INTRODUCTION

Salmonella Enteritidis and Salmonella Typhimurium are two of the ten most common serotypes confirmed in salmonellosis cases in humans, representing 81% of the isolates [1]. Non-typhoidal Salmonella infections generally result in mild to moderate self-limiting gastroenteritis. Antimicrobial treatment is only required in severe cases occurring in susceptible host population or to combat invasive infections. Antimicrobial resistance in S. enterica is a cause of serious concern in human medicine. Ampicillin, chloramphenicol, sulfamethoxazole, tetracycline and cotrimoxazole are used as first line therapy for enteric fever and other non typhoidal Salmonella infections [2]. However, the emergence of resistance to β-lactam antimicrobial agents is a great challenge in management of adult and pediatric infections [3]. Many of the recent isolates obtained worldwide are resistant to a number of antibiotics [4, 5]. A study by Onyango et al., [6] in western Kenya documented S. Typhimurium isolates from two hospitals were resistant to tetracycline, sulfamethoxazole, cotrimoxazole, ampicillin, chloramphenicol and streptomycin. Tabu et al., [7] reported 76% multidrug resistance to more than three antimicrobials tested (chloramphenicol, trimethoprim-sulfamethoxazole and ampicillin) in the same region. In a related study in Siaya District hospital in western Kenya, S. Typhimurium isolates were resistant to most commonly used antimicrobials in addition to the development of ceftriaxone resistance [8]. This shows the fluidity in resistance patterns of Salmonella worldwide and regionally. The aforementioned data implies that there is need for strategies to maintain spectrum of activity of existing antibiotics especially in resource poor settings in Kenya.

Antibiotics are the usual treatment for severe S. enterica associated disease, hence the emergence of multidrug resistance (MDR) S. enterica displaying this resistance is a cause of serious concern in human medicine. Antimicrobial susceptibility tests are carried out to confirm the type of drug resistant infection and to monitor the spread of drug resistant clones. In this study, antimicrobial susceptibility patterns of Salmonella enterica isolates obtained from the study area were investigated. The objective of this study was to assess the antimicrobial resistance patterns of clinical Salmonella enterica isolates from two district hospitals in Kenya.

KEYWORDS: Salmonella enterica, β- lactam, susceptibility, antimicrobial, resistance.
phenotype is a major public health problem [3]. Antibiotic resistance in bacteria can develop by accumulation of point mutation or by horizontal exchange of DNA between bacteria of the same or different species [9].

Exposure of bacterial pathogen to antibiotics enhances resistance by selecting for those cells that are able to tolerate them [10]. This creates positive correlation between level of antibiotic use and prevalence of antibiotic resistance in bacteria in the same human population both at national and regional levels [11]. The rate of emergence of resistance is also related to total antibiotic use, including widespread prescription of antibiotics for respiratory viral infection [10, 12] in addition to use of antibiotics in low doses as growth promoters in livestock farming [13]. In addition, increases in resistance in S. enterica may be at least partially due to clonal dissemination of resistant strains [9].

Characterization of resistance phenotypes and genotypes in clinically recovered isolates is significant in order to respond to ever changing antimicrobial resistance surveillance data in these regions. Knowledge of genotypes prevalence in a locality and region is important in surveillance of spread in antibiotic resistance since the genotypes of resistance differ from one geographic region to another.

Multidrug resistance has been associated with mobile genetic elements. The multidrug resistance phenotypes have been described by many authors S. Typhimurium [4] and S. enteritidis [14]. Resistance to β-lactams in S. enterica is mainly due to the production of acquired β-lactamases [15].

A bacterial pathogen is drug resistant because it has a plasmid bearing one or more resistance genes, the R-plasmid. Plasmid resistance genes code for enzymes that destroy or modify drugs for example the hydrolysis of penicillin or acetylation of chloramphenicol and aminoglycoside drugs [16]. Drug resistance may be caused by production of hydrolytic enzymes to destroy the antibiotic surrounding the environment, the mutations in the specific genes to avoid the action of antibiotics, decreased outer membrane permeability to prevent antibiotics from entering the bacterial cell itself and efflux systems to exclude antibiotics before they become effective.

MATERIALS AND METHODS
Study Sites and Design
This was a cross sectional study involving clinical Salmonella isolates obtained from patients treated for fever, defined as ≥ 38.0°C and diarrhoea (defined as presence of visible blood in stool) diarrhoea (≥ 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° 13’ 07”N, longitude 35° 08’ 35”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/Km² [17]. It is the main government hospital in Nandi Central of Nandi County as well as a referral hospital for the five administrative divisions (Figure 1).

Kisumu District Hospital
Kisumu District hospital is located in Kisumu town within Kisumu County. The town lies on latitude 0° 60’ 0”S, longitude 34° 45’ 0”E within Winam Gulf of Nyanza Province. It is the third largest city in Kenya with a population of 968,909 persons [17]. Kisumu is the headquarters of Kisumu District as well as Kisumu County. It serves as a communication and trading confluence for the Great Lakes region (Figure 1).

Patients
On arrival to hospital, both children and adult patients were examined by a medical physician and those found to have fever defined as ≥ 38°C, without acute respiratory illness, irrespective of malaria blood smear results and regardless of bloody diarrhoea (defined as presence of visible blood in stool) diarrhoea (≥ 3 bowel movements within 24hrs period during the preceding 5 days) were included in the study. Both 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 by the laboratory personnel 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 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 carry out the study in respective hospital was sort from hospital administration authority, KEMRI and Maseno University Ethical Review Committee.

**Culture and Isolation of Salmonella spp**

Stool samples were aerobically cultured at 37°C in Selenite F broth (Himedia Laboratories Pvt Ltd Mumbai, India) for 18-24hrs and subcultured when orange on plates of *Salmonella Shigella* agar, incubated for 18-24hrs at 37°C. Colonies suspected to be *Salmonella* were then subcultured onto plates of Xylose Lysine Deoxycholate (XLD) plate incubated for 18-24hrs at 37°C. Bacterial isolates were identified by biochemical tests using *Salmonella* API 20E strips (Biomerieux, Marcy L’etoli, France) and serotyped using agglutinating antiserum (Murex Diagnostics, Dartford, United Kingdom). *Salmonella* isolates were stored at -20°C in 15% Tryptic Soy Broth in 15% of glycerol stored in 2ml eppendorf tubes (Sarstedt Ltd, Germany) until they were analyzed.

**Antimicrobial Susceptibility Testing**

Salmonella isolates were tested for susceptibility to antimicrobials by Kirby Bauer disk diffusion technique. The antibiotic disks (all from Himedia Laboratories, Pvt Ltd Mumbai, India) contained ampicillin (AMP) (25mcg), gentamicin (GEN) (10mcg), kanamycin (K) (30mcg), tetracycline (TET) (25mcg), co-trimoxazole (COT) (25mcg), streptomycin (ST) (10mcg), sulfamethoxazole (SX) (200mcg) and chloramphenicol (C) (30mcg). Fresh *Salmonella* colonies were inoculated in 0.85% NaCl suspension to turbidity equivalent to 0.5 MacFarland standards which is equivalent to 1.0x10^8 corresponding approximate density of bacteria/ml. The culture was swabbed onto a Muller-Hinton agar from Himedia. Antibiotic discs were applied using sterile forceps onto the bacterial lawn in the plates after drying for 5 min under lamina airflow chamber, the plates were incubated at 37°C for 24hrs.

The MICs of these antibiotics were determined using Etest strips (AB Biodisk, Solna, Sweden) according to manufacturer’s instructions. *E. coli* ATCC 25922 was used as a control for potency of antibiotic discs. Disk sensitivity tests and MICs were interpreted according to guidelines provided by the Clinical and Laboratory Standard Institute [18]. Isolates resistant to two or more of antimicrobials tested were categorized as multidrug resistant.

**Salmonella DNA Extraction**

Pure *Salmonella* isolates obtained from a series of sub cultures in XLD medium (Himedia Pvt Ltd Mumbai, India) and stored in Tryptic Soy Broth from Himedia were allowed to thaw and then reconstituted in 200ml of 0.85% NaCl solution. *Salmonella* colonies were grown in nutrient agar (Himedia Pvt Ltd Mumbai, India ).The isolates were then suspended in 150µl of sterile distilled water in eppendorf tube (Sarstedt Ltd, Germany), gently vortexed and homogenate was then heated at 100°C for 10min in a water bath, then centrifuged at 10,000rpm (Spectrafuge 16M, Labnut International USA) for 5min at 4°C. The supernatant was aliquoted into eppendorf tubes and stored at -20°C for later used as a source of DNA template [19].

**Genetic Analysis of Antibiotic Resistance Genes**

The antibiotic resistance genes under investigation were blaoxEM (ampicillin), oxa1 (ampicillin), apfa (kanamycin), sul2 (sulfonamide), aadB (gentamicin) and cat (chloramphenicol). DNA amplification 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. Amplification was performed in ARKTIK thermocycler (Thermo Fisher Scientific Finland), using published primer pairs in Table 1.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. Amplicons were loaded onto casted 2% agarose gel (Eurobio, Les Ulis, France) and run in tris-borate EDTA buffer (89 mmol l^−1 Tris pH 8.3, 89 mmol l^−1 borate and 2 mmol l^−1 EDTA). The gel was stained with ethidium bromide solution (0.5 µg ml^−1) and run at 135V for 25 minutes, then photographed under UV (Daahkan Scientific, Wonju, Korea).

**RESULTS**

**Phenotypic Antibiotic Susceptibility of NTS from Kapsabet and Kisumu District hospitals**

A total of 174 NTS isolates were tested for susceptibility to 8 antimicrobials in both study areas. In Kapsabet (Table 2), all the isolates 100% (n=97) were susceptible to three antimicrobials, viz:- gentamicin (MIC<sub>90</sub>= 0.02µg/ml), kanamycin (MIC<sub>90</sub>= 0.01µg/ml) and chloramphenicol (MIC<sub>90</sub>= 0.12µg/ml). Resistance to ampicillin was 99% (MIC<sub>90</sub>=250 µg/ml), followed by sulfamethoxazole at 19% (MIC<sub>90</sub>=32µg/ml), resistance to tetracycline (MIC<sub>90</sub>=0.200µg/ml), cotrimoxazole (MIC<sub>90</sub>= 32µg/ml) and streptomycin (MIC<sub>90</sub>= 252µg/ml) was less often at 6%. In Kisumu (Table 3), all the isolates were resistant to ampicillin (MIC<sub>90</sub> = 250 µg/ml) at 100% (n=77). Resistance to chloramphenicol (MIC<sub>90</sub>=256µg/ml) and tetracycline (MIC<sub>90</sub>= 200µg/ml) was 96%, this was followed by resistance to streptomycin (MIC<sub>90</sub>= 252µg/ml) at 92%, sulfamethoxazole (MIC<sub>90</sub>= 32µg/ml) at 91%, gentamicin (MIC<sub>90</sub>= 215µg/ml) at 84%, kanamycin (MIC<sub>90</sub>= 214µg/ml) at 80% and cotrimoxazole (MIC<sub>90</sub>= 32µg/ml) displayed the least resistance at 76%. A total of 70% of *Salmonella* isolates from Kisumu and 50% of isolates from Kapsabet were multidrug resistant (MDR) phenotype that is resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT). Comparison of antimicrobial susceptibility data between Kapsabet and Kisumu revealed significantly high antimicrobial resistance in Kisumu than Kapsabet at p value <0.001 using chi square.

**Salmonella Antimicrobial Resistance Genes Analysis**

To confirm the phenotypic basis of resistance, several resistance genes were sorted; among them were those for beta-lactam inhibitors (blaoxEM and oxa1) sulphamamide resistance (sul2), chloramphenicol acetyltransferase (cat), kanamycin resistance (apfa) and gentamicin (aadB) (Table 1). Genetic analysis of *Salmonella* antimicrobial resistance genes was done by Polymerase Chain Reaction (PCR) and were found to correlate to those of phenotypic
antimicrobial susceptibility (Tables 2 and 3). Isolates displayed different resistance gene patterns; In Kapsabet, (99%) displayed presence of \(bla_{TEM}\) gene, (83%) displayed \(oxa1\) gene. The \(aphA\) gene was (0%) and \(aadB\) gene was (0%) \(sul2\) gene was (19%) while \(cat\) gene was (0%). Isolates from Kisumu displayed high resistance with the following resistance gene patterns; \(bla_{TEM}\) (100%), \(oxa1\) was (100%) and \(aadB\) 84%. The \(aphA\) displayed (80%), \(cat\) (96%) while \(sul2\) (91%).

**DISCUSSION**

Our study used 174 *Salmonella* isolates for the detection of antimicrobial susceptibility profile. These isolates were
recovered from patients in all age groups from infants to adults. Out of these, 97 (100%) of the isolates from Kapsabet were fully susceptible to three antimicrobials; gentamicin, kanamycin and chloramphenicol. Susceptibility to the same antimicrobials was relatively low in Kisumu at 12%, 20% and 4% for gentamicin, kanamycin, and chloramphenicol respectively.

This indicates a decline in NTS susceptibility to these commonly used antimicrobials in the study areas. Previous study by Velge et al., [26] reported that resistance to antibiotics within a population is linked to widespread prescription of antibiotics for other infections. The emergence of resistance to β-lactam antimicrobial agent (ampicillin) could be linked to widespread prescription. This is currently a challenge to management of adult and paediatric infections in resource poor settings in Kenya. In this study, 99% of the isolates in Kapsabet were resistant to ampicillin, followed by sulfamethoxazole at 19%, resistance to tetracycline, cotrimoxazole and streptomycin was low at 6%. In Kisumu, all the isolates 100% (n=77) were resistant to ampicillin, followed by resistance to chloramphenicol and tetracycline at 96%, streptomycin at 92%, sulfamethoxazole at 91%, cotrimoxazole was least resistant at 76%. This is contrary to study by Kariuki et al., [2] who reported a total of 23.4% (n=45) of NTS were fully susceptible to all antimicrobials tested including ampicillin, tetracycline, cotrimoxazole, chloramphenicol. In addition, only 5% (n=45) of NTS were resistant to ampicillin. The result implies that there is increase in resistance to commonly available antimicrobials, and the highest resistance was recorded in ampicillin. In this study, the resistance phenotype was ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT). Hence a total of 68.96% (120/174) of the isolates exhibited MDR phenotype. These results are consistent with findings by NARMS, [27], which found 4.3% (107/2474) of non typhoidal Salmonella isolates were ACSSuT- resistant, including 18.6% (68/366) of Salmonella enterica serovar Typhimurium. In addition, study by Kariuki et al., [2] reported that 34.2% (n=66) of NTS isolates from human subjects, were resistant to three or more antibiotics in Kenya. The most common resistant phenotypes were ampicillin, tetracycline and cotrimoxazole in 75% of the isolates. Resistance to tetracycline, gentamicin, chloramphenicol or ampicillin was low at 15%. This is contrary to this study in which the highest resistance was observed in ampicillin at 100% and 99% in Kisu and Kapsabet respectively. These shows increase in resistance compared to previous study. Comparatively, antimicrobial resistance was significantly high in Kisumu than Kapsabet at p< 0.001.

The presence of blaTEM and oxa1 plasmid oriented group of blaCMY2 genes confirmed the spread of clonally disseminated and horizontally transferred genes β-lactam penicillins (ampicillin) to which 90% of the isolates showed resistance. Resistance to sulfonamides was based on amplification of sul2 gene which is among Tn21 class 1 integron gene cassettes as documented by Cambray et al., [30]. This led to sulfamethoxazole resistance. Sulfonamide resistance in gram negative bacteria arises from acquisition of either of the two genes, sul1 or sul2, encoding forms of dihydropoteroate synthetase that are not inhibited by sulfamethoxazole. The presence of sul2 resistance genes may be as a result of successive pressure exerted by sulfonamides and other antimicrobial agents commonly used and may be mitigated by the fact that not all sulfonamide resistant determinants exert a fitness cost. Resistance to kanamycin was mediated by plasmid encoded aminoglycoside phosphotransferases, aphA gene while chloramphenicol and gentamicin resistance was mediated by cat and aadB genes respectively.

Monitoring phenotypic and genotypic resistance to antibiotics in Salmonella species isolated from animal is important for the protection of human and animal health [31, 32]. There is need for routine surveillance to detect emerging resistance trends and provide interventions within animal and public health. Training in personal hygiene, sanitation and provision of quality water cannot be overemphasized.

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