Characterization and antimicrobial susceptibility patterns of clinical salmonella isolates from Nandi County of rift valley, Kenya

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ABSTRACT
Salmonellosis, a water and foodborne infection is a major cause of high morbidity and mortality in Sub-Saharan Africa. Its prevalence and mortality has been associated with antibiotic resistance related strains that calls for specific strain identification. Data on prevalence and antimicrobial susceptibility patterns of Salmonella in Nandi County of Rift Valley is scarce despite the scourging effect of the disease. 200 stool samples were collected from patients treated for diarrhoea at Kapsabet District hospital between (February – November, 2011). 84%(n=168) were positive for various bacteria isolates as determined by standard microbiological culture techniques. 29%(n=48) were Salmonella. The distribution was; S.typhimurium 56%(n=27), S. enteritidis 29%(n=14), S.paratyphi C 6%(n=3), S. paratyphi A 4%(n=2), S. gallinarum 2%(n=1), S. dublin 2%(n=1). Isolates were confirmed by API 20E biochemical system in addition to invA gene sequencing. All Salmonella isolates were resistant to ampicillin, (19%) were resistant to sulfamethoxazole. Resistance to tetracycline, streptomycin and chloramphenicol was (6%). All ampicillin resistant isolates possessed blaTEM gene while sulfamethoxazole resistant isolates had sul2 gene both genes are associated with class1 integrons. The findings indicated a low antimicrobial resistance in relation to other regions within the country. This situation should be encouraged. Additional safety measures should include training in personal hygiene, sanitation and ensuring water quality.

Introduction
Infection with Salmonella enterica has been recognized as a major public health concern in developing countries including Kenya. In Sub-Saharan Africa, Salmonella are frequent cause of invasive bacteria disease. Salmonella infection is self-limiting and tends to be fatal in less than 1% of those infected. However, the new strain of invasive nontyphoidal Salmonella (iNTS) is fatal in up to 45% of the cases in Africa [1], in every 4 people infected with new strain has died [1].This strain causes a diverse range of symptoms including fever, respiratory problems and at times death. This is partly as a result of its resistance to antimicrobial drugs used for therapeutic purposes [2]. Nontyphoidal Salmonella are a common cause of bacterial gastroenteritis, which is usually a self-limited illness in previously healthy adults [3]. However, nontyphoidal Salmonella can also cause a variety of life-threatening extraintestinal infections. A number of host factors can predispose to extraintestinal Salmonella infections, including AIDS, hemolytic anaemias, and genetic defects that affect (interleukin -12) IL-12, (gamma interferon) IFN-γ, and the phagocyte (Nicotinamide diphosphate) NADPH oxidase [3]. Salmonella spp display high natural susceptibility levels to the most commonly used antimicrobial agents [4]. However the occurrence of isolated Salmonella strains exhibiting resistance to one or more antibacterial agents have steadily increased probably due to continuous antibiotic pressure [5,6]. There is direct evidence that antimicrobial use in animals selects for antimicrobial resistant NTS serotypes, which are then transmitted to human in food or through direct contact with animals. Withdrawal of antimicrobials often fails to have a significant effect on the prevalence of MDR Salmonella in food of animal origin [7], and in some cases new strains can persist despite a selective disadvantage from antimicrobials [8,9].

Thus, the emergence and dissemination of new strains does not necessarily hinge to antimicrobial selection pressure. Successful epidemic strains probably have other biological and ecological traits or genetic factors that increase bacterial virulence or fitness cost often accompanied by antimicrobial resistance [10-12], that allow more efficient dissemination of the strains in specific host population and environments relative to other coexisting strains.

In a community based study in Nairobi Kenya, bacterial isolates including non-typhoid Salmonella that exhibited antimicrobial resistance were reported to be; (25.5%), S. typhi (10.6%) and S.typhimurium(10.6%) [13]. A cross sectional study within two rural hospital settings in western Kenya to determine in vitro antibiotic susceptibilities found 90% of Salmonella typhi to be resistant to ampicillin and streptomycin. All S.typhimurium clinical isolates were 100% resistant to all the antibiotics tested except ciprofloxacin [14]. According to Kariuki et al.,[15,16] the prevalence of multidrug resistant (MDR) phenotype was reported to be on the increase and it is therefore thought that these serotypes have been spreading to other parts of Kenya and are gradually replacing the fully sensitive strains. DNA of clinical samples of Salmonella taken from patients with invasive non typhoidal Salmonella from Malawi, Kenya, Mozambique, Uganda and Democratic Republic of Congo, Nigeria and Mali were sequenced and results indicated a new strain of Salmonella, rarely isolated outside Sub-Saharan Africa[17]. Its prevalence and mortality has been...
associated with antibiotic resistance related strains that calls for specific strain identification. It is on this background that this study on characterization and antimicrobial susceptibility patterns of *Salmonella* in Nandi County of Rift Valley was weaved.

**Materials And Methods**

**Study Site**

Sample collection was done in Kapsabet District hospital in Nandi County. Kapsabet District hospital is located in Kapsabet town on latitude 0°13’07”N and longitude 35°08’35”E. Nandi County has a population of 752,965 persons (Kenya National census, 2009) and lies on the western side of the Rift valley. Kapsabet is the main government hospital in Nandi Central of Nandi county as well as a referral hospital for the five administrative divisions i.e: Kapsabet, Kilibwoni, kosirai, kabiyet and kipkaren (Fig.1).

![Fig.1: Map of Nandi District showing location of Kapsabet hospital](Adapted from 2012,Mars Group Kenya).

**Sampling procedure and study population**

Study population included male and female children aged one month and above and adults of 18 years and above who visited Kapsabet District hospital presenting with symptoms of fever >38°C and diarrhea (defined as ≥3 bowel movements in any 24hr period during the preceding 5 days) were enrolled for the study. Hospital laboratory personnel were issued with clean open mouthed disposal containers for collection of stool from any 24hr period during the preceding 5 days. Red colonies with black centers were carefully selected and streaked onto plates of Xylose Lysine Deoxycholate Agar (XLD) (Himedia laboratories Pvt Ltd Mumbai India) selective medium for *Salmonella* then cultured for 18-24 hrs at 37°C. Red colonies with black centers were selected and subjected to biochemical tests. Indole, Methyl Red, Voges Proskauer, Citrate (IMVIC) to identify specific bacteria genus based on their biochemical activities in appropriate culture media. Non lactose fermenting colonies were inoculated on Triple Sugar Iron (TSI) (Himedia laboratories Pvt limited Mumbai, India) in addition to API 20E system (Biomerieux, Marcy L’etoli, France) to confirm identity of *Salmonella*. Sample confirmed to be *Salmonella* were used to inoculate Tryptic Soy Broth (TSB in 15% glycerol), incubated for 18-24 hrs then frozen to -8°C in small eppendorf tubes for genetic analysis.

**Salmonella antimicrobial susceptibility testing**

*Salmonella* isolates were tested using standard Kirby-Bauer disks diffusion method (1996). Using Combi disk 34 (2/4) octodisks (Himedia laboratories Pvt limited Mumbai, India) for susceptibility to the following antimicrobial agents: Ampicillin (Amp) (25mcg), Gentamicin (GEN) (10mcg), Kanamycin (K) (30mcg), Tetracycline (TET) (25mcg), Co-trimoxazole (COT) (25mcg), Streptomycin (ST) (10mcg), Sulfamethoxazole (SX) (200mcg), Chloramphenicol (C) (30mcg) (Himedia Pvt Ltd Mumbai, India).

Fresh *Salmonella* colonies were inoculated in 0.85% NaCl suspension to turbidity equivalent to 0.5 MacFarland standards equivalent to 1.0x10^8 colonies. The culture was swabbed onto a Muller-Hinton agar (Himedia Pvt Ltd Mumbai India). Antibiotic discs were applied after drying the plates for 5min on working bench, the plates were incubated at 37°C for 24hrs. Diameters of zone of inhibition around the disc were measured to the nearest millimeter and isolates classified as sensitive, intermediate and resistant according to guidelines provided by clinical and laboratory standards institute [19].

**Molecular Characterization of Salmonella species**

**DNA extraction**

Pure *Salmonella* isolates obtained from a series of sub cultures in selective medium XLD and stored in Tryptic Soy Broth was allowed to thaw and reconstituted in 200ml of 0.9% NaCl solution.

*Salmonella* colonies were freshly grown in nutrient agar plates, suspended in 150ul of sterile distilled water in a micro centrifuge tube, gently vortexed and boiled for 10min in a water bath at 100°C. Micro tubes were then centrifuged at 1000rpm (Spectrafuge 16M, Labnut international, USA) for 5min at 4°C. Top supernatant were carefully aliquoted by micro pipette and used as a source of DNA template.
Amplification of invA, blaTEM, and sul2 genes was performed in Ready To Go PCR beads(GE Healthcare UK Ltd Chalfont Buckinghamshire UK) in a final volume of 25ul containing 6.25ul of each primer, 4ul of DNA template and 8.5ul of PCR water to make up the volume. Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific, Finland). The cycling conditions were as follows for invA: Denaturation 94°C for 5 min followed by 30 cycles of 94°C for 15 min, annealing at 57°C for 1 min and 72°C for 30 sec final extension 72°C for 7 min. For blatem: Denaturation 95°C for 10 min followed by 30 cycles of 95°C for 30 sec, 55°C for 1 min and 72°C for 1 min, final extension 72°C for 7 min. For sul2: Denaturation 95°C for 8 min followed by 30 cycles of 95°C for 30 sec, 56°C for 1 min and 72°C for 1 min, final extension 72°C for 7 min.

Amplicons were loaded onto casted 1.5% agarose gel alongside 100bp DNA ladder and resolved at a constant voltage of 100V for 35 min prior to UV visualization. PCR amplicons of invA were purified and sequence using the same primers. Sequencing was performed on an ABI 377 sequencer using the Big Dye sequencing kit (Applied Biosystems). Salmonella invA gene consensus sequences obtained were analyzed with the standard nucleotide-nucleotide BLAST(Basic Local Alignment Search Tool) search at NCBI(National Center for Biotechnology Information) obtained from http://www.ncbi.nlm.nih.gov/BLAST. The deduced sequences were matched with known invA Salmonella related gene sequences at NCBI using the BLAST algorithm. All the sequences including those retrieved from the database were then aligned in mega 5.1(http://update.megasoftware.net/download.php) and phylogenetic tree constructed based on the consensus nucleotide sequences with maximum likelihood method in mega 5.1 software.

Results

Out of 200 stool specimen collected from Kapsabet District hospital from patients presenting with diarrhoea during study period, 84% (n=168) yielded ≥ 1 bacterial pathogen as determined by standard microbiological techniques, out of which Shigella spp. 36% (n=61) was the most isolated Enterobacteriaceae. This was followed by 29% (n=48) Salmonella spp, 19% (n=32) E.coli, 10% (n=17%) Citrobacter and 6% (n=10) Proteus.

Antimicrobial susceptibility

100% (n=48) Salmonella strains were resistant to at least one antimicrobial agent. Resistance to ampicillin was 100% followed by sulfamethoxazole 19%. Resistance to tetracycline, streptomycin and chloramphenicol was observed less often (6%). All the isolates were susceptible to, co-trimoxazole, kanamycin and gentamicin. 25% of the isolates exhibited multiple antibiotic resistance.

Figure 3: Antimicrobial susceptibility pattern of Salmonella isolates from Kapsabet hospital

Antimicrobial Resistance genes

PCR results were consistent with antimicrobial susceptibility results (Fig.3). All ampicillin resistant isolates gave positive amplicons for blatem gene. A 643 bp sequence was obtained for all the isolates tested (fig 4). A band of 331bp was observed for sul2 gene in all sulfamethoxazole resistant isolates (fig 5). Nine isolates harboured both blatem and sul2 genes.

Discussion

Kenyan population is most vulnerable to waterborne diseases and death due to unsafe drinking water and consumption of contaminated food. Both human and animal excreta are always suspected sources of Salmonella, many potential routes are used for the transmission of these excreted enteric pathogens [20]. The present study detected moderately high prevalence of Salmonella 29% in Nandi County. Nandi County receives adequate rainfall and hence has robust drainage system comprising of a number of permanent rivers and streams [21]. These resources provide adequate water for domestic,
livelock and industrial use. However, there is limited access to safe and clean sources of water for domestic use.

The results of this study indicated a low antibiotic resistance contrary to previous studies [16] which observed multidrug resistant S. typhimurium infection as well as multiple resistance to commonly available antibiotics, including ampicillin, chloramphenicol, cotrimoxazole and tetracycline. Studies in western Kenya [14], documented all Salmonella isolates to be resistant to all antibiotics tested except ciprofloxin. In addition studies carried out in Asembo, a rural area along Winam Gulf in western Kenya indicated more than half of all the pathogens were not susceptible to empiric therapy [23]. Low antimicrobial resistance in the study area could be attributed to restricted antimicrobial use or low rate of acquisition of resistant genes from resistant serotypes. The results of the study indicated resistance to ampicillin, sulfamethoxazole and tetracycline. Drug resistance in most cases is as a result of a genetic change in the organism caused by either chromosomal mutation or acquisition of a plasmid or transposons [24,25]. The results of this study indicated resistance to ampicillin, sulfamethoxazole and tetracycline. Nandi County is an agricultural area hence ampicillin, tetracycline and sulfamethoxazole could be widely used by farmers for prophylaxis, chemotherapy and agricultural animal growth promotion due to relative low cost and availability. These results are consistent with previous studies [26] which indicated tetracycline was commonly used among poultry farmers and this may contribute to overall resistance in the community, hence there is need to emphasize on prudent use of antibiotics in order to delay emergence and minimize levels of resistance to antibiotics in addition to investigating sources of Salmonella transmission in study area.

Phenotypic testing i.e. minimum inhibition concentration (MIC), Kirby Bauer techniques may not detect ‘silent’ antimicrobial resistance genes that might be expressed in vivo or disseminated to other bacteria [27]. Molecular characterization of antimicrobial resistance genes as well as their location and diversity is important in identifying factors involved in resistance [28].

In the present study, blaTEM gene was amplified in ampicillin resistant isolates, while resistance to sulfamethoxazole was mediated by sul2 gene. Both genes are documented to be associated with class 1 integrons and other mobile genetic elements. blaTEM genes are located on transposon Tn3 [5,29]. The β-lactamases, coded for by the blaTEM genes are the commonest mechanisms by which facultative anaerobic gut flora resist β-lactam antibiotics. The blaTEM genes have a tendency to mutate and secrete enzymes with extended spectrum of activity, this could have accounted for the high resistance to ampicillin in the study population. Sulfonamide resistance in gram negative bacteria arises from acquisition of either of the two genes, sul1 or sul2, encoding forms of dihydropteroate synthase that are not inhibited by the drug [30]. In the present study, resistance to sulfamethoxazole was exclusively mediated by sul2 gene since sul1 gene failed to amplify. sul2 genes are located on small nonconjugative plasmids [31] or on large transmissible multiresistance plasmid. 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. The presence of Salmonellosis in the study area require intersectoral approach to contain the rate of prevalence. There is need for 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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Conflict of interest

No conflict of interest.

Ethical Considerations

The research project was approved by Maseno University ethical committee.

Reference

1. Christine H. Spread of lethal Salmonella strain that kills 1 in 4 linked to HIV. www.medicaldaily.com/articles/12434/20121001
2. Lindsay A. HIV appears responsible for new virulent strains of salmonella www.theatlantic.com/health/archive/2012/10
17. Kingsley R. Whole genome sequencing tracks spread of severe intestinal disease in Sub Saharan Africa. wellcome trust sanger institute, 2012