strains are the same or different [53].

Supporting Information

S1 Table. a. List of *S*. Typhimurium study isolates and their available basic metadata; b. List of *S*. Enteritidis study isolates and their available basic metadata.
(XLSX)

S2 Table. a. Serum bactericidal assay (SBA) results for *S*. Typhimurium isolates against pooled Malawian sera. b. Serum bactericidal assay (SBA) results for *S*. Enteritidis isolates against pooled Malawian sera.
(XLSX)

S3 Table. a. O-acetylation results for *S*. Typhimurium and *S*. Enteritidis isolates. b. Glucosylation results for *S*. Typhimurium and *S*. Enteritidis isolates.
(XLSX)

Acknowledgments

We thank the Directors, NVGH and KEMRI for permission to publish this work. We are grateful to Yunshan Goh for technical assistance in the laboratory.

Author Contributions

Conceived and designed the experiments: RSO FM LL RA AS CAM SK SR. Performed the experiments: RSO FM LL RA SR. Analyzed the data: RSO FM LL RA CKO AWM GR AS SK CAM SR. Contributed reagents/materials/analysis tools: RSO FM LL RA CKO AWM GR AS SK CAM SR. Wrote the paper: RSO FM LL RA CKO AWM GR AS SK CAM SR.

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Appendix 8: Onsare et al 2014_JIDC paper:

Letter to the editor

New genomics studies to understand the link between nontyphoidal *Salmonella* bacteremia and gastroenteritis in Africa

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Key words: nontyphoidal *Salmonella*; bacteremia; diarrhea; whole genome sequencing; genomics

J Infect Dev Ctries 2014; 8(2):252-253. doi:10.3855/jidc.4377

(Received 03 November 2013 – Accepted 29 January 2014)

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Dear Editor – Nontyphoidal *Salmonellae* (NTS) are attracting increasing attention as a cause of invasive disease among African children and HIV-infected individuals [1-4]. However, these invasive (hence iNTS) infections continue to cause confusion, partly because of the contrasting clinical presentations of NTS disease in high- and low-income countries [4]. NTS in US and Europe normally cause self-limiting gastroenteritis [5]. In sub-Saharan Africa, they cause bacteremia [2] with symptoms of gastroenteritis in under half of patients [1]. Case fatality rates for NTS bacteremia are 20-25%, multidrug resistance is increasing, and no vaccine is available [1,4].

Understanding iNTS disease in Africa is hindered by our limited knowledge of diarrheal NTS and its relationship to invasive disease. Better characterisation of African diarrheagenic isolates and comparison with invasive ones would help us understand whether the same strains are responsible for both clinical presentations or whether invasive disease is the consequence of different, potentially more virulent clades of NTS. This would also provide valuable insight into routes of transmission. Since healthcare resources and services in the continent have to be prioritised for the treatment of the most critically-ill patients, more is known about iNTS disease than NTS diarrhea. Microbiological facilities are limited, so few data exist on contemporaneous presence of NTS in stool and blood. Where blood culturing occurs, stool cultures are rare, and vice versa. This does not preclude *Salmonella* in blood of patients with NTS diarrhea nor NTS in the gastrointestinal tract of those with iNTS disease.

S. Typhimurium and Enteritidis, are responsible for most iNTS cases in Africa [1,2] and confusingly around 50% of NTS gastroenteritis in the US [5]. It is unclear why iNTS is so common in Africa. Possible explanations include differences in immunity, virulence, hygiene and transmission [4]. Recent whole genome sequencing (WGS) studies demonstrate that invasive African *S*. Typhimurium is genetically distinct [6], belonging to a new MLST type, ST313. This is characterized by marked genome degradation [7] and has spread throughout the continent [3]. Studies on the genotype/phenotype relationship of ST313, and the genomes of invasive African *S*. Enteritidis, are awaited.

The Global Enteric Multicenter Study provides important insights into diarrhea etiology in developing countries [8]. Of the four African sites, NTS only significantly associated with diarrhea in Kenya, possibly because of high levels of NTS in control subjects elsewhere. Of concern, a diarrhea study from Western Kenya found NTS in stool significantly associated with mortality. Most isolates were serovar Typhimurium or Enteritidis [9]. Neither study investigated bacteremia nor genotype, so it is unknown whether *S*. Typhimurium were ST313. In a Nairobi study comparing invasive and gastrointestinal NTS

isolates from hospitalized children, *S*. Typhimurium and Enteritidis again accounted for most isolates [10]. Pulsed-field gel electrophoresis (PFGE) detected no difference between invasive and gastrointestinal isolates. However, PFGE has limited ability to discriminate genetic differences compared with WGS.

Understanding the relationship between African invasive and diarrheagenic NTS disease requires new studies that recruit patients with bacteremia and gastroenteritis, investigate the presence of NTS in blood and stool of all participants, and use WGS to characterize isolates. Another approach to the genetic characterisation and discrimination of NTS isolates is transcriptomics [11]. Transcriptomics is complementary to WGS and can lead towards improved insight into how genetic differences relate to function. There is currently little evidence to support zoonotic transmission of NTS in Africa [12] and parallel studies to identify any environmental reservoirs of NTS are needed. Until such studies happen, understanding the pathogenesis and transmission of African NTS disease, and our ability to develop new interventions to improve clinical management, will remain limited.

Acknowledgements

This work was supported by a European Union FP7 Industry and Academia Partnerships and Pathways award, GENDRIVAX (Genome-driven vaccine development for bacterial infections). This is a collaboration between the Novartis Vaccines Institute for Global Health, Kenyan Medical Research Institute, Wellcome Trust Sanger Institute and Swiss Tropical and Public Health Institute [grant number 251522].

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Conflict of interests: CAM is an employee of the Novartis Vaccines Institute for Global Health and the recipient of a clinical research fellowship from GlaxoSmithKline.