RESEARCH ARTICLE

Genetic characterization and evaluation of antimicrobial resistance patterns of human salmonella typhi isolates in kenyatta national hospital in nairobi, kenya

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The rapid increase and subsequent management of Typhoid fever in Kenya has been complicated by the emergence and rise of antimicrobial resistance to the *Salmonella* Typhi pathogen. This situation is yet to be addressed due to the paucity of information regarding the circulating strains and their antimicrobial resistance profiles. This study was hence designed to genetically characterize and evaluate the antimicrobial resistance patterns of human Salmonella Typhi isolates circulating among patients in Kenyatta National Hospital the largest referral health facility in Kenya. Fifty (n=50) *Salmonella* Typhi archived sample isolates were obtained from stool samples of patients suffering from typhoid fever who attended the facility from 2010 to 2015. The isolates were evaluated for phylogenetic relationships targeting 16s rRNA gene sequences and antimicrobial susceptibilities against a panel of 12 drugs by disk diffusion method. Phylogenetically, the isolates clustered close to *Salmonella* Typhi strain CT18 and its plasmid pHCM1 and plasmid IncHI1 of *Salmonella* Typhi strain R27 both associated with drug resistance. Antimicrobial susceptibility tests showed that 73% of the isolates were resistant to the Aminopenicillin, Sulfonamide, Phenicol and Aminoglycoside classes of drugs. A resistance of between 7% to 19% was observed for the Beta-lactamase, Fluoroquinolone and Cephalosporin classes of drugs. Only one of the isolates was fully susceptible to all the antimicrobial drugs used. A total of 23% of the isolates were intermediately resistant to all the 12 drugs used. The high resistance patterns of *Salmonella* Typhi evidenced necessitates the re-evaluation of the currently used antibiotic regimen and also underscores the importance of continued drug resistance monitoring for this pathogen.

Keywords: *Salmonella* Typhi; Antimicrobial resistance; Phylogenetic; Susceptibility

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Introduction

Typhoid fever is a severe enteric disease caused by *Salmonella* Typhi [1]. Higher incidences of Typhoid fever have been mainly reported in urban areas due congestion and poor sanitation compared to the less populated rural areas of Kenya [2]. Diagnosis of this pathogen relies on laboratory confirmation for the presence of the bacteria in patients' blood, bone marrow or stool samples [3]. The currently available *Salmonella* Typhi evaluation techniques that use rapid tests and blood culture have shortcomings leading to misdiagnosis [4-7]. This results to inappropriate prescription for treatment which has often led to increased pressure for antibiotic resistance leading to complication during treatment with often recurring infection [8, 9]. Although PCR has the limitations of costs and availability in most public health facilities, it is higly sensitive and more specific [10] which can be overcome if governments weigh its merits against the demerits. Antimicrobials have been used for more than 50 years for the treatment of infections, including typhoid fever leading to recovery [11], however current available reports have also shown evidence of a steady rise in the prevalence of *Salmonella* Typhi that is resistant to some of the commonly used antimicrobial drugs [12]. In fact, globally, there is a major public health concern that drugs of greater potency such as ciprofloxacin have begun to exhibit patterns of resistance in *Salmonella* Typhi [13].

In Kenya, the occurrence of multidrug resistant *Salmonella* Typhi strains was first reported in the years 1997-1999 [14], with an initial prevalence rate of 50-65%, which has gradually risen recently [15]. Therefore, establishing phylogenetic relationships of the circulating *Salmonella* Typhi strains is paramount for the development of effective control strategies that enable to monitor the spread [16] and subsequently the implementation of proper diagnosis and treatment of typhoid fever. Additionally, continued genetic characterization and drug resistance surveillance of the bacteria is crucial for evaluating *Salmonella* Typhi's genetic structure and resistance patterns regarding specific antibiotics. The aim of this study was to genetically characterize *Salmonella* Typhi isolates from Kenyatta National Hospital the largest referral hospital in Kenya, and evaluate their antimicrobial resistance patterns.

Materials and methods

Ethical approval

Ethical approval was granted by the Kenyatta National Hospital/University of Nairobi Ethics and Research Committee (KNH/UoN-ERC).

Study design

The study design used was descriptive, cross-sectional.

Setting

Isolates were obtained from archived cultures of samples from Kenyatta National Hospital. The isolates were stool cultures of patients suspected to be suffering from typhoid fever who presented themselves at Kenyatta National Hospital for treatment.

Bacterial strains

Fifty (n=50) *Salmonella* Typhi archived sample isolates were obtained from clinical stool samples of patients with suspected cases of typhoid fever from Kenyatta National Hospital The samples were collected in the period of between 2010-2015. Control samples were archived isolates obtained from the Center for Microbiology Research at Kenya Medical Research Institute (KEMRI).

Culturing and identification of *salmonella* isolates

The isolates were cultured in nutrient broth for enrichment at 37˚C for 20 hrs, following the incubation a loopful of broth from each isolate was streaked on the surface of XLD (Xylose Lysine Deoxycholate Agar) plate and then incubated at 37˚C for 20hrs. Biochemical identification of the strains was done using the API 20E Kit (Biomerieux, Marcy, France). Control samples were subjected to the same treatment

Extraction of bacterial DNA and PCR amplification

Genomic DNA was extracted by Qiagen DNA extraction kit (Qiagen, Germany) according to manufacturers' instructions. Polymerase Chain Reaction was done targeting 16S rRNA gene sequences using a *Salmonella* specific set of primers (Table 1). The total volume for the PCR reaction of 50µl comprised of 25µl master mix, 15µl deionized H_20, 2.5µ Fprimer, 2.5µl Rprimer and 5µl template DNA. The thermo cycler conditions were as follows: An initial denaturation step at 95°C for 2min, followed by 30 cycles of 95°C for 30sec, annealing at 42°C for 30 sec, extension at 72°C for 45 sec and a final extension step of 72°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel in 1x TAE buffer with 0.5µg/ml ethidium bromide and visualized under a UV transilluminator (Herolab, Germany) to determine consensus, sizes and quality.

Table 1. Primers targeting 16S rRNA gene sequences for *Salmonella* sp

Gene	Primer	Sequences 5'-3'	Product size (bp)	Reference
16S rRNA	MINf FWD MINr REV	ACGGTAACAGGAAGCAG TATTAACCACAACACCT	402	[17]

Table 2. Antimicrobial resistant patterns of *Salmonella* Typhi isolates to 12 drugs used in the study

Salmonella Typhi isolates	Pattern of Antimicrobial Resistance	Proportion	Percentage (%)
6a, 11a, 13a, 14a, 38a	AMP^R, SXT^R, S^R, C^R	5/26	19
7a, 12a, 19a, 22a, 26a, 29a, 32a, 40a, 50a	AMP^R, TE^R, SXT^R, S^R, C^R	9/26	34
31a	AMP^R, CAZ^R, CRO^R, CXM^R, TE^R, SXT^R, CN^R, S^R, C^R	1/26	3
8a	AMP^R, CAZ^R, CRO^R, CXM^R, TE^R, CIP^R, SXT^R, CN^R, S^R, C^R	1/26	3
10a	AMP^R, CAZ^R, CRO^R, CXM^R, NA^R, TE^R, CIP^R, SXT^R, CN^R, S^R, C^R	1/26	3
16a	AMC^R, AMP^R, CRO^R, CXM^R, NA^R, TE^R, CIP^R, SXT^R, CN^R, S^R, CR	1/26	3
30a	AMC^R, AMP^R, CRO^R, CXM^R, TE^R, CIP^R, SXT^R, CN^R, S^R, C^R	1/26	3

Key: AMP(Ampicillin), SXT(Trimethoprime-Sulfamethoxazole), S(Streptomycin), C(Choramphenicol), TE(Tetracycline), CAZ(Ceftazidime), CRO(Ceftriaxone), CXM(Cefuroxime), CN(Gentamicin), NA (Nalixidic acid), CIP(Ciprofloxacin), AMC(Amoxicillin-Clavulinic acid).

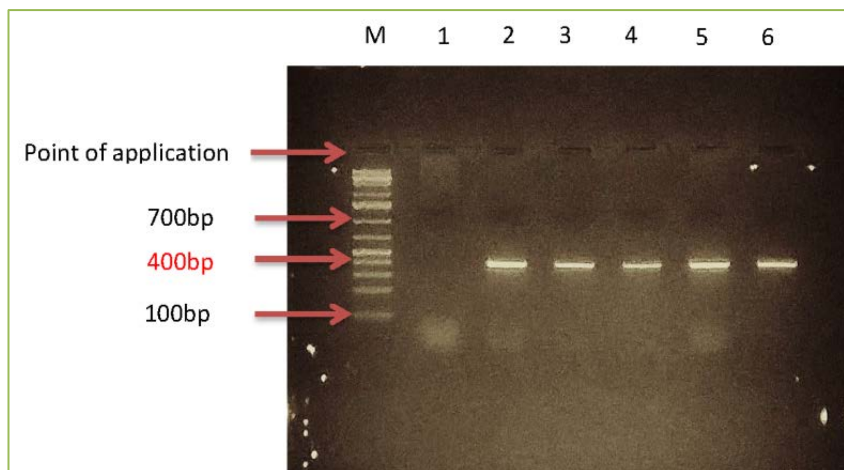

Figure 1. PCR product of the representative analyzed samples. PCR amplification of 16S rRNA gene. M (1kb DNA ladder), 1 (Negative control), 2 (Positive control-S. Typhi, KEMRI), 3-6 (Isolates 7a, 16a, 30a, 40a). A band of 402bp was obtained.

DNA sequencing

The PCR products were gel extracted and purified using Qiagen gel extraction kit (Qiagen, Germany) according to manufacturers'' instructions. The amplified products of the *Salmonella* Typhi isolates were sequenced by a commercial vendor, Macrogen (The Netherlands). The 16S rRNA sequences obtained were compared with known 16S rRNA sequences using Basic Local Alignment Search Tool (BLAST) algorithm form the National Centre for Biotechnology Information (NCBI) database.

Construction of phylogenetic tree

DNA sequences were edited and assembled using Bioedit software. Sequences were aligned using CLUSTAL W

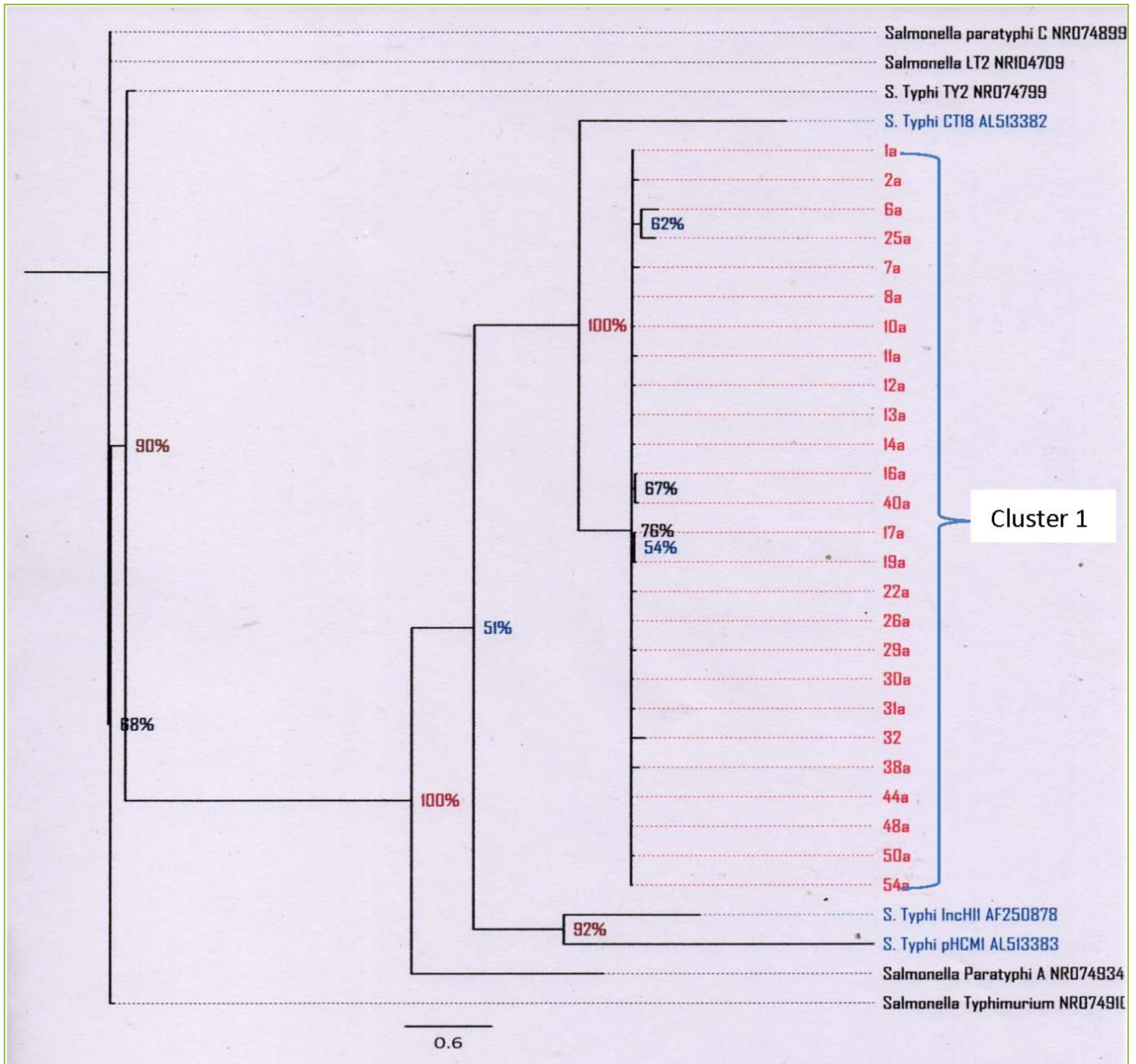

Figure 2: Phylogenetic tree based on 16S rRNA gene sequences. Phylogenetic tree based on 16S rRNA gene sequences. Cluster 1 shows *Salmonella* Typhi isolates. Numbers at the nodes show posterior probabilities indicating topological robustness of the tree. Reference sequences for Salmonella were obtained for the following strains: All isolates appeared to cluster close to *Salmonella* Typhi strain CT18 and its plasmid pHCM1 and plasmid IncHI1 of *Salmonella* Typhi strain R27.

algorithm. Phylogenetic trees were constructed based on 16S rRNA nucleotide sequences, with the Bayesian phylogenetic method, using MrBayes program for the Bayesian influence of phylogeny based on Markov Chain Monte Carlo (MCMC). The resulting trees were visualized using Fig tree software.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion protocol according to the Clinical Laboratory Standards Institute (CLSI) guidelines [18]. Test was done on Mueller Hinton agar, to 12 commonly used antibiotic drugs: Streptomycin, Gentamicin, Chloramphenicol, Nalixidic acid, Tetracycline, Ciprofloxacin, Trimethoprime-Sulfamethoxazole, Amoxicillin-Clavulinic acid, Ampicillin, Ceftazidime, Ceftriaxone and Cefuroxime. *Escherichia* coli strain ATCC 25922 was used as the standard for potency of the antibiotic discs. Antimicrobial activity of each drug was

Table 3. Response of *Salmonella* Typhi isolates to 12 antimicrobial drugs, shown in zones of inhibition (mm)

Isolate No.	AMC 20µg	AMP 10µg	CAZ 30µg	CRO 30µg	CXM 30µg	NA 30µg	TE 30µg	CIP 5µg	SXT 23.75µg	CN 10µg	S 10µg	C 30µg
1a	$>18^{S}$	26^{S}	30^{S}	32^{S}	23^{S}	21^{S}	22^{S}	28^{I}	27^{S}	26^{S}	14^{I}	29^{S}
2a	18^{S}	25^{S}	31^{S}	38^{S}	23^{S}	22^{S}	23^{S}	31^{S}	28^{S}	26^{S}	16^{S}	31^{S}
6a	14^{I}	6^{R}	27^{S}	29^{S}	23^{S}	20^{S}	19^{S}	32^{S}	6^{R}	23^{S}	6^{R}	6^{R}
7a	22^{S}	6^{R}	30^{S}	31^{S}	22^{S}	25^{S}	6^{R}	31^{S}	6^{R}	22^{S}	6^{R}	6^{R}
8a	18^{S}	6^{R}	10^{R}	6^{R}	6^{R}	15^{I}	7^{R}	19^{R}	6^{R}	12^{R}	6^{R}	6^{R}
10a	15^{I}	6^{R}	14^{R}	6^{R}	6^{R}	12^{R}	8^{R}	20^{R}	6^{R}	11^{R}	6^{R}	6^{R}
11a	17^{I}	6^{R}	23^{S}	27^{S}	22^{I}	22^{S}	17^{S}	31^{S}	6^{R}	20^{S}	6^{R}	6^{R}
12a	$>18^{S}$	6^{R}	31^{S}	35^{S}	22^{I}	26^{S}	6^{R}	34^{S}	6^{R}	23^{S}	6^{R}	6^{R}
13a	15^{I}	6^{R}	27^{S}	29^{S}	22^{I}	21^{S}	17^{S}	32^{S}	6^{R}	21^{S}	6^{R}	6^{R}
14a	16^{I}	6^{R}	29^{S}	30^{S}	23^{S}	25^{S}	6^{R}	32^{S}	6^{R}	21^{S}	6^{R}	6^{R}
16a	13^{R}	6^{R}	18^{I}	6^{R}	6^{R}	6^{R}	10^{R}	17^{R}	6^{R}	10^{R}	6^{R}	6^{R}
17a	$>18^{S}$	24^{S}	24^{S}	28^{S}	22^{I}	20^{S}	16^{S}	32^{S}	22^{S}	23^{S}	14^{I}	24^{S}
19a	14^{I}	6^{R}	21^{I}	27^{S}	22^{I}	21^{I}	6^{R}	37^{S}	6^{R}	22^{I}	6^{R}	6^{R}
22a	$>18^{S}$	6^{R}	39^{S}	40^{S}	31^{S}	36^{S}	7^{R}	41^{S}	6^{R}	31^{S}	6^{R}	6^{R}
25a	$>18^{S}$	28^{S}	29^{S}	29^{S}	20^{I}	24^{S}	20^{S}	30^{I}	25^{S}	20^{S}	15^{S}	30^{S}
26a	20^{S}	6^{R}	30^{S}	31^{S}	22^{I}	24^{S}	6^{R}	31^{S}	6^{R}	26^{S}	6^{R}	6^{R}
29a	22^{S}	6^{R}	29^{S}	28^{S}	20^{I}	21^{S}	6^{R}	33^{S}	6^{R}	22^{S}	6^{R}	6^{R}
30a	10^{R}	6^{R}	10^{R}	6^{R}	6^{R}	15^{I}	9^{R}	19^{R}	6^{R}	9^{R}	6^{R}	6^{R}
31a	17^{I}	6^{R}	13^{R}	6^{R}	6^{R}	15^{I}	10^{R}	22^{I}	6^{R}	10^{R}	6^{R}	6^{R}
32a	$>18^{S}$	6^{R}	27^{S}	29^{S}	26^{S}	24^{S}	6^{R}	27^{I}	6^{R}	24^{S}	6^{R}	6^{R}
38a	17^{I}	6^{R}	28^{S}	30^{S}	22^{I}	20^{S}	16^{S}	33^{S}	6^{R}	22^{S}	6^{R}	6^{R}
40a	22^{S}	6^{R}	30^{S}	30^{S}	23^{S}	22^{S}	6^{R}	30^{I}	6^{R}	25^{S}	6^{R}	6^{R}
44a	$>18^{S}$	22^{S}	27^{S}	30^{S}	21^{I}	24^{S}	24^{S}	35^{S}	23^{S}	24^{S}	17^{S}	24^{S}
48a	$>18^{S}$	20S	26^{S}	29^{S}	20^{I}	20^{S}	19^{S}	30^{I}	24^{S}	22^{S}	14^{I}	25^{S}
50	15^{I}	6^{R}	26^{S}	26^{S}	21^{I}	16^{I}	6^{R}	29^{I}	6^{R}	18^{S}	6^{R}	6^{R}
54a	$>18^{S}$	22^{S}	25^{S}	29^{S}	21^{I}	19^{S}	18^{S}	30^{I}	22^{S}	22^{S}	19^{S}	25^{S}
E. coli ATCC 25922	22^{S}	19^{S}	28^{S}	28^{S}	22^{S}	23^{S}	22^{S}	31S	28^{S}	22^{S}	15^{S}	28^{S}

Key: S=Susceptible, I=Intermediate and R=Resistant

tested for all isolates and results interpreted according to the guide lines provided for antimicrobial disc susceptibility tests by Clinical and Laboratory Standards Institute [18] and results recorded as Susceptible, Intermediate Resistant and Resistant.

Results

Fourty five (45) out of the 50 *Salmonella* isolates representing (90%) of the total samples evaluated were positively identified as *Salmonella* Typhi based on PCR using the 16S rRNA gene. The amplified product that was detected was 402 (Figure 1).

Phylogenetic analysis

Phylogenetically, the isolates clustered close to *Salmonella* Typhi strain CT18 and its plasmid pHCM1 and plasmid IncHI1 of *Salmonella* Typhi strain R27 both associated with drug resistance (Figure 2).

Evaluation of antimicrobial resistance

Resistance patterns for *Salmonella* Typhi are shown in Figure 3 and Table 2. Only one of the *Salmonella* Typhi isolates was fully susceptible to all the antimicrobial drugs used. In addition, only 23% of the isolates were susceptible to most of the drugs used. Most of the strains also showed intermediate resistance to one or more drugs (Table 3). Nalixidic acid and Amoxicillin-Clavulinic acid were the most effective drugs exhibiting the least resistance (7%) followed by ciprofloxacin and ceftazidime which exhibited 15% resistance. A total of 19% of the isolates were resistant to ceftriaxone, cefuroxime and Gentamicin while resistance to Tetraclycline was 57%. The highest resistance (<73%) was seen with Ampicillin, Trimethoprime-Sulfamethoxazole, Streptomycin and Chloramphenicol. Highly effective drugs Nalixidic acid and Amoxicillin-Clavulinate also exhibited an intermediate resistance, where 19% of the isolates were intermediately resistant to Nalixidic acid and 38% of were intermediately resistant to Amoxicillin-Clavulinic acid. A relatively high intermediate resistance was also seen in Ciprofloxacin and Cefuroxime, where 30% of the isolates were intermediately resistant to Ciprofloxacin and 50% of the isolates were intermediately resistant to Cefuroxime. The overall multidrug resistance was 73%, which was a resistance to four or more antimicrobial drugs

Discussion

Knowledge of the circulating *Salmonella* strains is important for crucial decision making in terms of diagnosis and treatment of the bacterial infection in humans. In the current study, molecular techniques combined with

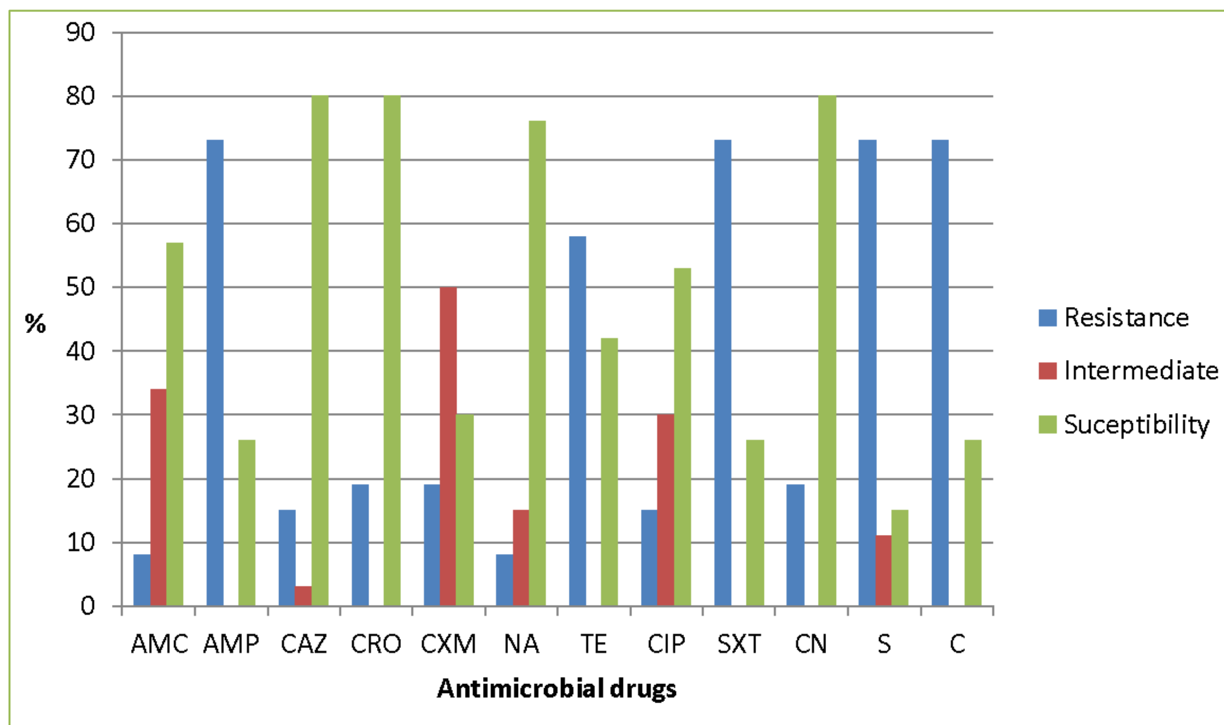

Figure 3. Antimicrobial susceptibility tests. Percentage resistance, intermediate resistance and susceptibility of *Salmonella* isolates against 12 antimicrobial drugs. AMC (Amoxicillin-Clavulinic acid), AMP (Ampicillin), CAZ (Ceftazidime), CRO (Ceftriaxone), CXM (Cefuroxime), NA (Nalixidic acid), TE (Tetracycline), CIP (Ciprofloxacin), SXT (Trimethoprime-Sulfamethoxazole), CN (Gentamicin), S (Streptomycin), C (Chloramphenicol). High susceptibility (>50%) was observed with AMC (Amoxicillin-Clavulinic acid), CAZ (Ceftazidime), CRO (Ceftriaxone), NA (Nalixidic acid), CIP (Ciprofloxacin) and CN (Gentamicin) groups. High resistance (>50%) was observed with TE (Tetracycline), AMP (Ampicillin), SXT (Trimethoprime-Sulfamethoxazole), S (Streptomycin) and C (Chloramphenicol).

phylogenetic analysis confirmed that 90% of the archived isolates were *Salmonella* Typhi strains. Phylogenetic mapping of the *Salmonella* Typhi isolates against reference *Salmonella* sequences showed that the isolates were related to *Salmonella* Typhi strain CT18 which is a characteristic of *Salmonella* Typhi CT18. Conjugative incH1 (pHCM1) plasmid has been shown to be a key factor that encodes resistance to all first line typhoid antimicrobials [19]. This resistant pattern is consistent with previous findings showing that H58 a multi-drug resistant lineage of *Salmonella* Typhi CT18 has already been detected in Kenya with a recent wave of transmission having been reported from the endemic region of South East Asia [20].

Antimicrobial susceptibility tests showed that there was an overall high resistance (73%) to the most commonly used antimicrobials including penicillins, sulfonamides, aminoglycosides and phenicols. This finding may necessitate a change of regimen against *Salmonella* Typhi in Kenya as has been already reported in Asia and India [21, 22]. Following development and rapid rise of resistance to Chloramphenicol, the drug was immediately withdrawn and substituted with second and third generation cephalosporins [23]. However, the 2nd and 3rd generation cephalosporins (Cefuroxime, Ceftriaxone and Ceftazidime) used in this study exhibited a resistance of between 15-19%. This clearly indicates a continued development and rise in resistance to even the most potent antimicrobial drugs by *Salmonella* Typhi. The overall Multidrug resistance of 73% in this study relates to an increase in resistance of approximately 13% (from 60.4%) in the last seven years [24]. This can be directly correlated to misuse of antibiotic drugs with a view of targeting multidrug resistant phenotypes while inadvertently using ineffective combinations of antibiotics. Some of the *Salmonella* Typhi isolates showed an intermediate resistance to Amoxicillin-Clavulinic acid, Cefuroxime, Nalixidic acid and Ciprofloxacin, an indication that these phenotypes may gain full resistance in the near future. In a previous similar study [15] resistance to Ceftriaxone drug was 6% compared to a 19% resistance while only 1% of the isolates were resistant to Gentamicin, compared to a 19% resistance in the current study indicating a steady rise in resistance by this bacteria. The rise in resistance to these drugs may indicate a direct correlation to the misuse of the antibiotic drugs in the time period between the two studies reinforcing the need to monitor and regulate the dissemination and use of these

drugs to curb the rise in resistance. The same study [15] also showed resistance to Nalixidic acid at 35%, compared to 7% resistance in the current study. This may indicate that the use of fluoroquinolones may have reduced in the past few years and that of 2nd and 3rd generation cephalosporins may have increased owing to their higher resistance rate in this study. The Kenyan government has given policy concerning the growing rate of antimicrobial resistance that needs to be implemented. The policy encourages research and the need for investment in order to develop new antibiotics, diagnostic tools as well as vaccines [25].

The high resistance patterns of *Salmonella* Typhi to various antimicrobials as evidenced necessitate re-evaluation of the currently used antibiotics and also underscore the importance of continued monitoring of drug resistance for this pathogen. Proper policies should also be put in place and enacted to regulate the use of antimicrobials in order to control the likely resistance that develops as a result indiscriminate use of antibiotics. It is imperative for future studies to evaluate resistant gene structures of *Salmonella* Typhi to establish whether there is resistant gene are transfer between animals and humans and between different *Salmonella* serotypes.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Abbreviations

KNH/UoN-ERC: Kenyatta National Hospital/University of Nairobi Ethics and Research Committee; KEMRI: Kenya Medical Research Institute.

Author Contributions

PG acquired, analyzed and interpreted the data, drafted and revised the manuscript, FK designed the study and acquired the data, NG acquired the data, evaluated and interpreted antimicrobial resistance, NO revised the manuscript, GJ conceived and designed the study and revised the manuscript, PK conceived and designed the study, AN conceived and designed the study, interpreted the data and revised the manuscript, JK conceived and designed the study, acquired and interpreted the data and revised the manuscript.

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