UNIVERSITY OF NAIROBI

DYNAMICS OF RESISTANCE PLASMIDS OF *Salmonella enterica* serovar Typhi IN KIBERA, AN URBAN SLUM WHERE HIGH BURDEN OF TYPHOID FEVER HAS BEEN DESCRIBED

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DECLARATION

I declare that this dissertation is original work and has not been submitted to the University of Nairobi or any other academic institution of higher learning before, for academic award.

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LIST OF ABBREVIATIONS

BP	Base Pairs
CGE	Center for Genomics Epidemiology
DNA	Deoxyribonucleic Acid
DTU	Technical University of Denmark
IATA	International Air Transport Association
Inc.	Incompatibility group
KEMRI	Kenya Medical Research Institute
KNH-UoN	Kenyatta National Hospital and University of Nairobi ethical committee
MLST	Multilocus Sequence Type
oriT	Origin of Transfer
PBIDS	Population-Based Infectious Diseases Surveillance system
pMLST	Plasmid Multilocus Sequence Type
PYO	Person-years of observation
SNP	Single Nucleotide Polymorphism
SPI	Salmonella pathogenicity islands
US-CDC	United States Centers for Disease Control and Prevention
WGS	Whole Genome Sequencing

OPERATIONAL DEFINITIONS

Helper plasmids	Conjugative plasmids which aid transfer of non-conjugative but mobilizable plasmids
Incompatibility group	Classification method of plasmids that is based on inability of plasmids sharing similar replication 'back-bone' proteins to be propagated stably in the same cell line, thus, plasmids which are stably propagated in a cell line belong to different incompatibility groups.
Mobile genetic elements	Suite of genetic molecules (e.g. transposons, integrons, plasmids) which can translocate freely within a bacterium or exchanged between the same or different species of bacteria.
Recombination	DNA hybridization process involving integration of foreign DNA segments into bacterial chromosome through breakup and rejoining of involved DNA strands. The foreign segment may originate from a different overlapping chromosome within same bacteria or from an acquired genetic molecule.
Secretion systems	Mechanisms used by bacteria to transfer protein and nucleic acids from one cell to another within and between species.

LIST OF APPEND1CES

Appendix 1: Copies of informed consent and assent forms

Appendix 2: KEMRI ethics approval letters for PBIDS platform

TABLE OF CONTENTS

SUPERVISORS ii
ACKNOWLEDGMENTS iii
LIST OF ABBREVIATIONS iv
OPERATIONAL DEFINITIONS v
LIST OF APPEND1CES vi
TABLE OF CONTENTS vii
ABSTRACT 1
CHAPTER 1 2
1. INTRODUCTION 2
1.1. PROBLEM STATEMENT 3
1.2. JUSTIFICATION 3
1.3. RESEARCH QUESTIONS 4
1.4. HYPOTHESES 4
1.5. OBJECTIVES 4
CHAPTER 2 6
2. LITERATURE REVIEW 6
2.1. Typhoid Fever 6
2.1.1. **Clinical overview** 6
2.1.2. **Burden (global and regional)** 6
2.1.3. Typhoid fever in Kibera 7
2.1.4. Adaptation of *S.* Typhi 8
2.2. Resistance mobile genetic elements of *S.* Typhi 11
2.2.1. Insertion sequences 11
2.2.2. Integrons 11
2.2.3. Transposons 12
2.2.4. Resistance Plasmids 12
CHAPTER 3 16
3. METHODS 16
3.1. Study design 16
3.2. Study Site 16
3.3. Study Population 17

3.4. Study procedures 17
3.4.1. Kibera Population-based infectious diseases Surveillance System 17
3.4.2. Sample size 18
3.4.3. Whole Genome Sequencing 19
3.4.4. Bioinformatics 21
3.4.5. PCR 21
3.4.6. Study variables 22
3.4.7. Statistical analysis 22
3.5. Ethics 23
CHAPTER 4 24
4. RESULTS 24
4.1. Resistance plasmid replicon families 24
4.2. Transposons and resistance genes 24
4.3. Changes in plasmids over time 26
4.3.1. Mutations within the QRDR region 36
4.4. Changes in disease burden and replicon families 36
CHAPTER 5 37
5. DISCUSSION 37
5.1. Limitation 39
5.2. Conclusion and recommendations 39
5.3. Future studies 40
6. REFERENCES 41
7. APPENDIX 46
7.1. Appendix 1: Consent and Assent Forms 46
8.2. Appendix 2: KEMRI and UoN_KNH ethics Approval Letters 51

ABSTRACT

Background

Multidrug resistance (MDR) in *Salmonella enterica* serovar Typhi is mainly plasmid-mediated, yet, little is known of the plasmid dynamics in typhoid endemic settings.

Methods

We used whole genome sequence data from 304 (81.9%, n=371) bacteremic *S.* Typhi, isolated during an 11-year period (2007-2017) in Kibera, an urban informal settlement in Nairobi, Kenya. Sequences were analyzed for plasmid replicon families, transposons, integrons, insertion sequences (IS) and resistance genes. Temporal changes in identified plasmid replicons were compared with typhoid incidence trends.

Results

A previously described IncHI1-PST 6 plasmid carrying an IS*1* composite transposon with a partial IncQ1 replicon, streptomycin *(aph(3'')-Ib)*, kanamycin *(aph(6)-Id)*, ampicillin *(bla*$_{\text{TEM-1b}}$), chloramphenicol *(catA1)*, trimethoprim *(dfrA7)*, and sulfonamide *(sul1* and *sul2)* resistance genes, dominated until 2013. Chromosomal integrations of the transposon were observed at *cyaA* and *uxuA* intergenic regions, and often coincided with loss of the IncHI1 plasmid. Plasmid-free strains were commonly identified during periods of decreased typhoid fever incidence.

Conclusion

We show expansion of plasmid-free strains with a chromosomally integrated MDR composite transposon. The strains coincided with periods of decreased typhoid incidence. Absence of the IncHI1 plasmid could potentially allow the strains to acquire additional plasmid-mediated resistance. Surveillance for resistance plasmids in areas with a high burden of typhoid fever can help inform efforts to control multidrug resistance.

CHAPTER 1

1. INTRODUCTION

Antimicrobial resistance in *S.* Typhi is expanding, strains resistant to multiple traditional first-line antibiotics (ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) have become common with the global expansion of *S.* Typhi genotype 4.3.1 (H58 subclade) (Wong et al., 2015), while those resistant to fluoroquinolones and third generation cephalosporins are emerging (Klemm et al., 2018). Increasing AMR has limited treatment options for typhoid fever risking adverse outcomes (Andrews, Qamar, Charles, & Ryan, 2018). Further, resistant strains persist longer and can increasingly transmit between individuals, which risks resurgence of typhoid fever burden (Pitzer et al., 2015).

Antimicrobial resistance in *S.* Typhi is mainly plasmid-mediated (Carattoli, 2003). Plasmids are naturally occurring extra-chromosomal DNA molecules that carry accessory genes (Carattoli, 2013). They can be shared within and between bacterial species, and thus play a key role in genetic expansion (Bolotin & Hershberg, 2017; Holt et al., 2011). Introduction of a plasmid in a bacterial population is often associated with co-adaptation processes such as chromosomal integration of beneficial plasmid regions and subsequent loss of other costly plasmid parts, as well as, compensatory mutations that often restore functions potentially lost or disrupted with the acquisition of plasmid (Porse, Schønning, Munck, & Sommer, 2016; San Millan et al., 2014). These processes are generally aimed at improving cost of plasmid carriage and optimizing plasmid vertical inheritance, and ultimately often frees the host population to acquire additional plasmids (Stalder et al., 2017). Other processes that can influence maintenance of plasmid by a bacterial host population include plasmid regulatory systems (plasmid replication and entry exclusion systems), which control coexistance of plasmids sharing similar replication proteins and copies of plasmids per cell (Kamruzzaman, Shoma, Thomas, Partridge, & Iredell, 2017; Sørensen, Bailey, Hansen, Kroer, & Wuertz, 2005). Understanding these dynamics can inform monitoring of antibiotic resistance risk such as, when new resistance plasmid can be acquired.

Resistance plasmids of *S.* Typhi are dynamic. Plasmid families varies across genotypes (Park et al., 2018), and within genotypes over time characterized by mutations and recombination processes, and new plasmid acquisitions. In the absence of bacterial sexual reproduction, they can be the primary force for evolution including in *S.* Typhi (Hendriksen et al., 2015; Klemm et al.,

2018; Partridge, Kwong, Firth, & Jensen, 2018). Various sub-strains of the globally dominant MDR *S*. Typhi genotype 4.3.1 have been described including strains carrying an MDR composite transposon on an IncHI1 plasmid; plasmid-free strains, with a chromosomally integrated copy of the MDR transposon; those with additional point mutations in the quinolone resistance determining region (QRDR) and XDR strains with a chromosomally integrated MDR transposon, QRDR mutations and an IncY plasmid with extended-spectrum β-lactamase (*bla*$_{\text{CTX-M-15}}$) and fluoroquinolone resistance (*qnrS*) genes (Hendriksen et al., 2015; Holt et al., 2011; Klemm et al., 2018; Park et al., 2018; Pham Thanh et al., 2016; Wong et al., 2015). Emergence and expansion of a sub-strain with a novel plasmid has often been followed by a change in typhoid fever incidence (Pitzer et al., 2015; Klemm et al., 2018). While much of our understanding of *S*. Typhi resistance plasmids dynamics comes from the study of outbreaks, little is known of the dynamics in endemic settings. Here we looked at the plasmids, their temporal variations and association with typhoid fever incidence, over a period of 11 years (2007-2017) in Kibera, an urban slum where high rates of typhoid fever have been described (Breiman et al., 2012).

1.1.PROBLEM STATEMENT

Approximately 11 million typhoid fever infections and 117 000 deaths were reported globally, in 2017 (Stanaway et al., 2019). The estimates were largely driven by typhoid burden data from poor settings in Asia and sub-Saharan Africa, where there is still limited access to clean water and sanitation facilities (Kim, Mogasale, Im, Ramani, & Marks, 2017; Marchello, Hong, & Crump, 2019). Antibiotic resistance is increasing (Park et al., 2018) and multi- and extensively-drug resistant *S*. Typhi strains have been described (Mogasale et al., 2014; Pitzer et al., 2015; Rasheed, Hasan, Babar, & Ahmed, 2019). These strains have limited treatment options for the disease and have been associated with adverse outcomes (Andrews et al., 2018). Resistance in *S*. Typhi is mainly plasmid mediated (Carattoli, 2003), however, little is known of the plasmid dynamics in typhoid endemic areas such as Kibera (Breiman et al., 2012).

1.2. JUSTIFICATION

Information on dynamics of resistance plasmids is critical for understanding emergence and spread of antimicrobial resistance in *S.* Typhi, and could guide public health action on prevention and control of resistant *S.* Typhi.

1.3.RESEARCH QUESTIONS

In this study we asked the following questions:

a. Which resistance plasmid are carried by *S.* Typhi strains circulating in the study area?

b. What are the genomic characteristics of the identified plasmids?
    i. Replicon families
    ii. Transposons and integrons
    iii. Resistance genes

c. How have the plasmids varied over time?
    i. Plasmid replicon types
    ii. Changes in nested mobile genetic elements
    iii. Changes in resistance gene profile over time

d. How does temporal changes in resistance plasmids relate with changes in typhoid fever incidence in the area?

1.4.HYPOTHESES

a. Plasmids of incompatibility group IncHI1 will be the most prevalent (Kariuki et al., 2010; Park et al., 2018)

b. Plasmid types and resistance genes will vary over time, and changes in the dominant plasmid types will coincide with changes in typhoid fever incidence in the population

1.5.OBJECTIVES

Main Objective

To characterize resistance plasmids of *S.* Typhi in Kibera, and describe their molecular variations (replicon types, plasmid sequence types, anchored transposons, integrons, insertion sequences and resistance genes) over time

Specific Objectives

i. Identify and characterize resistance plasmids (replicon types, plasmid sequence types, anchored transposons, integrons, insertion sequences and resistance genes) of *S.* Typhi in Kibera.

ii. Describe temporal changes in the resistance plasmids in relation to typhoid fever incidence in Kibera.

CHAPTER 2

2. LITERATURE REVIEW

2.1.Typhoid Fever

2.1.1. Clinical overview

Typhoid Fever is a systemic, potentially life-threatening infection caused by *Salmonella enterica* serovars Typhi and Paratyphi A (Baker et al., 2011). Classical symptoms of the disease include; acute high fever, rose spots (in light skinned persons), headache, malaise, bradycardia and abdominal pain. Complications may include bleeding, perforation, cholecystitis, and cholangitis (Crump, Sjölund-Karlsson, Gordon, & Parry, 2015).

2.1.2. Burden (global and regional)

Global estimates for typhoid fever burden have been variable, perhaps due to changes in typhoid epidemiology, or methodological differences in estimating typhoid fever burden. Consequently, recently published data have estimated annual global burden ranging between 10 and 24 million cases, with about 1% fatality (Antillón et al., 2017; Buckle, Walker, & Black, 2012; Crump, 2014; Mogasale et al., 2014).

Until recently, limited information existed on typhoid burden in Africa. Early global estimates were largely influenced by population-level data from Asia, however recent improvements in typhoid surveillance in Africa have now provided better insight of global and regional estimates. It is now evident that typhoid incidence in many settings in Africa parallels those of endemic settings in Asia (Figure 1) (Mogasale et al., 2014; Steele, Hay Burgess, Diaz, Carey, & Zaidi, 2016).

In East Africa, about 1.1 million cases of typhoid fever occur annually (Kim et al., 2017). The burden is particularly high among children aged 2-9 years. A typhoid study led by Mogasale, estimated adjusted incidence in children 2-4 years and 5-9 years at 2248 cases and 1788 cases per 100,000 persons respectively (Akullian et al., 2015; Mogasale et al., 2014). These rates, however, were largely influenced by previous rates from Kibera, a slum in Nairobi, Kenya (Figure 2).

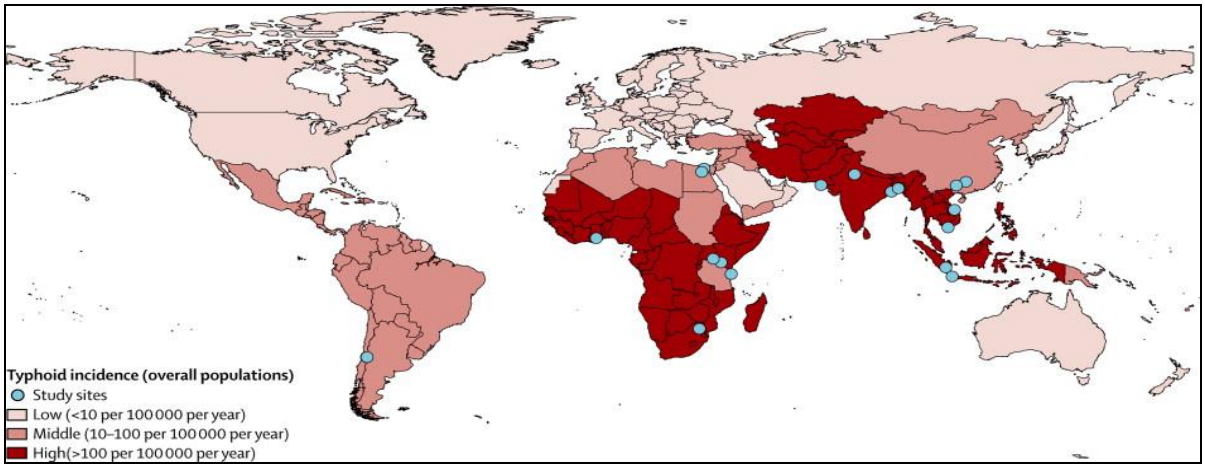


Figure 1: Global distribution of typhoid fever burden in Low-and Middle-income countries, as of 2014. (M.J. Carvalho and J. Tyrrell1, 2015; Mogasale et al., 2014; Tyrrell, 2015)

2.1.3. Typhoid fever in Kibera

A population-based typhoid fever surveillance, led by Breiman *et al*, estimated an overall crude burden of 247 cases per 100,000 person-years of observation (pyo), in Kibera (Breiman et al., 2012). Highest rates were reported in children 5-9 years old and 2-4 years old (596 cases and 521 cases per 100000 pyo respectively), age groups considered at risk due to naïve immunity and increased exposure to environment (Akullian et al., 2015). However, the rates have since declined. Review of typhoid rates between 2007 and 2017 revealed a fluctuating but declining trend of the disease. Current crude rates in children 2-4 and 5-9 years are estimated at 215 cases per 100,000 pyo and 203 cases per 100,000 pyo respectively (Ng'eno DE, unpublished). In contrast, increasingly resistant strains emerged and persisted during this period. By 2017, approximately 75% of isolated *S.* Typhi were resistant to ampicillin, chloramphenicol and trimethoprimsulfamethoxazole. Resistance to nalidixic acid rose from 7% in 2012 to 11% in 2017. Prevalence of ciprofloxacin and ceftriaxone resistance were estimated at 5% and 3% respectively (Ng'eno DE, unpublished). Mechanisms underlying emergence and spread of resistance in the area remain unclear, though a previous study on resistant *S.* Typhi isolated from hospitals in Nairobi, pointed at plasmids of incompatibility group (Inc) HI1 (Kariuki et al., 2010)

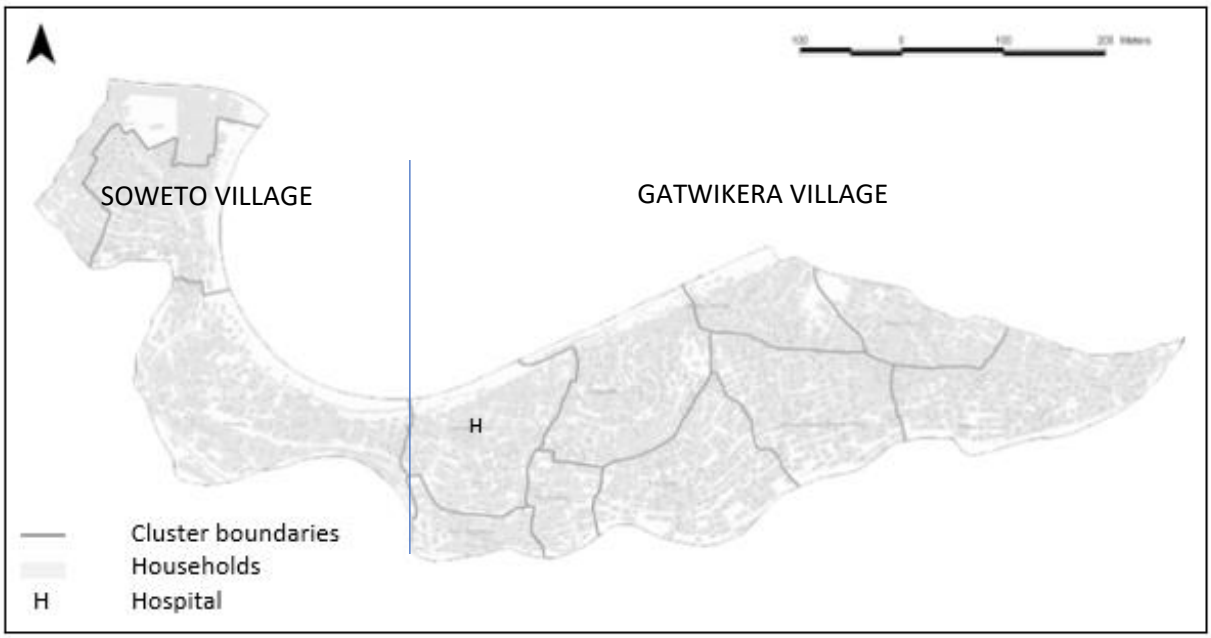


Figure 2: Population-based infectious diseases surveillance area

2.1.4. Adaptation of *S.* Typhi

Ability of *S.* Typhi to quickly adapt to changing environments has enabled its persistence and global distribution. At the core of the adaptation are genetic transfer systems which aid movement of survival genes from one DNA molecule to another within and between bacteria (Ochman & Moran, 2001). Within transfers are regulated through non-conjugative transposons and integron systems, by recombination mechanisms, while between transfers involve conjugative transposons and plasmids. Other active genetic transfer systems include bacteriophages (transposing bacteriophages) (Bennett, 2008; Hallstrom & McCormick, 2015).

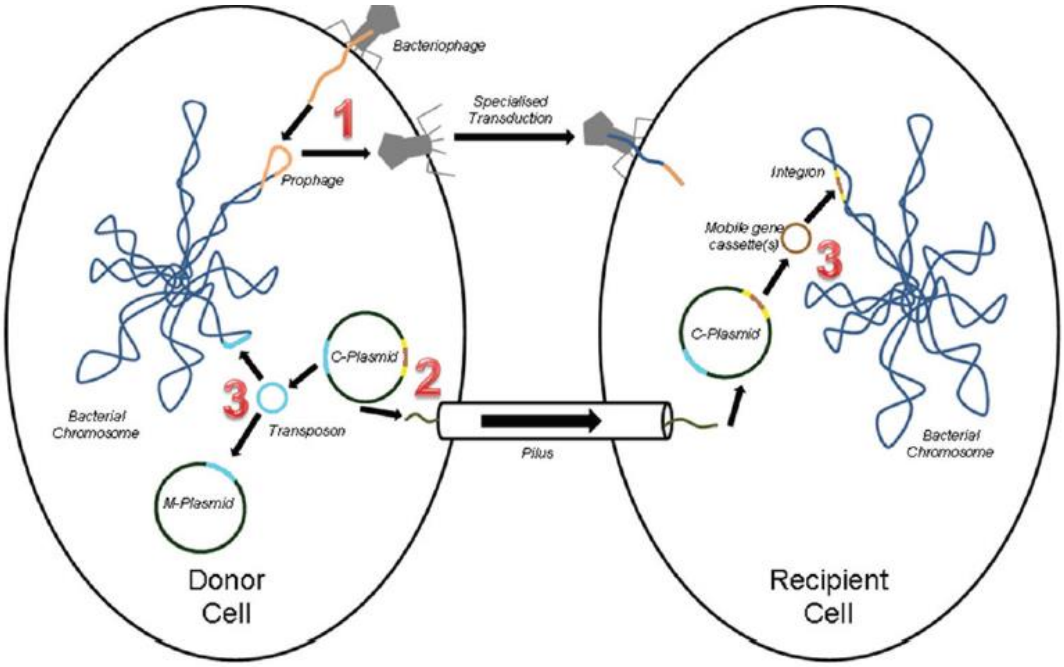


Figure 3: Summary of bacterial genetic transfer systems: System 1; transduction, System 2; conjugation, System 3; transposon and integron systems with illustration of recombination events

Conjugative systems involve pairing of donor and recipient cells, and transfer of plasmids or integrative and conjugative elements through bacterial secretion systems. Transduction is a bacteriophage dependent transfer system, while transformation involves random uptake, by a competent bacterial cell, of DNA material from dead cells (figure 4) (Ellison et al., 2018; Wozniak & Waldor, 2010). A recent study comparing the three horizontal gene transfer mechanisms identified conjugation as the dominant gene transfer mode (Halary, Leigh, Cheaib, Lopez, & Bapteste, 2010).

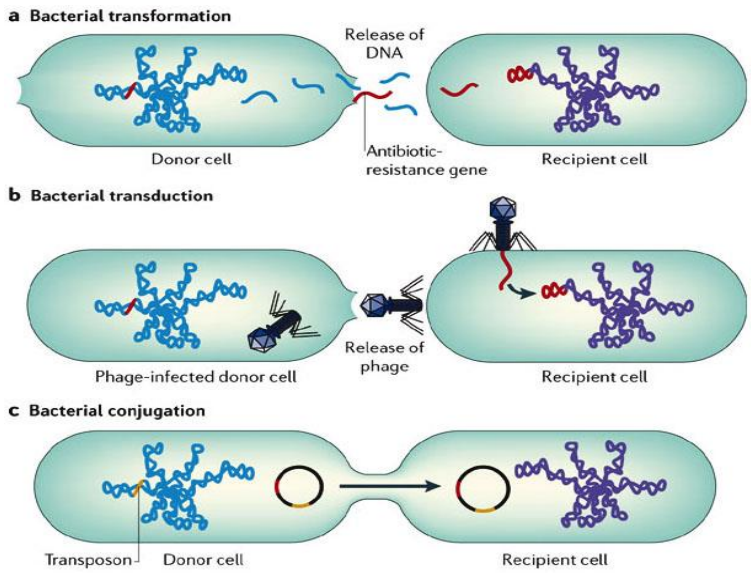


Figure 4: Horizontal gene transfers systems. Transformation: occurs through direct uptake of DNA fragments by competent bacteria from environment. Transduction: involves transference of DNA from one bacterium into another through bacteriophages. Conjugation: occurs through movement of DNA from one bacterium into another through sexual pilus (Furuya & Lowy, 2006).

Two types of conjugative systems have been described; Single stranded DNA (ssDNA) conjugation systems, and double stranded DNA (dsDNA) conjugation systems (Llosa, Gomis-Rüth, Coll, & de la Cruz Fd, 2002). Single stranded DNA conjugation is the most common transfer system in Gram negative bacteria while dsDNA conjugation systems are limited to filamentous bacteria such as Actinobacteria. In ssDNA, a single strand of conjugative DNA is transferred from donor to recipient. It involves nicking of transferable DNA at the origin of transfer (*oriT*) by relaxases proteins encoded by conjugative plasmids or transposons. The proteins then bind the detached circular DNA fragment forming a nucleotide-protein complex. The complex attaches to the type IV secretion system (T4SS) and is transported across the cell membrane into the cytoplasm of the recipient cell (Llosa et al., 2002). Replication of single stranded copies of DNA in donor and recipient cells then occurs, resulting in formation of double stranded DNA molecules which may exist autonomous of host chromosomal DNA or integrate into the host chromosomal DNA in the case of conjugative transposons (Cury, Touchon, & Rocha, 2017).

This study described genetic elements of *S.* Typhi, with an emphasis on resistance plasmids, which have been shown to play a critical role in the recent evolution of *S.* Typhi strains (Hendriksen et al., 2015; Holt et al., 2011).

2.2.Resistance mobile genetic elements of *S.* Typhi

Currently described resistance mobile genetic elements of *S.* Typhi include insertion sequences, integrons, transposons and plasmids.

2.2.1. Insertion sequences

Insertion sequences (ISs) are the smallest transposable elements defined by flanking inverted repeat ends and a gene encoding transposase. Typically, they do not to carry adaptive genes, however they can aid translocation of resistance genes in a composite transposon (a transposon flanked by same or related IS elements (Partridge et al., 2018)). An example of a composite IS is the IS*1* which translocate Tn*9* carrying *catA1* which confers chloramphenicol resistance (Partridge et al., 2018).

2.2.2. Integrons

Integrons (In) are gene acquisition systems for bacteria. The basic In structure consists of an integrase gene (*intI*), a promoter (Pc), a recombination site (*attI*), and gene cassettes (Gillings, 2014). Gene cassettes are compact DNA elements made of a single open reading frame (ORF) which often encodes a resistance gene, expressed from the Pc promoter and a recombination site (*attC*). In acquire new gene cassettes through recombination mechanisms between the *attC* of a circular cassette and the *attI* site of the In as shown in figure 5. Different classes of In have been described based on differences and divergence in the sequences of *intI*. Class 1 integrons are mostly associated antibiotic resistance.

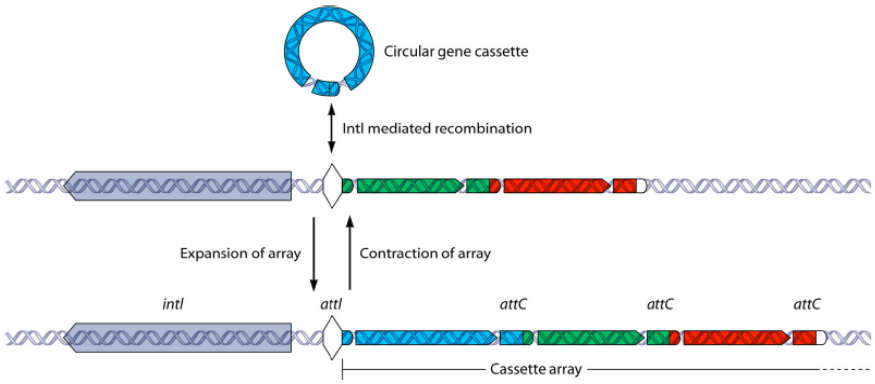


Figure 5: A diagram illustrating gene acquisition by recombination mechanisms in integrons (Gillings, 2014)

2.2.3. Transposons

Transposons (Tn) are characterized by inverted repeats terminals, a transposase gene (*tnpA*) and a gene or genes encoding an adaptive phenotype (Figure 6). Majority of the transposons also carry a resolves gene (*tnpR*) which encodes resolvase enzyme that aids site-specific recombination (Partridge et al., 2018). Unlike ISs, Tn have larger or multiple transposase. They move genetic material within and between chromosome, from a chromosome to a plasmid and contrariwise, and from one plasmid to another (Bennett, 2008). The transfers can occur indiscriminately but many show preference for certain nucleotide sequences.

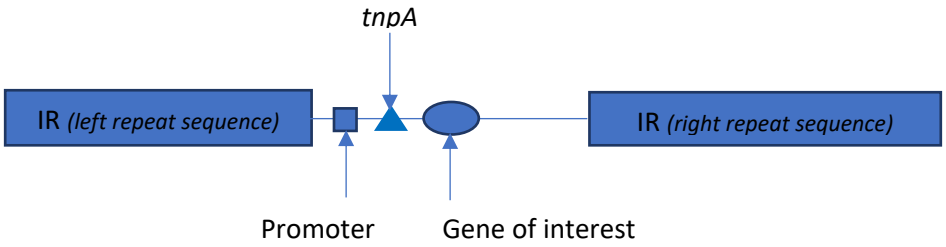


Figure 6: A map of a classical transposon structure

Some of the described Tn families which carry resistance genes in *S*. Typhi include Tn*3* family of transposons comprising of Tn*1* and Tn*2* carrying bla_{TEM} genes, encoding extended-spectrum *β*-lactamases (ESBL), Tn*5393* carrying *strAB* genes conferring streptomycin resistance, Tn *21* subfamily of transposon carrying *tetA* (tetracycline resistance gene) as well as Tn*7* family comprising of Tn*5053*-like transposons carrying a class 1 integron with *dfrA1* (a trimethoprim resistance gene) and *sul1* encoding resistance to sulfonamides (Partridge et al., 2018), Tn*9* carrying *catA1* which confers chloramphenicol resistance and Tn*10* carrying *tetB*, also a tetracycline resistance gene (Holt et al., 2011).

2.2.4. Resistance Plasmids

Plasmids are extra chromosomal mobile genetic molecules. They can be transferred both horizontally between bacteria and vertically during cell division. Horizontal transmission often occurs within members of the same species but can also occur between species and families. Two broad categories of plasmids have been described; conjugative plasmids which are capable of self-propagating, the plasmids carry complete set of genes regulating self-transfer

including; origin of transfer (*oriT*), relaxase protein, type IV coupling protein (T4CP) and T4SS, and mobilizable plasmids which do not encode T4SS but encode a suite of mobilization proteins (MOB) that enable them to hitchhike on conjugative plasmids (helper plasmids) for their transfer (Garcillán-Barcia, Alvarado, & de la Cruz, 2011; Smillie, Garcillán-Barcia, Francia, Rocha, & de la Cruz, 2010). Consequently, mobilizable plasmids are often relatively smaller in size compared to conjugative plasmids.

Plasmids often act as scaffolds on which other genetic elements are nested including Tn and In, and are thus the main players among mobile genetic elements (Halary et al., 2010). Structurally plasmids are usually circular and double stranded. They comprise core and non-core genes. Core genes are essential for regulating their transfers while non-core genes confer survival benefits to the host (figure 7).

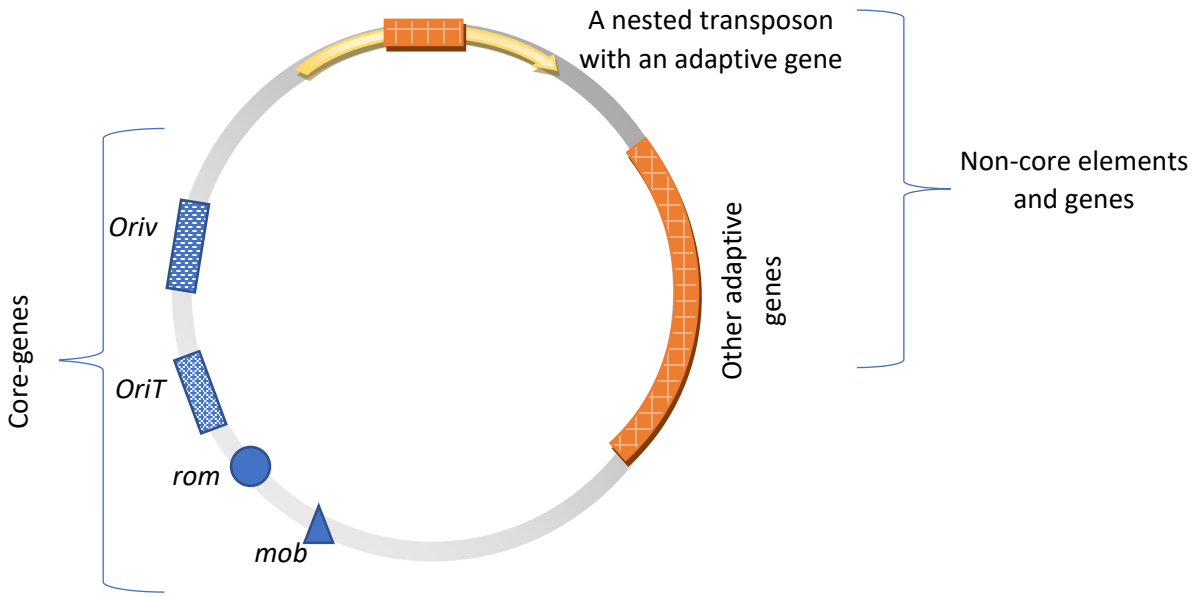


Figure 7: Genetic map of a typical plasmid; *mob* represents genes coding for mobilization protein, *rom* codes for proteins regulating copy number, OriT, origin of transfer, Oriv, origin of replication

Some of the non-core genes include resistance genes, virulence genes, and genes essential for survival in the environment, among others. Some of these genes and their carrier plasmids have been shown to integrate into sites on chromosomal DNA (Hendriksen et al., 2015). An example of a typical sequenced plasmid of *S.* Typhi is pHCM1 which is highly related (> 99% sequence

identity) to plasmid R27 of *Salmonella* serotype Typhimurium. The plasmid carries genes conferring antibiotic resistance, including; *dhfr1b* (trimethoprim), *sul2* (sulphonamide), *catA1* (chloramphenicol), $bla_{\text{TEM-1b}}$ (ampicillin) and *aph(3')-Ib* (streptomycin) (Holt et al., 2007; Parkhill et al., 2001).

2.2.4.1. Classification of plasmids

Different schemes have been used to classify plasmids. Among the earliest schemes are phenotypic classification based on conjugative properties of plasmids and incompatibility grouping (Novick & Hoppensteadt, 1978). Incompatibility refers to the failure of plasmids sharing similar replication 'back-bone' proteins to be propagated stably in the same cell line. Unknown plasmids were introduced to a cell line with known plasmids (by conjugation or transduction methods) and if the unknown plasmid was not stable maintained, it was then included in the same incompatibility group as the plasmid initially in the cell line (Carattoli et al., 2005). Examples of Inc groups common to *S.* Typhi include IncF, IncH, IncQ and IncN. (Park et al., 2018; Partridge et al., 2018).

A more recent scheme uses a molecular approach to characterize conserved replication initiating genes (plasmids replicon typing) (Carattoli et al., 2005). The system has been largely successful. However, some plasmids carry multiple replicons as such, it is challenging to determine the dominant replicon. It is also less sensitive to divergent replicons within incompatibility groups. Multilocus sequence typing (plasmid MLST) methods and whole genome sequencing (WGS), can provide higher resolution typing for plasmids (Carattoli, 2009). In this study we used WGS approach to examine evolutionary changes in *S.* Typhi plasmids in this population.

2.2.4.2. Intrinsic systems regulating plasmid niches in bacterial population

A number of systems regulating plasmid niche in bacterial population have been described. Plasmid incompatibility inhibits co-occurrence of related plasmid types in the same bacteria, entry exclusion systems (EES) impede transfer of a plasmid into a cell that already has a resident plasmid of the same exclusion group (Kamruzzaman et al., 2017) while post-segregational killing (`addiction') systems, poisons any bacterial cells that loses an addictive plasmid, as way to guarantee survival of already existing plasmid (Tsang, 2017).

Addictive plasmids carry an operon which encoded an antitoxin (upstream) and a toxin with long shelf life (downstream). The toxin is synthesized in higher levels. In presence of the plasmid the toxin is neutralized while in its absence, the toxin persists longer than the antitoxin leading to cell death. Interaction of these regulatory systems and external selection pressure, shapes the dynamics of plasmids population, and could be used to better understand epidemiology of plasmids in an area.

2.2.4.3. Evolution and global distribution of resistance plasmids of *S*. Typhi

Resistance plasmids of *S.* Typhi are dynamic, varying between genotypes and within genotypes over time (Holt et al., 2011; Park et al., 2018). A wide range of resistance plasmid families have been identified in *S.* Typhi, however resistance in *S.* Typhi has mainly been shaped by plasmids of the incompatibility group IncHI1(Holt et al., 2011). Chloramphenicol resistance in the 1950s was mainly associated with IncHI1 sequence type 5 (IncHI1-PST5) plasmid. Emergence of IncHI1-PST2 in Southeast Asia in the 1970s then introduced resistance to multiple antibiotics including chloramphenicol (*catA*), tetracycline (*tet*) and sulfonamide (*sul*) (Holt et al., 2011). IncHI1-PST8 identified in 1980s in South America carried streptomycin (*straAB*) in addition to the resistance genes identified on IncHI1-PST2. IncHI1-PST6, carrying tetracycline *(tetA and tetB),* streptomycin *(aph(3'')-Ib),* kanamycin *(aph(6)-Id),* ampicillin *(bla*$_{\text{TEM-1b}}$), chloramphenicol *(catA1),* trimethoprim *(dfrA7),* and sulfonamide *(sul1* and *sul2)* resistance genes, emerged about 1992 and quickly dominated in Oceania and Asia before transmission to East and South Africa during the first decade of 2000 (Kariuki et al., 2010; Wong et al., 2015). Global distribution of the IncHI1 plasmid has often been influenced by the circulating genotypes. IncHI1-PST6 plasmids are found in regions with genotype 4.3.1 while IncHI1-PST2 often occur in areas dominated by genotype 3.1.1(H56 subclade)(Park et al., 2018).

CHAPTER 3

3. METHODS

3.1.Study design

We used isolates collected from a longitudinal study

3.2.Study Site

The isolates were obtained from the ongoing Population-Based Infectious Disease Surveillance (PBIDS) system in Kibera, a collaboration between the U.S. Centers for Disease Control and Prevention (CDC) and the Kenya Medical Research Institute, Centre for Global Health Research (KEMRI-CGHR). The surveillance is conducted in two villages of Kibera slum; Gatwikira and Soweto West, which cover approximately 0.38km^2. The study area is sub-divided into 10 geographic areas referred to as clusters (figure 8). The area is densely populated (~77000 persons/km^2) and has poor sanitation infrastructure as indicated by open sewers and often broken or leaky drinking water piping systems (Akullian et al., 2015; Breiman et al., 2012; Njuguna et al., 2013; Worrell et al., 2016). Two seasons define annual weather in Kibera; wet long rains season running between March – May and short rains season (October–November); and dry seasons, which runs between June–September and December–February.

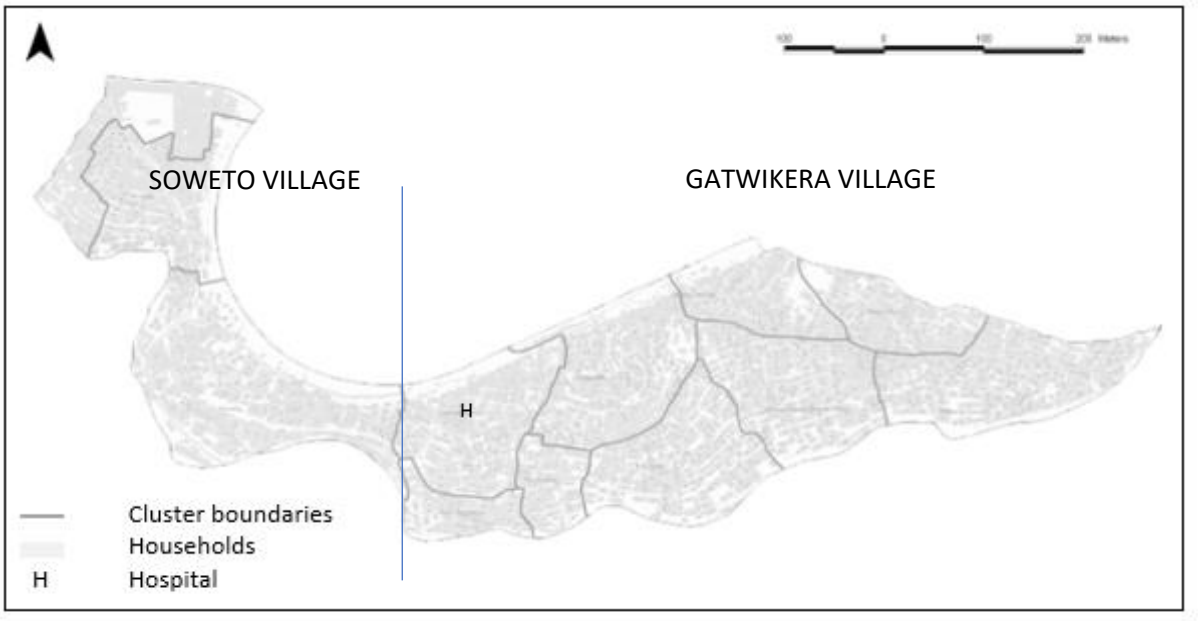


Figure 8: Population-based infectious diseases surveillance area

3.3.Study Population

The surveillance follows a cohort of ~23,000 individuals, of all ages and gender, living in Kibera. High burdens of infectious diseases have been described in the population, including adult HIV prevalence of ~13% (Dalal et al., 2013). PBIDS participants in Kibera reside within 1 km of Tabitha Clinic, where PBIDS participants receive free care for infectious diseases (Breiman et al., 2011).

3.4.Study procedures

3.4.1. Kibera Population-based infectious diseases Surveillance System

PBIDS implements both household and clinic level surveys.

3.4.1.1. *Household level survey*

Between January 2007 and September 2014, household surveys were conducted every two weeks by trained community interviewers (Breiman et al., 2012). This frequency was revised to bi-annually in April 2015. Self reported morbidity and health care usage data were recorded during the interviews. Those with fever, diarrhea or both at the time of the home visit were advised to seek medical care at the surveillance clinic in the study area.

3.4.1.2. *Clinic Level survey*

Blood for culture was collected from consenting PBIDS participants who presented at the clinic with fever (axillary temperature ≥ 37.5°C) or acute lower respiratory illness (ALRI). For children <5 years old, ALRI was defined as cough or difficulty breathing plus chest indrawing, or oxygen saturation <90%, or any of one the danger sign (lethargy, convulsions, unable to drink fluids/breastfeed, vomiting everything, or stridor). For individuals ≥5 years old, ALRI was defined as cough or difficulty breathing or chest pain plus axillary temperature ≥38.0°C or oxygen saturation <90%. About 1-3 ml of blood was collected from children less than 5 years, and 8-10 ml from children and adults 5 years and older.

3.4.1.3. *Microbiology*

Collected blood was inoculated into commercially produced BACTEC Ped Plus/F bottle (for children <5 years) and BACTEC Plus/F bottles (for children and adults ≥5 years).

Blood samples from positive BACTEC bottles were cultured on chocolate Agar Plates, blood agar plates and MacConkey plates using standard microbiological procedures. The plates were then incubated aerobically at 37.0°C for 24 hours. Suspect colonies of *Salmonella* based on morphology were confirmed using standard biochemical tests and were characterized by agglutination assays using commercially produced group D and Vi antisera (Breiman et al., 2012). Confirmation of isolates that could not be typed through agglutination methods was performed using the API 20E system (Appareils et Procedes d'Identification, Montalieu Vercieu, France) following manufacturers' instructions. Identified *S.* Typhi were inoculated in Trypticase Soy Broth with 20% glycerol and preserved at -80.0°C.

3.4.2. Sample size

Between January 2007 and December 2017, 371 *S.* Typhi were isolated. Of these, 317 (83.2%) were successfully re-cultured for sequencing. We considered the sample size adequate for intended analyses because this is a descriptive study with no testing of *a priori* hypothesis. Further, isolates which could not be revived were randomly distributed over the study period and likely did not introduce substantial bias (Table 1).

Table 1: Distribution of blood cultures performed, isolated and sequenced *S.* Typhi by year, in the Kibera population-based surveillance system

Year	Cultures done	*S.* Typhi isolated	Sequenced *S.* Typhi	% sequenced
2007	702	66	42	63.6
2008	996	60	48	80.0
2009	2,968	30	19	63.3
2010	2,530	70	60	85.7
2011	1,543	43	42	97.7
2012	1,173	64	58	90.6
2013	1,214	9	8	88.9
2014	1,350	10	10	100.0
2015	898	13	13	100.0
2016	725	4	2	50.0
2017	665	2	2	100.0
Total	**14,764**	**371**	**304**	**81.9**

3.4.3. Whole Genome Sequencing

Whole genome sequencing was performed on all revived isolates. Subsets of the isolates were sequenced at Technical University of Denmark, Wellcome Sanger Institute, and locally following establishment of sequencing capacity.

3.4.3.1. ***Revival of archived S.* Typhi *Isolates***

Frozen *S.* Typhi isolates were thawed by gentle agitation in a water bath set at 25.0°C, before colonies were re-cultured on blood agar media and incubated at 37.0°C for 24 hours. Some of the revived colonies were inoculated into Trypticase Soy Broth with 20% glycerol and shipped to Denmark and the United Kingdom in dry ice according to standard IATA regulations. Locally, revived colonies were processed as described below.

3.4.3.2. ***DNA extraction and preparation***

3.4.3.2.1. Genomic DNA extraction

DNA extraction was performed using Invitrogen Easy-DNA kit (Invitrogen, Carlsbad, CA, USA). Tissue lysis buffer (180 µl) was dispensed into 1.5 ml microcentrifuge tubes. A loop (1µl loop) full of each revived culture was added into the lysis buffers and was vortexed at high speed for 10 seconds. Proteinase K (20 µl) was then added into each sample tube and was also vortexed at high speed for 10 seconds. The sample tubes were then incubated at 56°C for 2 hours with vortexing every 20 minutes through the incubation period. RNase A solution (4 µl) was added to the samples and was vortexed at medium speed for 10 seconds. The samples were then incubated at room temperature for 5 minutes. Lysis buffer (200 µl) was then added and the samples were vortexed for 10 seconds. Another 200 µl of 100% ethanol was added and vortexed for 10 seconds.

The mixture was poured into a DNeasy spin column set in a 2 ml collection tube. The set (spin column and collection tube) was centrifuged at 10000 rates per minute (rpm) for 1 minute and the flow-through and collection tube were discarded. DNeasy silica gel membrane binds nucleic acid. The DNeasy spin column was placed in a new 2 ml collection tube and 500 µl of AW1 (wash 1 buffer) was added. The set was centrifuged

at 10000 rpm for 1 minute. The flow-through and collection tube were discarded. The DNeasy spin column was again placed in a new 2 ml collection tube and 500 µl of AW2 (wash 2 buffer) was added. The set was then centrifuged at 14000 rpm for 3 minutes and the flow-through and collection tube discarded. The DNeasy spin column was placed in a clean, labeled 1.5 ml microcentrifuge tube and 100 µl of pre-warmed 10 mM Tris-HCL pH 8.0 (re-suspension buffer) was added onto the DNeasy membrane. The mixture was incubated at room temperature for 5 minutes, and then centrifuged at 11000 rpm for 1 minute to collect the eluate (DNA) in a new tube.

3.4.3.2.2. DNA concentration

DNA concentrations was determined using Qubit double-stranded DNA BR assay kit (Invitrogen, Carlsbad, CA, USA) consistent with manufacturer's instructions. A Qubit working solution (1:200 ratio of Qubit Reagent to Qubit Buffer) for each sample and 2 standards were prepared. A working solution of 198 µl and 2 µl of each extracted sample DNA was dispensed into labelled assay tubes. Standard tubes were loaded with 190 µl of working solution and 10 µl of Qubit standards. All tubes were vortexed for 3 seconds and incubated for 10 minutes. Qubit 2.0 fluorometer was then set at DNA sample type and dsDNA broad range assay type. Samples were loaded and DNA concentration value were recorded. All samples had more than 10 ng/µl

3.4.3.2.3. Library preparation

Extracted DNA was prepared for Illumina (Illumina, Inc., San Diego, CA) pair-end sequencing using Illumina Nextera XT library preparation kit reference guide 15027987 (https://support.illumina.com/downloads/nextera-dna-library-prep-reference-guide-15027987.html). Library preparation involved Tagmentation using Amplicon Tagment Mix, amplification of tagmented DNA using index primers, PCR product clean-up using AMPure XP beads, beads washing and DNA quantification. The latter is a QC step aimed at ensuring that fragments constituting the library range between 500 to 1000 bp.

3.4.3.2.4. Sequencing

A sample of the pooled Nextera XT libraries was loaded onto an Illumina MiSeq reagent cartridge using MiSeq reagent kit v2 and 500 cycles with a standard flow cell. The libraries were sequenced using an Illumina platform and MiSeq control software 2.3.0.3. Quality of raw reads was assessed using the FastQC quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality metrics of reads were computed using CG pipeline against in-house standards (coverage ≥30X).

3.4.4. Bioinformatics

Assemblies were generated using Shovill (v1.0.4), excluding contigs with coverage below 10% average genome coverage). Assembled sequences were analyzed for plasmid replicon families and antimicrobial resistance determinants using an in-house database adapted from PlasmidFinder (https://github.com/StaPH-B/resistanceDetectionCDC; 60% coverage, 90% identity) in Abricate (v0.8.10), and staramr (https://github.com/phac-nml/staramr; 50% coverage, 90% identity), respectively. Mobile genetic elements were detected using Galileo AMR (http://galileoamr.arcbio.com/mara/login/auth). Putative chromosomal insertion sites (*cyaY, uxuA*, *galU*) were screened using megaBLAST (ncbi-blast+ v2.9.0).

3.4.5. PCR

PCR was used to confirm chromosomal integration of an IS*1*-mediated composite transposon (Figure 10b). One isolate per quarter per year (2007-2017), from among resistant isolates with putative chromosomal insertion sites (determined by megaBLAST screening), were included. PCR primers for suspected integration sites were designed using Primer3 software. They covered regions flanking the insertion site on the IncHI1 plasmid, putative insertion sites on the chromosome and internal regions within the composite transposon (Table 2). Primer selection criteria included; size ~20 bp, melting temperature ~60ºC, self-dimer <2. PCR was performed using Gene Amp PCR System 9700 Thermal cycler. Amplification reactions were performed using 12.5 µl of 2X MyTaq red mix, 0.5 µl of each primer (10 mM), 2 µl of DNA template and 9.5 µl of MyTaq PCR buffer. Total single reaction volume

was 25 µl. Cycling conditions were: 1 minute at 95ºC; 35 cycles of 30 sec at 95ºC, 30 sec at 59ºC and 45 sec at 72ºC with final extension of 10 mins at 72ºC.

Table 2: PCR primers for detection of insertion of IS*1*-mediated composite transposon

Site name	Site	Forward 5'-3'	Reverse 5'-3'
cyaY F1	Chromosome	ACGAAAACAATGAAGGACGCG	GCGCCTTATCCAGCCTACAT
Tn21	Transposon	TGGAAACCGTGCTGTCTGAA	GGAACCTCTTACGTGCCGAT
Plasmid	IncHI1 plasmid	CAGATCCCGGTGCTGCAATA	CCCTACCAGAGACGCACTTG
uxuA	Chromosome	CTTTTCTAACGCAGGCGGTG	TGCCATAACCATACGTTCACCA
galU	Chromosome	GCACCCCAGCCACATAGTTA	CCTGCTGGCGAGTCAAAAAC
cyaY F2	Replaces cyaY F1 for isolates with deletion of target region	CAAAAACCGTAGCGTTGCCA	

3.4.6. Study variables

Variables generated from this study included; plasmid Inc. groups, antimicrobial resistance genes, chromosomal integration sites of the MDR transposon and annual incidence rates

3.4.7. Statistical analysis

Annual unadjusted typhoid incidence rates were estimated as number of typhoid cases (numerator) divided by annual person years of observation (sum of participants person-time of residency, in years, in the study area) (Breiman et al., 2012). Fisher's exact test was used to compare proportion of sub-strains with quinolone resistance determining region (QRDR) mutations, between MDR plasmid free strains with chromosomally integrated IS*1* composite transposon and strains with the IncHI1 plasmid. Analysis were performed using STATA software (version 14.1.)

3.5.Ethics

This study was approved by Kenya Medical Research Institute Scientific and Ethics Review Committee (protocol number SSC 1899 and SSC 2761 (appendix 1), Institutional Review Board of US Centers and Ethical Review Committee of University of Nairobi and Kenyatta National Hospital (Protocol number P186/03/2019 (appendix 2). *S.* Typhi isolates were obtained from individuals who provided written informed consent to participate in the surveillance and to additional testing of their samples (appendix 3).

CHAPTER 4

4. RESULTS

Of the 371 sequenced isolates, 304 (95.9%) were *S.* Typhi, and 11 (3.5%) were found to be non-typhoidal *Salmonella* (NTS). Only *S.* Typhi genomes were included in the analysis.

4.1.Resistance plasmid replicon families

Plasmid replicons were detected in 239 (78.6%) of the 304 screened *S.* Typhi genome assemblies. Of these, IncHI1A, IncHI1B and partial (Δ) IncQ1 replicons were identified in 225 (94.1%) isolates and a partial IncQ1 replicon only was identified in 14 (5.9%) isolates (Figure 9).

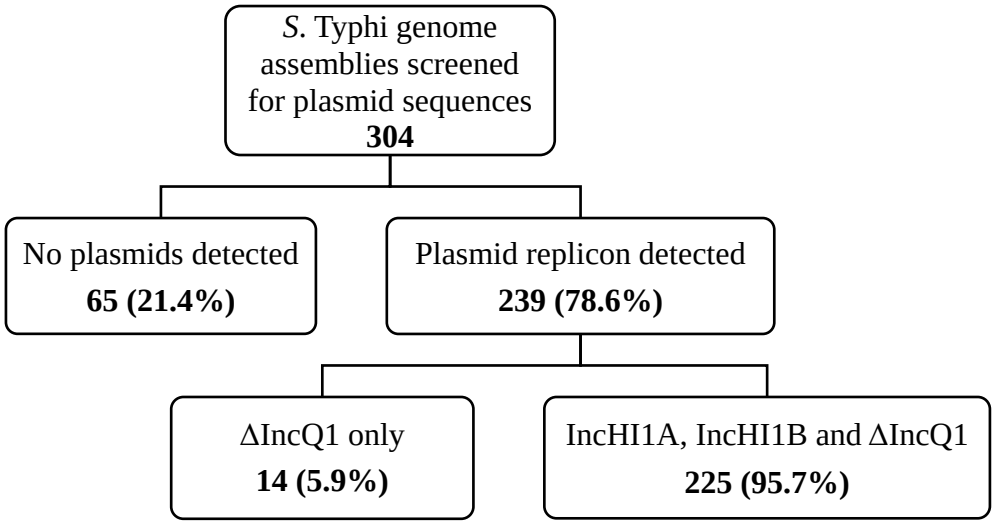
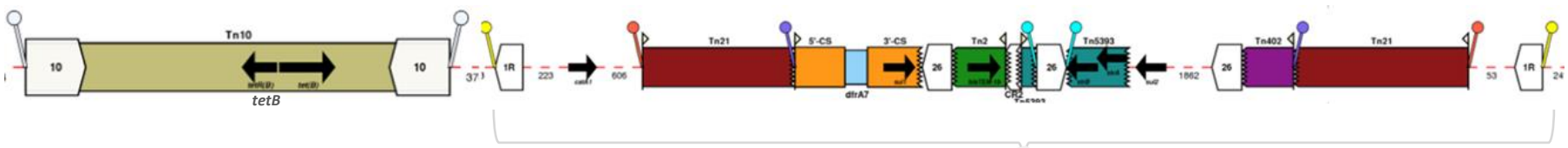


Figure 9: Flow diagram of plasmid replicon families identified in *S.* Typhi genomes from Kibera, 2007-2017. Majority of detected plasmid sequences belonged to the IncHI1 replicon family

4.2.Transposons and resistance genes

The IncHI1 plasmid (comprising IncHI1A, IncHI1B and partial IncQ1 replicons) was PST 6 and carried a Tn*10* transposon with *tet(B),* a tetracycline resistance gene; an IS*1*-mediated composite transposon, carrying a Tn*21*-like transposon, harboring an In/Tn (Tn*402*- class 1 integron) with *bla*$_{\text{TEM-1B,}}$ an ampicillin resistance gene (in a partial Tn*2*), *dfrA7* and *sul1* genes encoding trimethoprim and sulfonamide resistance, and *catA1*, a chloramphenicol resistance gene, and a partial Tn*5393* with *aph(3'')-Ib* and *aph(6)-Id* genes encoding streptomycin and kanamycin resistance, and *sul2*, a sulfonamide resistance gene, adjacent to a partial IncQ1 replicon (Figure 10a).

a)



b)

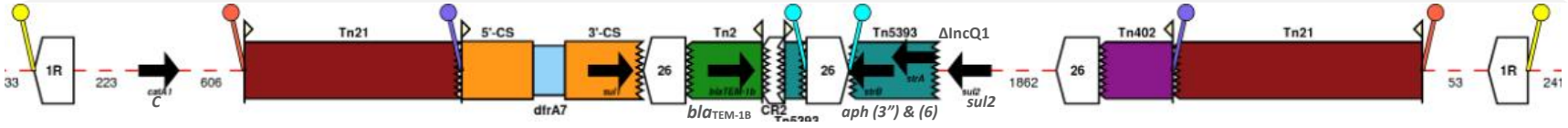


Figure 10:(a) IncHI1 plasmid with tetB carrying Tn*10*, a Tn*21*-like composite transposon with *bla*$_{TEM-1B}$, *dfrA7*, *sul1* and *catA1* resistance genes and a partial IncQ1 replicon carrying a partial Tn*5393*, with *aph(3'')-Ib* and *aph(6)-Id* resistance genes. (b) An IS*1*-mediated composite transposon. Contains ΔIncQ1 replicon, Tn*21*, ΔTn*5393* and their corresponding resistance genes. Images generated by Galileo AMR detection software (http://galileoamr.arcbio.com/mara/).

4.3.Changes in plasmids over time

Plasmid replicon families varied over time. IncHI1 was the dominant replicon family identified until 2013. However, in 2014, a distinct partial IncQ1 became the primary detected replicon, with sporadic emergence of IncHI1 (Figure 11).

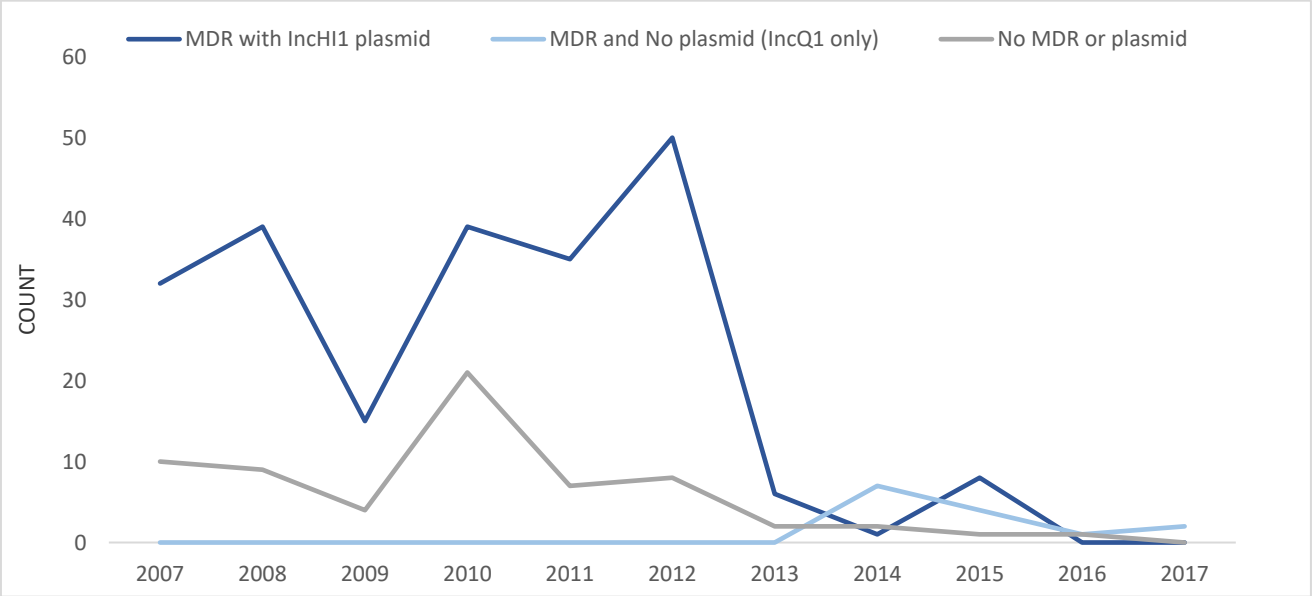


Figure 11: Annual counts of *S.* Typhi plasmid replicon families in Kibera, 2007-2017.

All resistance genes persisted except *tet(B)* which was not located in the IS*1* composite transposon (figure 10).

Multiple chromosomal integrations of the IS*1*-composite transposon were identified over time (Figure 12). Integration at the previously reported *cyaA* and *cyaY* intergenic region was detected in 2014 (Wong et al., 2015). Other putative integration locations were identified in 2015, in an intergenic region adjacent to an alcohol dehydrogenase gene (*uxuA*), and within a thymidine kinase gene (*galU*).

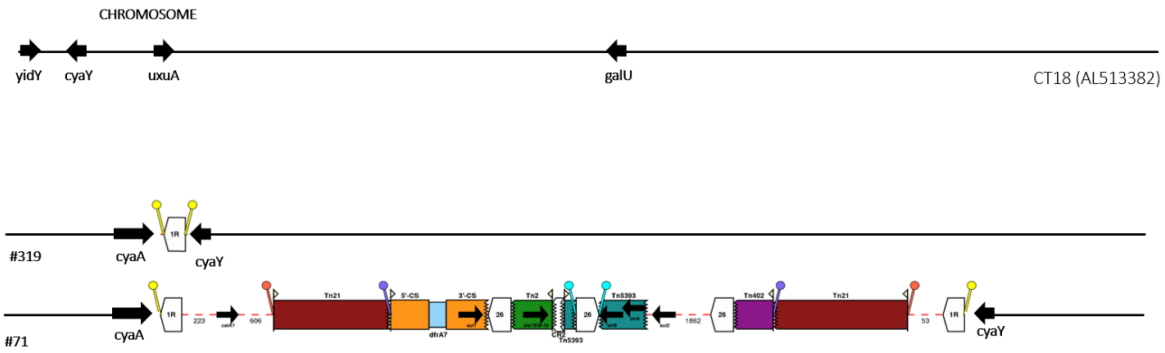


Figure 12: a diagram of a CT18 showing the insertion sites. Isolate number 319 shows integration of an IS*1* at the *cyaA-cyaY* intergenic region. Isolate number 71 shows integration of the IS*1* composite transposon at the same insertion location (*cyaA-cyaY*).

PCR suggested the composite transposon (Figure 10b) was integrated into the *cyaY* and *uxuA* sites only, and that an IS*1* alone was present at the *galU* location (Figure 13 and Table 3).

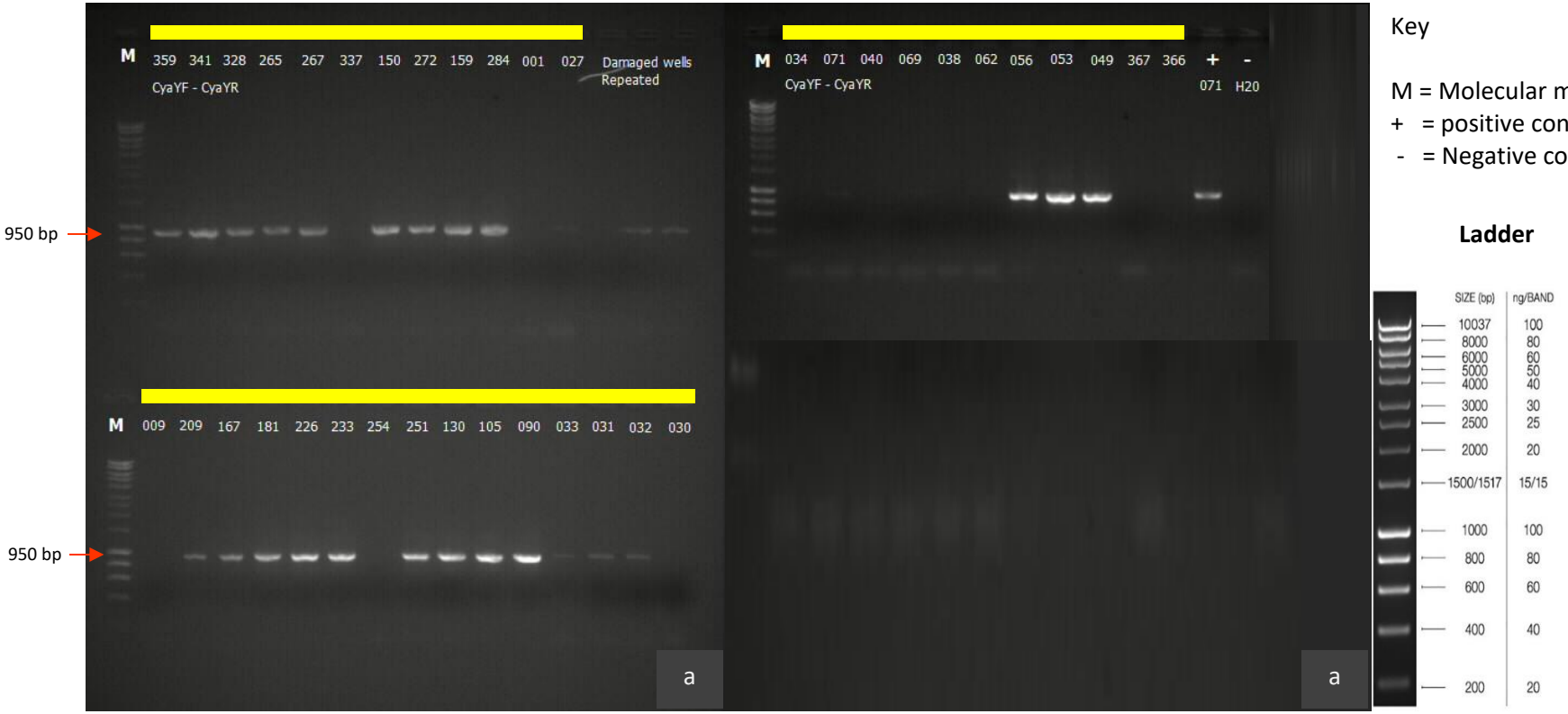


Figure 13a: *cyaY* forward1-*cyaY* reverse PCR (yellow band), checked for integration of IS*1* alone (~950bp) at the *cyaA*-*cyaY* intergenic region in the chromosome.

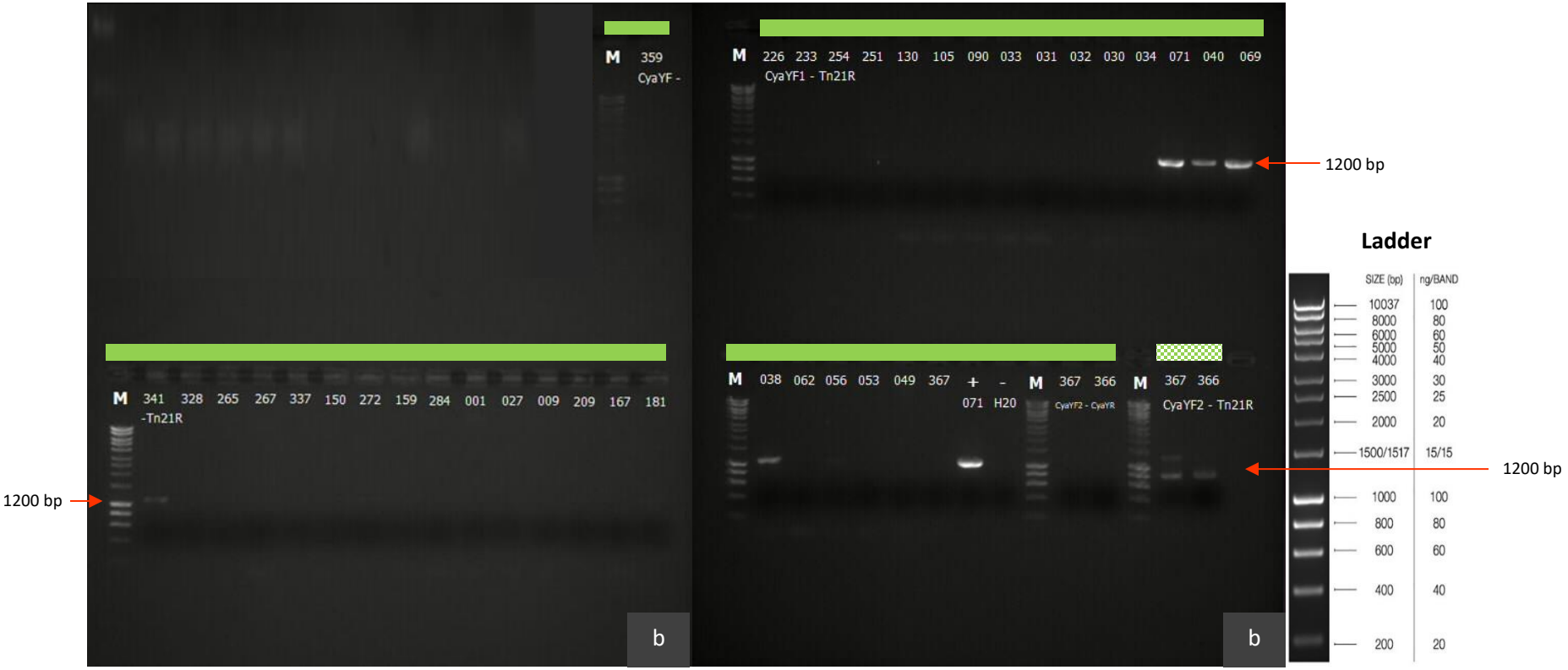


Figure 13b: *cyaY* forward1-Tn21 reverse PCR, checked for chromosomal integration of the IS*1*-mediated composite transposon at the *cyaA-cyaY* intergenic region. The IS*1* composite transposon is ~25Kb, thus, two nested PCRs amplifying its flanking regions and with product size of ~1200bp, were used to confirm chromosomal integration (see diagram illustrating the PCRs performed below (Figure 13i). The textured light green bad shows PCR for samples 367 and 366 performed using *cyaY* forward2 primer.

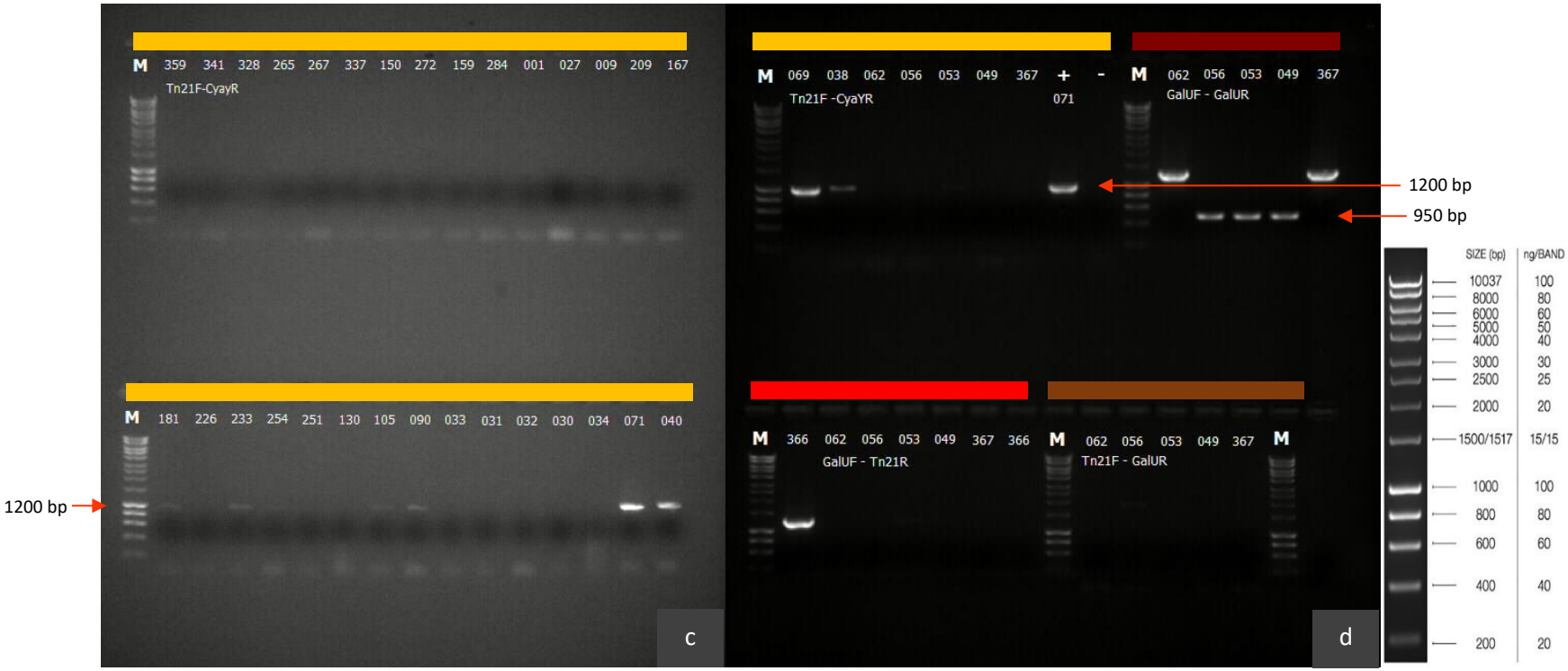


Figure 13c-d: Tn21 forward- *cyaY* reverse (orange band), also checked for chromosomal integration of the IS*1*-mediated composite transposon at the *cyaA*-*cyaY* intergenic region. Figure 13d) *galU* forward-*galU* reverse PCR (maroon band), checked for integration of IS*1* alone (~950 bp) within a thymidine kinase gene. *galU* forward-Tn21 reverse PCR (red band), checked for chromosomal integration of the IS*1*-mediated composite transposon in this region. Tn21 forward- *galU* reverse (brown band), also checked for chromosomal integration of the IS*1*-mediated composite transposon at the *galU* intergenic region

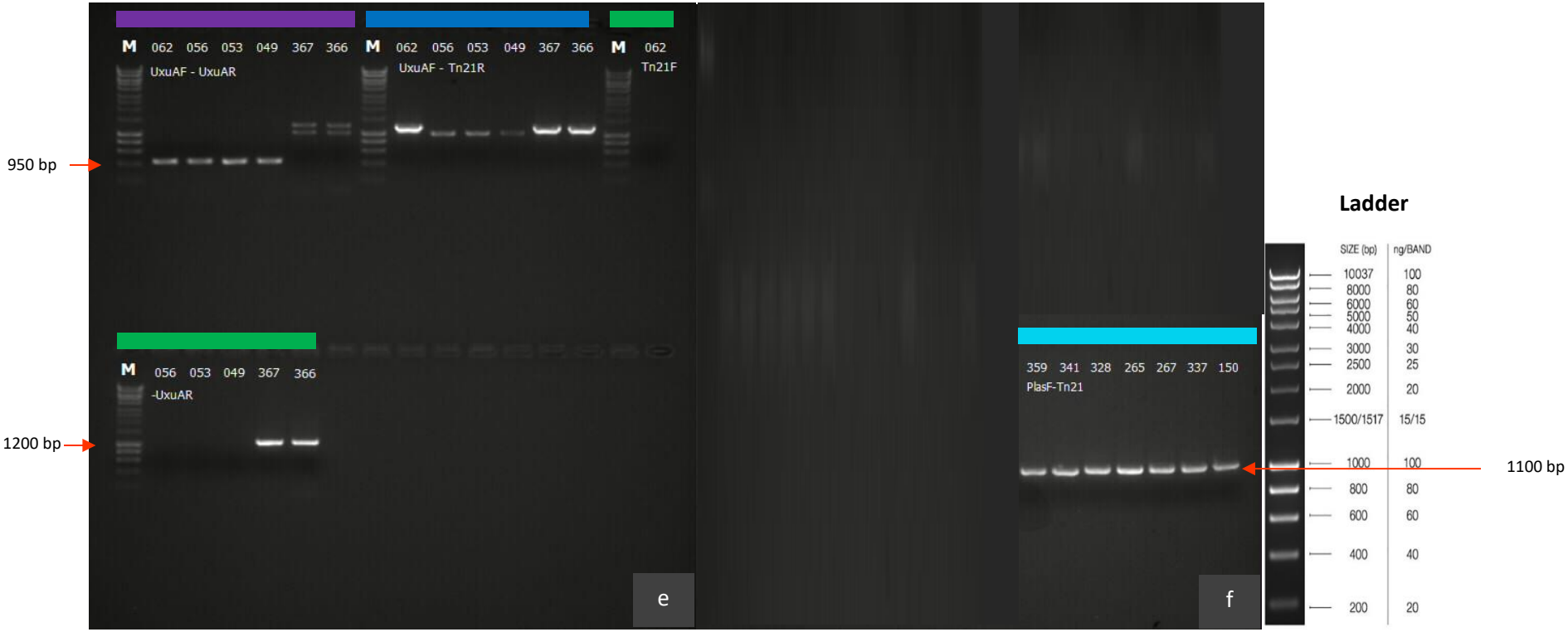


Figure 13e-f: *uxuA* forward- *uxuA* reverse PCR (purple band) checked for integration of IS*1* alone in an intergenic region adjacent to an alcohol dehydrogenase gene. *uxuA* forward-Tn21 reverse PCR (blue band), checked for chromosomal integration of the IS*1*-mediated composite transposon in this region. Tn21 forward- *uxuA* reverse (green band), also checked for chromosomal integration of the IS*1*-mediated composite transposon at the *uxuA* intergenic region. Figure13f) Plasmid forward and Tn21 reverse (pink band), checked presence of the IS*1*-mediated composite transposon in the IncHI1 plasmid

Figure13h-i: Plasmid forward and Tn21 reverse (light blue band) and Tn21 forward and plasmid reverse (grey band) PCRs checked presence of the IS*1*-mediated composite transposon in the IncHI1 plasmid

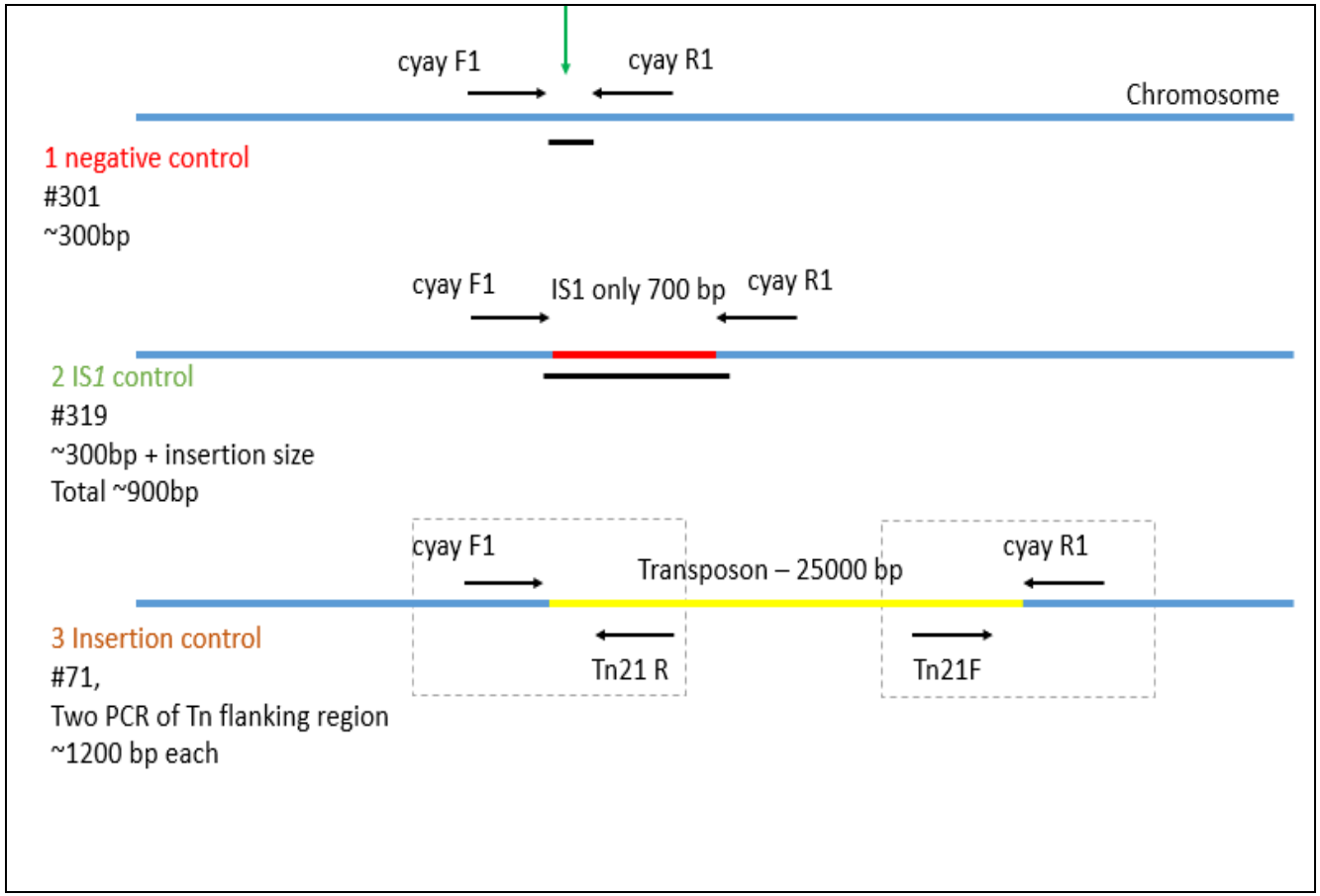


Figure 13i: diagrammatic illustration of the PCR experiments. PCR 2 checked for integration of IS*1* only. PCR3, nested PCR checked for integration of the composite transposon

Table 3: Summary table of PCR experiments results and their interpretation. Y indicates region of interest was detected (positive PCR), N indicates no region detected (negative PCR) and D indicates indeterminate PCR results (partial region detected)

ISOLATE NUMBER	YEAR	PCRS											RESULTS INTERPRETATION				
		cyaYF-cyaYR	cyaYF-Tn21R	Tn21F-cyaYR	PlasmidF-Tn21R	Tn21F-PlasmidR	GalUF-GalUR	GalUF-Tn21R	Tn21F-GalUR	UxuAF-UxuAR	UxuAF-Tn21R	Tn21F-UxuAR	insertion at cyaY	insertion in plasmid	both in plasmid and cyaY	insertion at galU	insertion at uxuA
359	2007	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
341	2007	Y	N	Y	Y	Y	NA	NA	NA	NA	NA	NA	D	Y	D	NA	NA
328	2007	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
265	2007	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
267	2008	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
337	2008	N	N	N	Y	N	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
150	2008	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
272	2008	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
159	2009	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
284	2009	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
001	2009	N	N	N	N	Y	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
027	2009	Y	N	N	N	Y	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
009	2010	N	N	N	Y	N	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
209	2010	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
167	2010	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
181	2010	Y	Y	N	Y	Y	NA	NA	NA	NA	NA	NA	D	Y	D	NA	NA
226	2011	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
233	2011	Y	Y	N	Y	Y	NA	NA	NA	NA	NA	NA	D	Y	D	NA	NA
254	2011	N	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
251	2011	Y	N	N	N	Y	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
130	2012	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA-	NA
105	2012	Y	Y	N	Y	Y	NA	NA	NA	NA	NA	NA	D	Y	D	NA	NA
090	2012	Y	Y	N	Y	Y	NA	NA	NA	NA	NA	NA	D	Y	D	NA	NA
033	2012	Y	N	N	N	Y	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
031	2013	Y	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
032	2013	Y	N	Y	N	N	NA	NA	NA	NA	NA	NA	D	N	D	NA	NA
030	2013	N	N	N	Y	N	NA	NA	NA	NA	NA	NA	N	D	N	NA	NA
034	2013	N	N	N	Y	Y	NA	NA	NA	NA	NA	NA	N	Y	N	NA	NA
071	2014	N	Y	Y	NA	NA	NA	NA	NA	NA	NA	NA	**Y**	NA	NA	NA	NA
040	2014	N	Y	Y	NA	NA	NA	NA	NA	NA	NA	NA	**Y**	NA	NA	NA	NA

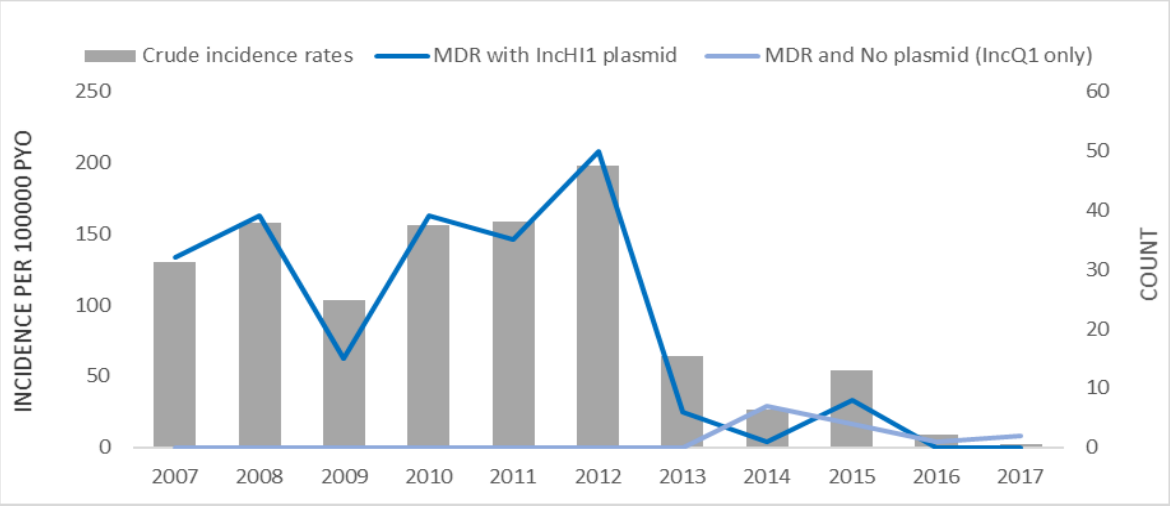
069	2014	N	Y	Y	NA	NA	NA	NA	NA	NA	NA	NA	**Y**	NA	NA	NA	NA
038	2014	N	Y	Y	NA	NA	NA	NA	NA	NA	NA	NA	**Y**	NA	NA	NA	NA
062	2015	N	N	N	Y	N	Y	N	N	Y	Y	N	N	D	N	N	D
056	2015	Y	N	N	Y	N	Y	Y	N	Y	Y	N	N	D	N	D	D
053	2015	Y	N	Y	N	N	Y	N	Y	Y	Y	N	D	N	D	D	D
049	2016	Y	N	N	NA	NA	Y	N	N	Y	Y	N	N	NA	NA	N	D
367	2017	N	Y	N	NA	NA	Y	N	N	Y	Y	Y	D	NA	NA	N	**Y**
366	2017	N	Y	N	NA	NA	Y	N	N	Y	Y	Y	D	NA	NA	N	**Y**

4.3.1. Mutations within the QRDR region

Thirty-seven (16.4%, n=225) isolates with an IncHI1 plasmid and 7(50.0%, n=14) with a chromosomally integrated IS*1* composite transposon had mutations in the QRDRs of *gyrA* and *gyrB*. A serine to phenylalanine amino acid substitution at codon 464 of *gyrB*(S464F) was the most common (62.2%, n=23/37) in strains with IncHI1 plasmid, followed by a serine to tyrosine substitution at codon 83 of *gyrA* (S83Y) (32.4%, n=12/37). All QRDR mutation in plasmid free strains with chromosomally integrated IS*1* composite transposon were S83Y substitution in *gyrA*. Mutations in the QRDR were more common in plasmid free strains with chromosomally integrated IS*1* composite transposon (7 [50%]; P=0.006) compared to strain carrying IncHI1 plasmid

4.4.Changes in disease burden and replicon families

Incidence of typhoid fever in Kibera declined over time, characterized by a considerable decline in 2013 that remained low, but with subsequent periodic peaks (Figure 14). Periods of low typhoid incidence coincided with the period when plasmid-free strains with the chromosomally-located resistance region were detected, while incidence increased with relative increase in IncHI1-carrying strains.



Years	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
***S*. Typhi**	59	53	21	69	42	62	8	9	9	4	2
PYO	21401	21941	24224	25912	24971	23831	22983	22888	17608	20132	14138

Figure 14: Annual counts of plasmid replicon families and crude typhoid fever incidences overlaid, Kibera (2007-2017). Crude annual typhoid incidence rates estimated by dividing annual number of typhoid cases into annual person years of observation (PYO).

CHAPTER 5

5. DISCUSSION

Our findings show expansion of plasmid-free strains with a chromosomally integrated MDR transposon, which coincided with periods of decreased typhoid fever incidence. The transposon was integrated on multiple sites on the chromosome including a site not previously described. Prior expansion of the strains, relatively increased annual incidences of typhoid fever were described in the study area, largely characterized by strains carrying these IncHI1-PST6 plasmid. While plasmid-free strains with chromosomally integrated MDR transposon have been described along strains carrying the IncHI1 plasmid in typhoid endemic settings, our data highlights expansion of these strains over time, and gives clues into evolution of plasmid-mediated antibiotic resistance in *S.* Typhi. Movement of the MDR transposon into the chromosome and subsequent loss of the IncHI1 plasmid could potentially allow both stabilization of the MDR phenotype and acquisition of additional plasmid-mediated resistance. With recent emergence of strains with a chromosomally integrated MDR transposon and IncY plasmid with extended-spectrum β-lactamase ($bla_{CTX\text{-}M\text{-}15}$) and fluoroquinolone resistance (*qnrS*) genes, this finding indicates the importance for a comprehensive understanding of plasmid dynamics underpinning emergence of increasingly resistant *S.* Typhi strains.

Expansion of MDR *S.* Typhi across Southeast Asia and sub-Saharan Africa has mainly been dominated by genotype 4.3.1 which carries the IncHI1-PST6 resistance plasmid (Park et al., 2018; Wong et al., 2015). The plasmid confers resistance to multiple traditional antibiotics (ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) and osmotolerance, which likely increases transmissibility of the genotype (Holt et al., 2011; Kariuki et al., 2010; Pitzer et al., 2015). However, the establishment of the genotype in different settings has been followed by emergence of plasmid-free strains, with chromosomal integration of an IS*1*-mediated multi-drug resistance composite transposon, originally hosted on the IncHI1-PST6 plasmid (Chiou et al., 2014; Hendriksen et al., 2015; Park et al., 2018; Wong et al., 2015). In this setting where genotype 4.3.1 with IncHI1-PST6 plasmid dominates (Holt et al., 2011; Park et al., 2018), we show chromosomal integrations at the *cyaA* location, and one additional location, *uxuA*. Recombination of the resistance transposon occurs at chromosomal sites with existing copies of IS*1* but shows preference for the *cyaA* location whose IS*1* could have been acquired from the IncHI1-PST6 plasmid (Wong

et al., 2015). The site has been described in India, Bangladesh, Afghanistan, Tanzania, Malawi, Zambia and South Africa which share common ancestor of genotype 4.3.1 with Kenya. Other described chromosomal integrations regions include, *yidA* in India, Bangladesh, Iraq and Pakistan, *fbp* in India and STY4438 region in Fiji (Klemm et al., 2018; Wong et al., 2015).Variations of chromosomal integration locations across settings, and within settings over time, support the observation by Wong *et al* (Wong et al., 2015), that chromosomal integrations are relatively frequent in *S*. Typhi 4.3.1., and that this phenomenon may offer a selective advantage to these variants. However, we did not test for selective advantages of the variant, in this work.

Unlike previous studies we show expansion of the plasmid-free strains carrying the chromosomally integrated composite transposon. We speculate that antibiotic usage could be driving the expansion. Like in other settings (Akhtar et al., 2015; Britto, Wong, Dougan, & Pollard, 2018; Emary et al., 2012), MDR *S*. Typhi strains in this population are exposed to a wide spectrum of antibiotics during treatment, including self-prescribed penicillin and cotrimoxazole at onset of symptoms (Bagnis et al., 2020; Omulo et al., 2017), and fluoroquinolones and ceftriaxone, which are used at the surveillance clinic for infections resistant the traditional antibiotics (Breiman et al., 2012). Chromosomal integration enables stable maintenance and likely expression of genes conferring resistance to the commonly used antibiotics, while loss of the IncHI1 plasmid may mediate the potential fitness burden associated with carriage of large plasmids (Dahlberg & Chao, 2003; Helling, Kinney, & Adams, 1981) which potentially improve overall 'resistance efficiency' of the variant in environment with wide range of antibiotic selection pressure. Potential benefit of hosting the resistance genes in the chromosome is supported by the observation that plasmid-free strain with chromosomally integrated resistance transposon were more likely to carry additional QRDR resistance mutations compared to strain carrying the IncHI1 plasmid. Further, loss of the IncHI1 plasmid may also allow for acquisition of other plasmids harboring additional resistance mechanisms. The phenomenon has recently been observed in Pakistan where an extensively drug resistant strain with chromosomally integrated IS*1* MDR composite transposon and an IncY plasmid with fluoroquinolone resistance (*qnrS*) and extended-spectrum β-lactamase genes ($bla_{\text{CTX-M-15}}$) has been described (Klemm et al., 2018).

As mentioned earlier, the identified IncHI1 plasmid not only confers antibiotic resistance, but is also associated with increased osmotolerance which likely increases survival of host bacteria in

inhibitive, high osmolarity environments (Holt et al., 2011). *S.* Typhi is exposed to a wide range of limiting environments during its infection cycle including physical environment, gastric acid, bile and urine (Holt et al., 2011; Mitscherlich, 1984; Tiwari, Sachdeva, Hoondal, & Grewal, 2004). The ability to survive these environments is essential for its transmission. The IncHI1 plasmid, which has been associated with environmental bacteria, is thought to enhance survival of *S*. Typhi outside the human host (Alonso, Baptista, Ngo, & Taylor, 2005; Holt et al., 2011). It has also been associated with increased *S*. Typhi concentration in the blood, suggesting potential growth benefit within the human host (Wain et al., 1998). High bacteria concentration in the blood influences disease severity, and potentially *S*. Typhi shedding in stool (Gibani et al., 2019; Wain et al., 1998). Thus, while chromosomal transfer of the resistant IS*1* composite transposon stably maintains the MDR phenotype, loss of the IncHI1 plasmid potentially lowers disease severity and transmissibility of the strain. This may explain the dominance of these plasmid-free strains during periods of relatively decreased incidence of *S.* Typhi. Further, in active disease surveillance systems, such strains may circulate undetected, and thus untreated, for longer compared to highly transmissible and virulent strains.

5.1.Limitation

These findings are reported with the following limitations. We were unable to confirm by PCR concurrent carriage of the composite transposon in both the IncHI1 plasmid and the chromosomal *cyaY* location (Table 3). Some PCR were indeterminate. We suspect that potential mutations in the insertion sites of the screened genomes could have interfered with the binding of primers, which were designed from a published genome (Accession LT904878, Salmonella enterica subsp. enterica serovar Typhi strain ty3-193). Confirmation of the integration locations could be achieved by long read sequencing in future. We also did not test for association between temporal changes in antibiotic prescriptions, and resistance plasmids in the area.

5.2.Conclusion and recommendations

Our data, gathered in a typhoid endemic setting, over a period of 11 years, characterized by varied epidemiological scenarios of the disease, reveals previously unappreciated expansion of the plasmid-free strain with chromosomally integrated MDR genes. Exposure of MDR *S*. Typhi to wide range of antibiotics during treatment, could be selecting for these variants.

Chromosomal integration of the resistance genes and subsequent loss of plasmid potentially enhances resistance efficiency (maintenance and expression of multiple resistance genes), which allows the bacteria to acquire additional resistance including plasmid mediated resistance, and could mark the beginning of a new wave of extensively drug resistant variants. With the global spread of genotype 4.3.1 and the frequent occurrence of the integrations, there is risk for rapid expansion of these variants. Typhoid vaccination, improved access to clean water and sanitation, and antimicrobial stewardship could help reduce selection pressure driving emergence of this increasingly resistant strains.

5.3.Future studies

Our findings set the stage for additional studies, such as, competitive experiments to assess the fitness cost of chromosomal carriage of the resistant IS*1* composite transposon. Such experiments may provide information which could be used to determine persistence, and thus dissemination potential of these strains. It would also be important to determine *in-vitro* and *in-vivo* conditions under which this strain may acquire new resistance plasmids and the population dynamics underpinning the emergence and persistence of these strains. With this information we can monitor for antibiotic resistance risk in *S.* Typhi and guide antibiotic resistance public health control actions such as antimicrobial stewardship, improvements of water and sanitation facilities, and resistance plasmid curing.

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7. APPENDIX

7.1.Appendix 1: Consent and Assent Forms

Version 2, June 2012

Informed Consent for collection of specimens for adults (15years and older) and children (6years and younger) for Kibera study (Flesch-Kincaid readability score 7.2)

Today's date	☐☐/☐☐/☐☐	**DSS Permanent ID**	☐☐☐/☐☐☐,☐
Name of ill person			
Name of parent if child			

Your house has agreed to be part of a research study in Kibera. The study looks at the common illnesses that occur in the area. As part of this, a field worker visits your house every week to ask about illnesses in people in your family. We want to look at what are the main germs causing these illnesses and how they can best be treated and prevented. Since you (or your child) are ill today with one of these illnesses, we would like to ask you (or your child) to give some samples to find out what germ is making you (or your child) sick today. Even if you refuse to give samples today, you can still be part of the study. The samples we are asking you (or your child) to give are the following:

FOR PNEUMONIA. A malaria blood smear, a blood sample (a teaspoon or less) from a vein in your arm or by sticking your finger. Also, we want to collect a nose sample by sticking a cotton swab inside your nose, a throat swab, and a cup of urine. We would also like to collect another blood sample from you in 4-6 weeks. We will also collect a urine sample. If the clinical officer or nurse feels it is needed, he/she might do a test for TB with your sputum (spit). The clinical officer or nurse may also recommend a chest x-ray to find if there is any abnormality in the chest

FOR INFLUENZA-LIKE ILLNESS. We want to collect a nose sample by sticking a swab inside your nose, and a throat swab.

FOR DIARRHOEA. A stool sample. For persons with diarrhea unable to have a stool in clinic, we will ask if the health care worker can take a swab from the rectum. The swab is made from soft cotton that is placed just inside your rectum for a few seconds.

FOR JAUNDICE. A malaria blood smear, a blood sample (a teaspoon or less) from a vein in your arm or by sticking your finger. We would also like to collect another blood sample from you in 3-6 weeks. You will be paid for your transport to come back to the clinic for that sample.

FOR FEVER; A blood specimen (approximately 2 teaspoons) will be taken from a vein. Some of the blood will be placed into a bottle where it will be tested to see if bacteria are present in your blood and causing fever. Some blood will be tested with a malaria smear, and the remaining blood will be evaluated for to see if you have been exposed to some of the most important germs (viruses, bacteria, parasites) known to cause fever. We would also like to collect another blood sample from you in 4-6 weeks. If you agree, you will be paid for your transport to come back to the clinic for that specimen.

These specimens will be tested at the KEMRI/CDC laboratories in Kisumu or Nairobi or at other reference labs outside of Kenya. We are asking permission to link the results of these lab tests with the facts we collect about you or your child at the household visits every two weeks.

Benefit from being in this study:

If you agree to have samples taken today, some of the tests being done at the clinic might help the medical staff in treating you (or your child) better.

Risks from being in this study:

Nose swabs and throat swabs cause brief pain. The nose swab might rarely cause brief bleeding from the nose. Drawing blood can also cause brief pain. Rarely it might cause bleeding and bruising. Serious injury due to taking swabs or drawing blood is very rare. Giving a stool or urine sample can be embarrassing, but poses no medical risk.

Giving samples today is your choice. You can choose not to give samples today and still continue to be part of the rest of the study – the household visits and the use of Tabitha Medical Clinic for your health care.

The facts about you and your family from this study will be kept private as much as allowed by law. No names will be used on any of the study reports. If you want to discuss this study with a doctor not involved in the study, contact Dr. William Macharia at Kenyatta National Hospital. His phone number (xxxx). These include problems from the study that you may want a doctor to look at or any questions about your rights as a study member. Should any more questions arise, if you feel like you or your family might have been harmed by being in the study, or if you want to quit the study, please contact Beatrice Olack or Dr. Joel Montgomery at the CDC office in Nairobi (0202713008).

If anyone has any questions about you or your child's rights as a study member, or if you want to talk with someone who is not part of this research project, please contact Secretary or Chairman of the KEMRI Ethical Review Committee, PO Box 54840 00100, Nairobi, Kenya: Telephone: +254-20-2722541 or email ERC@kemri.org.

The consent form has been explained to me and I agree for myself (or my child) to give specimens today. I understand that I am free to choose not to take part in this study at any time and that saying "NO" will have no effect on my family or me.

Name of ill person	**Name: .....**	**Signature:.....**	**date☐☐/☐☐/☐☐**
Name of parent (if child)	**Name: .....**	**Signature:.....**	**date☐☐/☐☐/☐☐**
Witness*	**Name: .....**	**Signature:.....**	**date☐☐/☐☐/☐☐**

* Subject may sign or provide verbal consent in the presence of a witness. The witness (by his/her signature) verifies that the consent form has been accurately translated to the subject and this is the subject's signature or that he/she has provided verbal consent.

Consent to store specimens

We would also like to ask if we could store the samples to do more tests at a later time if necessary to determine the cause of your fever. These stored specimens will be linked to you only by an identification number. There will be no name on them these tests would be to look for other germs that might be causing the illness that we do not have the ability to test for at this time. Your specimen will be stored at KEMRI/CDC at Nairobi. Any information obtained from future tests that will be important for your health will be communicated to you through project staff.

If you do not want to have your specimen stored, it will be discarded after the initial testing is done. This will not affect your being in the study. It will not affect the medical care you get here at the clinic. If you agree to this, but then have questions or later decide you don't want your specimen stored anymore, you can contact Beatrice Olack or Dr. Joel Montgomery at the CDC office in Nairobi (254202713008).

If anyone has any questions about you or your child's rights as a study member, or if you want to talk with someone who is not part of this research project, please contact Secretary or Chairman of the KEMRI Ethical Review Committee, PO Box 54840 00100, Nairobi, Kenya: Telephone: +254-20-2722541 or email ERC@kemri.org.

The consent form has been explained to me and I agree for my (or my child's) specimen to be stored. I understand that I am free to change my mind at any time and that saying "NO" will have no effect on my family or me.

Name of ill person	**Name: .....**	**Signature:.....**	**date☐☐☐☐☐☐**
Name of parent (if child)	**Name: .....**	**Signature:.....**	**date☐☐☐☐☐☐**
Witness*	**Name: .....**	**Signature:.....**	**date☐☐☐☐☐☐**

Assent form for children aged 7-14 years old for giving specimens for Kibera study (Flesch-Kincaid readability score 5.3)

The investigator will read this consent to the child at the time of enrollment.

Introduction

We are asking you to give samples for a study about what germs cause illnesses in people in this area. We want to find out how big a problem these germs are and how to treat them. The compound head for your house has already agreed to be part of this study. Today we are just asking you if you will give some samples to find out what germs are making you sick today.

The samples we want to get are these:

FOR PNEUMONIA. drawing blood from a finger for malaria blood smear and tests for germs causing pneumonia (a teaspoon or less) and a sample from your nose and throat by sticking a cotton swab inside your nose and throat. We would also like to draw blood again from you in 4-6 weeks. . If the clinical officer or nurse feels it is needed, he/she might do a test for TB with your sputum (spit). The clinical officer or nurse may also recommend a chest x-ray to find if there is any abnormality in the chest

FOR INFLUENZA-LIKE ILLNESS. We want to collect a nose sample by sticking a swab inside your nose, and a throat swab.

FOR DIARRHEA. a small piece of your stool or if you can't have a stool in clinic, we will ask if the health care worker can take a small piece of stool from your rectum. The swab is made from soft cotton that is placed just inside your rectum for a few seconds.

FOR JAUNDICE. a blood smear for malaria and a blood sample (approximately 2 teaspoons) from a vein in your arm or by sticking your finger. We would also like to collect another blood sample from you in 3 weeks.

For FEVER; A blood specimen (approximately 2 teaspoons) will be taken from a vein. Some of the blood will be placed into a bottle where it will be tested to see if bacteria are present in your blood and causing fever. Some blood will be tested with a malaria smear, and the remaining blood will be evaluated for to see if you have been exposed to some of the most important germs (viruses, bacteria, parasites) known to cause fever. We would also like to collect another blood specimen from you in 4-6 weeks.

Benefit from being in this study:

If you agree to have samples taken today, some of the tests being done at the clinic might help the medical staff in treating you better.

Risks from being in this study:

FOR PNEUMONIA/ILI. Nose swabs and throat swabs cause temporary discomfort. The nose swab might rarely cause brief bleeding from irritation of the nose. Drawing blood can also cause brief pain. Rarely it might cause bleeding and bruising. Serious injury due to taking swabs or drawing blood is very rare.
FOR DIARRHEA. Have a rectal swab or giving a stool sample can be embarrassing, but poses no medical risk.
FOR JAUNDICE OR FEVER. Drawing blood can also cause brief pain. Rarely it might cause bleeding and bruising. Serious injury due to taking swabs or drawing blood is very rare.

To give samples today is your free choice. If you do not want to, you will still get the best possible medical care here at the clinic. If you do not want to, nobody will be mad at you. If you agree to give samples, but then change your mind, you can stop at any time.

We have already asked your parents about this and they said it was okay to ask you if you wanted to do this. If you have any further questions about this study, please ask your parents or me.

Will you be a part of this study and give samples? ☐ Yes ☐ No

Name of child (Print) _____

_____ Date_____Child Signature (Signature or mark of consent) _____
To be signed by witness:
The above statement has been read to the child and the child agrees to participate in the research project.
Name of witness (Print) _____

Date_____Witness Signature (Signature or mark of consent) _____

8.2. Appendix 2: KEMRI and UoN_KNH ethics Approval Letters

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel:(254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1 November 16, 2018

TO: **DR. JOEL MONTGOMERY,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGHR,**
KISUMU

Dear Sir,

RE: **SSC PROTOCOL NO. 2761 *(REQUEST FOR ANNUAL RENEWAL)*: ESTABLISHING A PLATFORM TO EVALUATE INTERVENTIONS AIMED AT REDUCING THE DISEASE BURDEN IN KIBERA AND KEMRI/CDC HEALTH DEMOGRAPHIC SURVEILLANCE SITE IN WESTERN KENYA**

Thank you for the continuing review report for the period **November 22, 2017** to **October 2, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval** for continuation.

This approval is valid from **November 22, 2018** through to **November 21, 2019**. Please note that authorization to conduct this study will automatically expire on **November 21, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU by **October 10, 2019.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
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Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

KENYATTA NATIONAL HOSPITAL APPROVED 09 MAY 2019 KNH/UoN-ERC P. O. BOX 20723 - 00202 NAIROBI

Ref: KNH-ERC/A/164

6th May, 2019

Eric D. Ng'eno
Reg. No. H56/89324/2016
Dept.of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Eric

Research proposal: Analysis of Plasmids of *Salmonella enterica serovar* Typhi and their variations over time in Kibera, an urban slum where high rates of Typhoid Fever were previously described (P186/03/2019)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 6th May 2019 – 5th May 2020.

This approval is subject to compliance with the following requirements:

a. Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b. All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH-UoN ERC before implementation.
c. Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d. Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e. Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
f. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
g. Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.