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Genotyping virulence and resistance profiles in *salmonella* isolated from diarrheic children in Nairobi city, Kenya

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ABSTRACT

Aim: To characterize *salmonella* virulent and antibiotic resistance genes in children with diarrhea in Nairobi city, Kenya.

Background: *Salmonella* species carry virulent genes whose expression correlate with severity of salmonellosis. Effective treatment of salmonellosis by antibiotics is threatened by expression of antibiotic resistant genes.

Methods: In a cross-sectional study, a total of 374 children below five years of age presenting with diarrhea at Mbagathi County Hospital were recruited. Stool microbiology test was used to detect *Salmonella* species. Polymerase chain reaction was employed to detect virulent and antibiotic resistant genes.

Results: *Salmonella* species was isolated in 9 (2.4%) children. A total of 9 (100.0%), 7 (77.8%), 9 (100.0%) and 6 (66.6%) of the isolates harbored *invA*, *Hila*, *sopB*, and *Stn* virulent genes, respectively. None (0.0%) of the isolates was resistant to gentamycin but 7 (77.8%), 7 (77.8%), 9 (100.0%), 8 (88.9%), 7 (77.8%), 6 (66.7%) and 5 (55.6%) of *Salmonella* species were resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin, and tetracycline, respectively. Ampicillin (*citm*), ceftriaxone (*bla CMY*), streptomycin (*aadA1*), gentamycin (*aac(3)-IV*), ciprofloxacin (*qnr*), chloramphenicol (*catA1*), erythromycin (*ereA*), and tetracycline (*tetA*) resistant gene was detected in 6 (85.7%), 6 (85.7%), 9 (100.0%), 8 (100.0%), 6 (85.7%), 6 (100.0%), and 5 (100.0%) of *Salmonella* isolates which were phenotypic resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively.

Conclusion: *Salmonella* species expressing virulent and antibiotic resistant genes is an important cause of gastroenteritis in children in Kenya.

Keywords: Antimicrobial resistance profile, Virulence profile, Salmonella.

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Introduction

Salmonella is one of the most common pathogens of infectious diarrhea. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017 estimated that salmonella species resulted in over 95.1 million cases (95% uncertainty interval [UI] 41.6–184.8), 50 771 deaths (2824–129 736), and 3.10 million DALYs (0.39–7.39) in 2017 (1). Rates of *Salmonella* morbidity and mortality are highest in children under 5 years in particular those living in sub-Saharan Africa (SSA) and South Asia (1). Treatment of salmonellosis is complicated by emergence of virulence genes and antimicrobial resistance which is associated with higher costs, requiring additional investigations and longer hospitalizations (2). Thus, continuous epidemiological surveillance is critical to detect virulent genes and the extent of resistance.

Salmonella can carry different virulent genes, whose expression may determine the course of the infection by favoring the interaction of the microorganism with the host. These numerous virulence genes comprising *invA*, *Hila*, *sopB*, and *Stn* are located in the *Salmonella* chromosomal pathogenicity islands (3). The *invA* gene is involved in invasion of host epithelial cells (4). *SopB* gene has been found to affect cytoskeletal rearrangement forming cage-like structures that surround salmonella containing vacuoles, facilitating bacterial replication and survival during cell membrane fission (5). The *hila* gene

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is an important feature of *salmonella* pathogenicity as it is required for bacterial colonization of extracellular luminal compartment of the host intestine (6). Enterotoxin production is mediated by the *stn*; thus it plays a significant role in causing gastroenteritis by producing enterotoxin (3). As such, virulence genes have different effects on the pathogenicity of *Salmonella*. Most importantly, some studies have revealed virulence genes are related to *Salmonella* serotypes and antibiotic resistance (7–9). However, to my knowledge, no study has reported *Salmonella* virulent genes in Kenya.

Antimicrobial-resistant (AMR) strains of *Salmonella* are a major concern for public health in Kenya. Previous studies analyzing *Salmonella* human isolates have reported higher antibiotic resistance rates of between 70%-80% to amoxiclav, ampicillin, amikacin, ciprofloxacin, ceftriaxone, and ceftazidime (10, 11) and a more recent study has shown reduced resistant rates of between 39%-53%, mediated by antimicrobial resistant genes, to commonly available antimicrobials (12). Interestingly, antimicrobial resistant *Salmonella* isolates harboring antibiotic resistant genes have been detected in animal feeds and agriculture as well a food animals in Kenya (13–15). These create a massive source of antibiotic resistant bacteria or antibiotic resistant genes that can be transmitted to people through both the consumption of contaminated food or derived food products as well as contact with colonized/infected animals or biological substances such as blood, urine, and feces among others (16, 17), underscoring the need for continuous antimicrobial resistance surveillance. Accordingly, this study characterized *salmonella* virulent and antibiotic resistance genes in children with diarrhea in Nairobi city, Kenya.

Methods

Study site and study design

This cross-sectional study was conducted on children with diarrhea under five years of age seeking treatment at Mbagathi County Referral hospital in Nairobi city, Kenya. Detailed description of recruitment, sample, and data collection method is presented elsewhere (18–20).

Identification of *salmonella* species

About 25 g stool sample was dissolved in about 200 mL of sterilized buffered peptone water (BPW), and incubated at 37°C for 16-20 hours. About 10 mL from the incubated BPW culture was selectively enriched into the 100 mL sterilized Selenite Cystine Broth and incubated again at 37°C for 24-48 hours. Following incubation, 1 loop full inoculum from the selective enrichment culture was streaked onto the pre-incubated Bismuth Sulfiite Agar (BSA) and Xylose Lysine Deoxycholate (XLD) agar plate. Black colonies on Bismuth Sulfiite Agar (BSA) and red to pink (since the background is red) with black center on Xylose Lysine Deoxycholate (XLD) agar were identified as *Salmonella* species. Presumptive *Salmonella* colonies were purified and maintained on Tryptic Soy Agar (TSA) slant for biochemical, virulotyping, and drug sensitivity tests. Biochemical tests using Gram's stain, nutrient broth, lysine iron agar, Methyl Red (MR) and Voges-Proskauer (VP) broth, Simmons citrate agar, Kligler iron agar (KIA), Sulfide-Indole-Motility (SIM), Christensen urea agar, and Motility Indole Ornithine Medium (MIO) were performed to confirm *Salmonella* species. Further confirmation of biochemical reactive cultures was done by agglutination test with *Salmonella* polyvalent (O) somatic antisera.

Salmonella virulotyping

DNA was extracted from 24-hour old colonies using the QIAamp DNA Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instruction. All *Salmonella* isolates were screened for the presence of *invA*, *hilA*, *sopB*, and *Stn* virulent genes using primer pairs presented in Table 1. Optimized multiplex PCR reaction was performed in a volume of 25 µL containing 2 µL of DNA template, 5 µL of

Table 1. Virulotyping primers

Primer sequences	Virulent gene	Species	Fragment size
F: ACCACGCTCTTTCGTCTGG R: GAACTGACTACGTAGACGCTC	*invA,*	*Salmonella*	942bp
F: TGTTTCCGGGCTTGTGCT R: CAGGGCATTTGCTGATTCTTCC	*hilA,*	*Salmonella*	854bp
F: -AGCATCTCTAAACGCTACTG R: GCTTCTATCACTCAGCTTCA	*sopB,*	*Salmonella*	470bp
F: ATTGAGCGCTTTAATCTCCT R: GCTGTTGAATCTGTACCTGA	*Stn*	*Salmonella*	543bp

5 × PCR buffer, 2.5 µL of 25 mM MgCl2, 0.5 µL of 10 mM deoxynucleotide triphosphate (dNTP), 0.5 µL of 1.2 µM primer mix, and 14.2 µL of deionized water and 0.3 µL (1.5 U) Taq DNA polymerase. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were electrophoresed in a 2% agarose gel and visualized under ultraviolet light.

Antimicrobial resistance phenotyping

Antibiotic susceptibility was performed using Kirby-Bauer disk diffusion method on Mueller Hinton agar by incubation at 37°C for 18 hours (Humphries et al., 2018). Antibiotic discs of ampicillin (10µg), ceftriaxone (30µg), streptomycin (10µg), gentamycin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), and tetracycline (30 µg) were used. Broth turbidity was made to match 0.5 McFarland standards. According to the size of the zone of inhibition, the organisms were classified as sensitive, intermediately sensitive, or resistant to each antibiotic based on Clinical Laboratory Standard Institute interpretation guideline (21). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeroginosa* ATCC 27853 were used as quality control strains.

Genotyping antimicrobial resistance

The isolates were grouped on the basis of resistance phenotype and determined for the presence of corresponding antibiotic resistance genes. The presence of resistance genes to ampicillin: *citm*, ceftriaxone: *bla CMY*, streptomycin: *aadA1*, gentamycin: *aac(3)-IV*, ciprofloxacin: *qnrA1*, chloramphenicol: *catA1*, erythromycin: *ere(A)* and tetracycline: *tet(A)* was detected by single PCR using primers reported elsewhere (20). All reactions were prepared using 2 µl template DNA, 12.5 µl 2X PCR master mix (0.04 U/µl Taq DNA polymerase, PCR buffer, 3 mM MgCl2, 0.4 mM of each dNTP), and 0.4 µM of each primer in a volume of 25 µl. Amplification reactions were carried out as follows: Five min at 95°C, 35 cycles each consisting of 1 min at 94°C, 30 s at ~55°C and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. Amplified samples were analyzed via electrophoresis in 2% agarose gel and stained by ethidium bromide.

Results

Salmonella virulent genes are reported in Table 2. The stool samples collected from 9 (2.4%) out of 374 children were positive for *Salmonella* species in the present study. Virulotyping of *Salmonella* species revealed that 9 (100.0%), 7 (77.8%), 9 (100.0%), and 6 (66.6%) of the isolates harbored *invA*, *Hila*, *sopB*, and *Stn* virulent genes, respectively.

Data are presented as number and proportions (%) of enteric bacteria and virulent genotypes.

Phenotypic and genotypic antimicrobial susceptibility patterns

Phenotypic and genotypic antimicrobial susceptibility patterns are outlined in Table 3. Although none of the

Table 2. Frequency of virulence genes of Salmonella species

Isolate	Phenotype number n (%)	Virulent genotype	Genotype number n (%)
Salmonella species	9 (2.4)	*invA*	9 (100.0)
		Hila	7 (77.8)
		sopB	9 (100.0)
		Stn	6 (66.6)

Data are presented as number and proportions (%) of enteric bacteria and virulent genotypes.

Table 3. Phenotypic and genotypic antimicrobial susceptibility patterns of isolates

Antibiotic	Phenotypic resistance			Genotypic resistance	
	Sensitive	Intermediate	Resistant	Genotype	Number (%)
Ampicillin	0 (0.0)	2 (22.2)	7 (77.8)	*Citm*	6 (85.7)
Ceftriaxone	2 (22.2)	0 (0.0)	7 (77.8)	*bla CMY*	6 (85.7)
Streptomycin	0 (0.0)	0 (0.0)	9 (100.0)	*aadA1*	9 (100.0)
Gentamycin	9 (100.0)	0 (0.0)	0 (0.0)	*aac(3)-IV*	-
Ciprofloxacin	0 (0.0)	1 (11.1)	8 (88.9)	*Qnr*	8 (100.0)
Chloramphenicol	2 (22.2)	0 (0.0)	7 (77.8)	*catA1*	6 (85.7)
Erythromycin	1 (11.1)	2 (22.2)	6 (66.7)	*ere(A)*	6 (100.0)
Tetracycline	3 (33.3)	1 (11.1)	5 (55.6)	*tet(A)*	5 (100.0)

Salmonella isolates were resistant to gentamycin, 7 (77.8%), 7 (77.8%), 9 (100.0%), 8 (88.9%), 7 (77.8%), 6 (66.7%), and 5 (55.6%) of *Salmonella* species were resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin, and tetracycline, respectively. Additionally, ampicillin (*citm*), ceftriaxone (*bla CMY*), streptomycin (*aadA1*), gentamycin (*aac(3)-IV*), ciprofloxacin (*qnr*), chloramphenicol (*catA1*), erythromycin (*ereA*), and tetracycline (*tetA*) resistant gene were detected in 6 (85.7%), 6 (85.7%), 9 (100.0%), 8 (100.0%), 6 (85.7%), 6 (100.0%), and 5 (100.0%) of *Salmonella* isolates which were phenotypic resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin, and tetracycline, respectively.

Discussion

The prevalence of *Salmonella* among children under five years old seeking healthcare for diarrhea at the facility in this study was low, indicating that *Salmonella* is not a common cause of diarrhea across this population. Viral and other bacterial gastroenteritis have been documented as the leading cause of diarrhea in the same study area (19, 22). Lower rates of *Salmonella* have been reported in diarrheic children younger than five years seeking healthcare in Turkana County, Kenya (11), Tanzania (23), Nigeria (24), West-central Ethiopia (25), and those dwelling in a Mkuru slum, an urban informal settlement, in Nairobi city, Kenya (26).

However, the findings of this study disagree with previous studies in Murangá county, Kenya (27) and South Eastern Ethiopia (28) reporting nearly five and three times, respectively, higher *Salmonella* infection rates in diarrheic children younger than five years with diarrhea seeking healthcare service. These geographic and regional variations in infection rates may reflect direct association of *Salmonella* with social economic status, hygiene practices, environmental, behavioral and biological drivers (29). For instance, previous studies have demonstrated that rainfall and temperature have the potential to act as environmental drivers while seasonal variation in host susceptibility to infections act as biological drivers of *Salmonella* transmission (30, 31). Therefore, the geographic and regional variation in prevalence of *salmonella* could be employed in diarrhea management to ensure optimal usage of limited resources in preventing diarrhea transmission thus reducing indiscriminate use of antibiotics.

This is the first study exploring *Salmonella* virulent genes in clinical isolates in Kenya. *InvA* gene, responsible for invasion of epithelial cells, is highly conserved and is used as a genetic target in molecular identification of *Salmonella* at the genus level (32). This is an explanation that is consistent with the observation of the present and previous studies detecting *InvA* gene in all *salmonella* human isolates (3, 33, 34). Similar to *invA* gene, all isolates expressed *sopB* gene which was in agreement with a previous study that detected *sopB* in all human *salmonella* isolates (35). The *stn* gene which is a virulence factor having enterotoxicity activity causing diarrhea was isolated in some strains, a finding that is similar with previous studies isolating *Salmonella* from humans (3, 36). However, Hila was detected in some isolates which is inconsistent with a previous study that detected Hila in all human *salmonella* isolates (37). Variations in detecting virulent genes may be attributed to the fact that different plasmid incompatibility types carry distinct pathogenicity genes. For example, molecular analysis of *pSLT* plasmid carrying *Salmonella* plasmid virulent (*spv*) genes *spvA*, *spvB*, and *spvC* in 72 *Salmonella* Typhimurium revealed that only 4 isolates tested positive for all (*spvA*, *spvB*, and *spvC*) the *spv* genes (38). Thus, *salmonella* strains with plasmid borne virulence characters are causing gastroenteritis and systemic infection in Kenyan children.

The present study found higher susceptibility to gentamycin agreeing with previous studies involving patient isolates in Nairobi (39), while disagreeing with a study in Murang'a county, Kenya, which reported higher gentamycin resistant rates of *salmonella* isolated from stool samples of children younger than five years (27). Interestingly, it should be noted that high resistant rates to gentamycin has also been reported in *salmonella* isolated from food samples in Embu county, Kenya (40), highlighting substantial risk of flow of gentamycin resistant genes to humans. Sadly, high resistance rates towards ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin, and tetracycline antibiotics were observed in the present study. This observation is partly in line with a study in Murang'a county, Kenya, which reported high resistance to ampicillin, ceftriaxone, ciprofloxacin, and chloramphenicol with concomitant lower resistance to streptomycin, erythromycin, and tetracycline among diarrheic children in Murangá county, Kenya (27).

However, the findings are inconsistent with a study in Murang'a county which reported higher resistant rates of *Salmonella* isolates to gentamycin (27). The differences in resistance rates may be linked to antimicrobial prescription practices at the healthcare settings as well as animal husbandry which show regional variation but drive resistance (42, 43). Therefore, resistant rates may be due to widespread dependency on antibiotics, where complex interactions between human health, animal husbandry and veterinary medicine have contributed to the propagation and spread of resistant organisms.

The detection rate of antibiotic resistant genes was basically consistent with resistant phenotypes. The trend in our study is on the higher end of the spectrum with 85.7% of the resistant phenotypes harboring ampicillin, ceftriaxone, and chloramphenicol resistant genes. This suggests that there may be other antibiotic resistant genes as well as intrinsic factors driving resistance. This observation is consistent with a study in Nigeria (44) and Pakistan (45) detecting higher rates of *catA1*, *Qnr*, and *tet* genes in phenotypic resistant isolates. In the present study *aadA1*, *Qnr*, *ere(A),* and *tet(A)* were detected in all the streptomycin, ciprofloxacin, erythromycin, and tetracycline resistant phenotypes, which is in agreement with previous studies in Iran (46, 47) and Denmark (48), detecting *aadA*, *qnr*, and *tetA* in all phenotypic resistant *Salmonella* isolated from patients. However, the findings of this study are inconsistent with a study in China (49) which did not detect *qnr* and another study in Central African Republic (50) detecting *tetA* and aadA1 in one and catA1 in all *Salmonella* isolates. Plasmid incompatibility and antimicrobial resistant gene accessory content as well as geographic distribution may explain the variations in detection rates of antibiotic resistant genes observed in this and previous studies.

Plasmid typing revealed that plasmid types vary in their antibiotic resistant gene content and may show compatibility or incompatibility during horizontal gene transfer between bacteria species (51). For example, the *incF* plasmid encodes for Extended-Spectrum β-Lactamase (ESBL) Genes, carbapenemases genes, aminoglycoside-modifying enzymes, and plasmid-mediated quinolone resistance (PMQR) genes while *IncI* plasmid, which is compatible with *incF* plasmid, encodes for ESBL and plasmid-mediated *AmpC* resistant genes (51). Resistant gene content of plasmids varies geographically with studies reporting that *blaCTX-M* that encodes class A extended-spectrum β-lactamases is associated with IncF plasmids in Korea and France, while in Spain this gene is mainly located on *IncK* plasmids (51). Furthermore, whole-genome sequencing of drug-resistant human *Salmonella* enterica serovar Dublin isolates in Washington State and New York State demonstrated that *strA*, *aadB*, and *cmlA* antibiotic resistant genes were strongly associated with Washington State (52). The study further demonstrated that IncI1 plasmid, which is associated with extended-spectrum cephalosporin resistance in S. typhimurium (51), was associated with isolates from New York State (52). Thus, the proportion and quantity of antibiotic resistant genes in *salmonella* isolates is high and it may be driving antibiotic resistance issues in *salmonella* gastroenteritis in children.

There have been a few limitations of this study. *Salmonella* isolates were not serotyped. Additional limitations in this study included few numbers of *Salmonella* isolates, few classes of virulent genes genotype, and being conducted at a single health facility.

Conclusion

In conclusion, *Salmonella* species causing gastroenteritis in children in Kenya express virulent genes. In addition, majority of phenotypic resistant isolates harbor antibiotic resistant genes. This study can inform the antimicrobial policy for tertiary care centers including preparing the management of hospital infections, treatment protocol, and diagnostic procedure.

Conflict of interests

The authors declare that they have no competing interests.

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