

Genetic Diversity of Clinical *S. Typhimurium* *Salmonella* Isolates from Two District Hospitals in Kenya

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ABSTRACT

Invasive *Salmonella Typhimurium* infection is in a flux in resource poor settings in Kenya. However, sequence analysis of the strains in the study areas have not been determined. This study aimed to determine the distribution and genetic diversity of *S. Typhimurium*. Single locus sequence typing (SLST) based on *fliC* gene indicated *S. Typhimurium* isolates exhibited geographic restriction in addition to intraspecies diversity. The dominant strain isolated belonged to *S. Typhimurium* strain UK-1 lineage in both study areas. Data on genetic diversity is vital in monitoring the disease in the study areas.

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1. Introduction

Salmonellosis, a water and foodborne infection is a major cause of high morbidity and mortality in sub Saharan Africa to human (Feasey *et al.*, 2012). This invasive mechanism has been associated with the type of serovar, patient's age and geographical location (Gordon, 2012). Global statistics of non typhoidal *Salmonella* (NTS) gastroenteritis is estimated at 93.8 million illnesses and 155,000 deaths each year (Crump *et al.*, 2010; Majowicz *et al.*, 2010).

Most human *Salmonella* infections are caused by three serovars viz:- *S. Typhimurium*, *S. Typhi* and *S. Enteritidis* and a limited number of serovars for examples *S. paratyphi A*, *S. choleraesuis*, *S. schottmuelleri*, which are found in varied geographic locations. Amongst these serovars, *Salmonella enterica* serovar *Typhimurium* is highly associated with invasive salmonellosis due to its adaptability to various environments (Helms and Ethelberg, 2005). This strain of *Salmonella* seemed to have been contained until in the 1990s due to emergence of global epidemic of multidrug resistant *S. Typhimurium* definitive type 104 (DT 104) in animals and humans (Helms and Ethelberg, 2005). The microevolution of virulence gene expression has been associated with environmental stress (Boor, 2006). Due to climatic changes, countries in sub Saharan Africa were prone to extreme drought followed by rain storms, hot days and then followed by cool nights, these conditions could have led to the emergence of successful clone of *S. Typhimurium* (Boor, 2006).

Re-emergence of ST 313 as a possible human adapted strain of *S. Typhimurium* in several sub Saharan countries between 1997 and 2010 in Malawi cannot be over emphasized (Okoro *et al.*, 2012). The clone has advanced from ST19 previously associated with gastroenteritis and has evolved to LT2, SI 1344 and DT 104, NCTC 13348 and ST313 of phage type DT 56 var which is the latest strain as at 2012 (Kingsley *et al.*, 2009; Okoro *et al.*, 2012). This is

indicative of intraspecies genetic evolution of the isolate based on environmental cues.

The *S. Typhi* genome, compared to that of *S. Typhimurium*, harbors many inactivated or disrupted genes (de jong *et al.*, 2012), this indicates that ST313 is evolving along similar lines as the typhoid bacteria. It is characterized by loss of gene function, including genes previously implicated in the virulence of *S. Typhimurium* in the murine model of infection, such as *sseI*, encoding a type III-secreted effector protein and *ratB*, encoding a secreted protein associated with intestinal persistence, and of the 44 novel pseudogenes or deletions in the strain relative to LT2, 26 are pseudogenes or deletions in *S. Typhi* or *S. Paratyphi A* (Kingsley *et al.*, 2009). These observations indicate that a similar process of adaptation to the human host may be occurring in African *S. Typhimurium* as has been observed in *S. Typhi*.

In addition, *Salmonella* pathogenicity islands (SPIs), plasmids, functional prophages and phage remnants has also contributed significantly to the genetic diversity among *S. enterica* strains (Boyd and Brussow, 2002). *fliC* gene influence *Salmonella* virulence, *fliC* gene encodes the major component of the flagellum which plays a key role for the Type III secretion system, a mechanism used to secrete proteins from cytoplasm of bacterial cell (Yonekura *et al.*, 2003; McQuiston *et al.*, 2004; Phillip *et al.*, 2006).

Salmonella modifies *fliC* gene expression during invasion, dissemination and colonization of intestinal mucosa (Yonekura *et al.*, 2003; Cummings *et al.*, 2006). This enhances virulence by generating genetic diversity, selected upon by environmental or host pressures, hence the best adapted phenotype survives during infection (Arunavas Das *et al.*, 2012).

In Kenya, multidrug resistant *S. Typhimurium* clone causing invasive disease is reported to be in a flux due to selective pressure in resource poor urban and rural settings (Tabu *et al.*, 2012). However, sequence analysis of

S. Typhimurium strains in these study areas has not been determined. In this regard, this study set to determine the genetic diversity of *S. Typhimurium* found within these two geographically different micro-environments with a view of unraveling any possible evolutionary changes that might have occurred.

II. Materials and Methods

Study Design and Sites

This was a cross sectional study involving clinical *Salmonella* isolates obtained from patients treated for fever, defined as $\geq 38.0^{\circ}\text{C}$ and diarrhoea defined as, ≥ 3 bowel movements within 24hrs period during the preceding 5 days at Kapsabet and Kisumu District hospitals in Kenya between June 2011 to November 2013.

Kapsabet District Hospital

Kapsabet District hospital is located in Kapsabet town of Nandi County in Rift Valley Province in Kenya. Kapsabet town lies on latitude $0^{\circ} 13' 07''\text{N}$, longitude $35^{\circ} 08' 35''\text{E}$, along Kisumu- Eldoret road. Kapsabet is both a Division and County headquarter, it is the Division with the highest population density with approximately 276 persons/ Km^2 (KNBS, 2009). It is the main government hospital in Nandi Central of Nandi County as well as a referral hospital for the five administrative divisions (Fig. 1).

Kisumu District Hospital

Kisumu District hospital is located in Kisumu town within Kisumu County. The town lies on latitude $0^{\circ} 60' 0''\text{S}$, longitude $34^{\circ} 45' 0''\text{E}$ within Winam Gulf of Nyanza Province. It is the third largest city in Kenya with a population of 968,909 persons (KNBS, 2009). Kisumu is the headquarter of Kisumu District as well as Kisumu County. It serves as a communication and trading confluence for the Great Lakes region (Fig. 1).

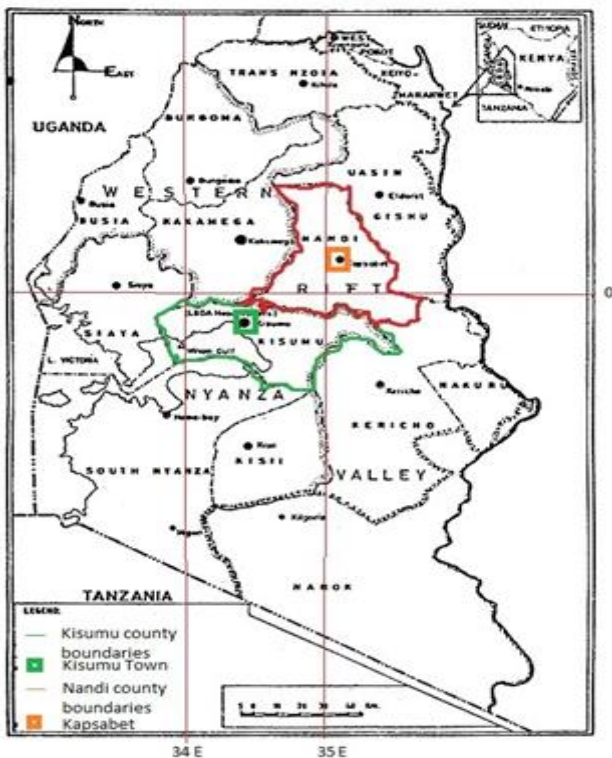


Figure 1. Map of Kenya showing location of study areas: Kapsabet and Kisumu towns (Marked in box)(Adapted from Google Map 2014).

Patients

On arrival to hospital, both children and adult patients were examined by a medical physician and those found to

have fever defined as $\geq 38.0^{\circ}\text{C}$, without acute respiratory illness, irrespective of malaria blood smear results and regardless of bloody diarrhoea, defined as presence of visible blood in stool and diarrhea, defined as ≥ 3 bowel movements within 24hr period during the preceding 5 days were included in the study. Both swabs of whole stool and rectal swabs from patients enrolled for the study were received at the laboratory reception area and immediately placed in Cary Blaire transport medium and transported within 6 to 12hrs in iced cool box at 8°C for culture and isolation of *Salmonella*. Only the first three (3) children and first three (3) adult patients, both in and out patients who met the above criterion per day were enrolled for the study. For children less than 15 years old, parents or guardian gave consent to permit their participation. Consent to carryout study in respective hospital was sort from hospital administrative authority and KEMRI and Maseno University Ethical Review Committee.

Culturing and isolation of Salmonella species

Stool samples were aerobically cultured at 37°C in Selenite F broth for 18 - 24hrs for enrichment after which the cultures were streaked onto plates of *Salmonella Shigella* agar from Himedia, for 18-24hrs at 37°C . Isolates from *Salmonella Shigella* agar were subcultured on plates of Xylose lysine Deoxycholate (XLD) agar and incubated for 18-24 hrs at 37°C . All culture media were from Himedia Laboratories Pvt Ltd Mumbai India. Bacteria isolates were identified by biochemical tests using *Salmonella* API 20E strips (Biomerieux, Marcy L'etoli, France).

Salmonella isolates were inoculated in Tryptic Soy Broth (TSB in 15% glycerol) from Himedia, incubated for 18-24hr then frozen at -20°C in 2ml eppendorff tubes (Sarstedt Ltd, Germany) for genetic analysis.

Salmonella DNA extraction

Pure *Salmonella* isolates obtained from a series of sub cultures in XLD medium and stored in Tryptic Soy Broth (Himedia Pvt Ltd Mumbai, India) were allowed to thaw and then reconstituted in 200ml of 0.9% NaCl solution.

Salmonella colonies were freshly grown in nutrient agar (Himedia pvt Ltd Mumbai, India).The isolates were then suspended in 150 μl of sterile distilled water in eppendorf tube (Sarstedt Ltd, Germany), gently vortexed and homogenate was heated at 100°C for 10min in a water bath, then centrifuged at 10000rpm (Spectrafuge 16M, Labnut International USA) for 5min at 4°C . The supernatant was aliquoted into eppendorff tubes and stored at -20°C for later used as a source of DNA template (Amini et al., 2010).

Salmonella DNA amplification

DNA amplification of *invA* and *fliC* genes was performed in a final volume of 25 μl , consisting of Ready To Go PCR beads, 4 μl of 5ng DNA template, 6.25 μl of 12.5 pmol concentration of each primer and 8.5 μl of PCR water. In ARKTIK thermocycler (Thermofisher Scientific Finland), using published primer pairs for *invA* gene; *F* – ACAGTGCTCGTTTACGACCTGAAT and *R* – AGACGACTGGTACTGATCTAT (Chui et al., 2006). Amplification conditions were ; 30 cycles of 94°C for 5min, 94°C for 1min, 57°C for 1min and 72°C for 30s with final extension extension step of 72°C for 7 min.

Primer sequence for *fliC* gene: *fli15* – CGGTGTTGCCAGGTTGGTAAT and Tym- ACTCTTGCTGGCGGTGCGACTT (Kilger and Grimont, 1993).

Amplification conditions were ; 30 cycles of 95⁰C for 5min, 95⁰C for 1min, 57⁰C for 1min and 72⁰C for 2min with final extension extension step of 72⁰C for 7 min.

PCR products were resolved by electrophoresis on 1.5% agarose gel at 100V for 35min.

DNA sequencing

invA and fliC PCR products were purified using Gene-Jet PCR purification kit (Inqaba biotechnology, South Africa). Purified gene fragments were subjected to direct sequencing in the forward and reverse directions using the same forward and reverse primers as in the PCR , using the Big Dye Terminator 3.1 cycle sequencing Ready Reaction kit (Applied Biosystems), in an automated DNA sequencer, ABI Prism 377 (Perkin Elmer, Warrington, United Kingdom). The automated sequence data was analyzed using the SEQUENCHER v.3.0 software (Gene Codes Corporation, Inc.). Chromatographs were visually inspected and consensus sequences were aligned manually prior to further analysis. Chromatographs were edited using Bioedit software. Multiple alignments of the contigs was done using Muscle 3.8.31(<http://update.musclesoftware.net/download.php>) multiple alignment software.

Nucleotide sequence data for invA, and fliC gene sequences from all clusters were submitted to Basic Local Alignment Search Tool (BLAST),www.ncbi.nlm.nih.gov/blast for similarity searches in Gen Bank. Phylogenetic trees for S.Typhimurium using fliC gene was constructed based on mrBayes software (<http://mrbayes.net>).

III. Results

A total of 400 stool specimens were collected from each study site. Of these, 55.75% (97/174) and 44.25% (77/174) *Salmonella* species were isolated from patients under going treatment at Kapsabet and Kisumu District hospitals respectively. Based on invA gene BLAST search, the distribution of *Salmonella* isolates from Kapsabet District hospital was as in Fig. 2: *Salmonella* serovar Typhimurium 52.6% (51/97), Enteritidis 26.8% (26/97), Paratyphi A, 4.1% (4/97), Paratyphi C 6.2% (6/97), Gallinarum 2.0% (2/97), S.dublin2.0% (2/97), S.Thompson 6.2% (6/97), S. Bareilley 0% (0/97) .The highest *Salmonella* isolate from Kisumu District hospital was *Salmonella* serovar Typhimurium at 57.1%(44/77), as depicted in Fig 3, this was followed by serovar Enteritidis 24.6% (19/77), Paratyphi A, 0% (0/77), S.Paratyphi C, 7.8 (6/77), S.Dublin 0% (0/77), S.Gallinarum 0% (0/77), S. Thompson 3.9% (3/77), S.Bareilley 6.5% (5/77).

Distribution of Salmonella strains

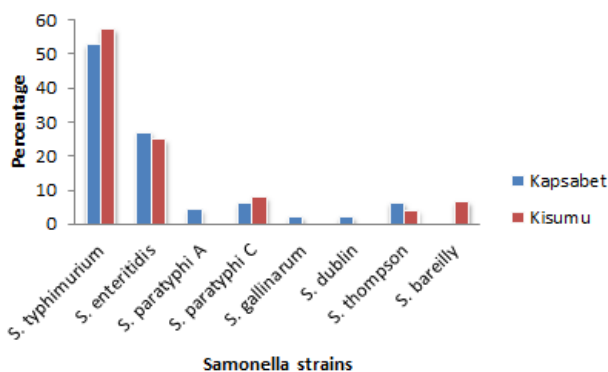


Figure 2 . Distribution of Salmonella isolates from Kapsabet and Kisumu District hospital

Distribution of Salmonella strains in Kapsabet and Kisumu District hospitals

Kisumu had the highest distribution of *Salmonella* Typhimurium strain as shown in Fig.2, while Kapsabet had the highest distribution of *S. Enteritidis*. Kapsabet had significantly high distribution of different strains of *Salmonella* at p-value 0.0288 ($\alpha < 0.05$) (one tailed), using the spearman correlation coefficient.

Phylogenetic analysis of *S.Typhimurium* strains from Kapsabet and Kisumu District hospitals

Salmonella Typhimurium strains from Kapsabet and Kisumu were clustered within separate clades with bootstrap value of 55.92%. Strains KPH 017,001,011 are clustered within the same clade with strains from Kisumu with a bootstrap value of 72.47%, an indication that they are closely related and belong to the same ancestral lineage.

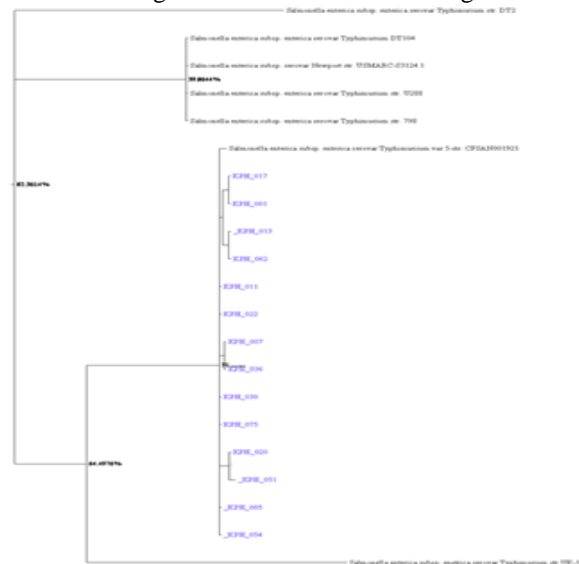


Figure 3. Phylogenetic tree for *S. Typhimurium* isolates from Kapsabet District hospital based on *fliC* gene sequences. The tree was constructed in mrBayes, a program for inference of phylogeny that is based on the Markov Chain Monte Carlos (MCMC) method. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.



Figure 4. Phylogenetic tree for *S. Typhimurium* isolates from Kisumu District hospital based on *fliC* gene

sequences. The tree was constructed in mrBayes, a program for inference of phylogeny that is based on the Markov Chain Monte Carlos (MCMC) method. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.



Fig. 5 . Combined phylogenetic tree for *S. Typhimurium* isolates from Kapsabet and Kisumu District hospitals based on *fliC* gene sequences. The tree was constructed in mrBayes, a program for inference of phylogeny. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.

Legend

KPH- DNA sequences generated from Kapsabet samples

KSH- DNA sequences generated from Kisumu samples

Discussion

This study documents DNA sequence based analysis of *S. Typhimurium* strain associated with non typhoidal infection in Kapsabet and Kisumu regions in Kenya. *Salmonella* serovar Typhimurium was the highest isolate followed by *S. Enteritidis*, the other *Salmonella* strains formed a small proportion (Fig. 2).

The principal findings of this study indicated that *S. Typhimurium* isolates from the two study areas are genetically diverse with significant difference in interclade diversity (p value < 0.001). The results based on *fliC* gene sequences using Bayesian method of phylogeny indicated that there was intra and inter genetic diversity (Figs. 3,4 and 5).

Phylogenetic analysis of *S. Typhimurium* strains from Kapsabet (Fig.3) comprised of both monophyletic and paraphyletic groups. This is due to genetic diversity within the isolates resulting to interclade diversity. All the clades are rooted on *S. Typhimurium* strain UK-1 with bootstrap consensus value of 64.49%, this implies that they belong to the same ancestral lineage.

Phylogenetic tree (Fig.4) depicts *S. Typhimurium* strains from Kisumu. The strains comprises of monophyletic group, implying that tree includes most recent common ancestor with all its descendants. All the clades are rooted on *S. enterica* serovar Typhimurium str. UK-1 with bootstrap consensus value of 66.06% hence they belong to the same ancestral lineage but are new members of the lineage.

Gain and loss of genes during evolution of *Salmonella* is one of the mechanisms that drives diversification within the

bacteria as documented by Retchless and Lawrence (2007). In this study phylogenetic tree was based on *fliC* gene, a gene that encode surface structure and hence under significant recombination pressure that contributes to genetic diversity.

The low consensus bootstrap values indicates that recombination has occurred at a frequency sufficiently high to limit phylogenetic signal as documented by Octavia and Lan (2006).

This could probably be linked to recombination mechanism that produce genomic diversity as documented by Baker *et al.*, (2010). *Salmonella* subspecies I strains share a common niche, one restricted to warm blooded animals, and endowed with compatible restriction modification systems that allow incorporation of larger gene segments of DNA among closely related *Salmonella* pathogens. The results of this study also concurs with findings by Brown *et al.*, (2003), Soyer *et al.*, (2009) and Didelot *et al.*, (2011) who found that *Salmonella* genomes undergo frequent intergenome recombinations and thereby disrupt the clonal inheritance pattern resulting to genetic diversity.

Comparative phylogenetic analysis of *S. Typhimurium* isolates from Kapsabet and Kisumu indicated that all the clades are rooted on *S. entericaserovarTyphimurium*str. UK-1 with bootstrap consensus value of 55.92% ($<97\%$), thus the strains are highly diverse but have evolved from common ancestral lineage.

Of significant importance is that the phylogenetic tree consisted of two sub-clades, one with a cluster of *S. Typhimurium* isolates from Kapsabet and another with clusters of isolates from Kisumu. This clearly proved that the *S. Typhimurium* isolates have a geographic restriction. This results indicates that *S. Typhimurium* isolates from Kapsabet and Kisumu diverged from each other at the strain level of evolution of *S. Typhimurium* and that they have evolved largely independently.

However, *S. Typhimurium* isolates (strains KPH 001,011,017) from Kapsabet had a closer phylogenetic relationship with strains from Kisumu, it is probable that mobility of human population or environmental reservoirs may have been a source of these strains, however surveillance data do not exist to confirm this.

In conclusion, our data demonstrated that *S. Typhimurium* strain is under selective pressure and genetic modification to adapt to changing environments, this accounted for diversity of *S. Typhimurium* strains from the two study sites. In addition, the *S. Typhimurium* strains from the study areas have not evolved to ST313 strain reported in urban Nairobi and rural Kilifi.

IV. Acknowledgement

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