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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 bla_{TEM} 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.

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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

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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 0^0 13' 07"N and longitude 35^0 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 $>38^0$C and diarrhea (defined as $\geq$ 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 randomly selected in and out patients.

Study participants inclusion criteria

This study did not seek consent from patients at the health faculties. Rather, all stool samples received at Kapsabet District laboratory hospitals for routine daily diagnosis process were used for analysis. Laboratory personnel were provided with data collection form to fill in secondary data (record) on anthropometric indices like age, sex, health status of patients, hence consent from hospital administration was sort.

Assumption

For the purpose of this study, it was assumed that most patients visiting Kapsabet District hospital were residents of Nandi county or would have stayed in the respective area for a period not less than three weeks. . It was also assumed that all the clinically asymptomatic diarrhoea patients of any cause were directed to the laboratory for stool collection and ascertaining the cause of diarrhoea.

Sample collection

All fresh stool samples received at Kapsabet hospital laboratory during the study period (February to November 2011) regardless of patients history of Salmonellosis, were collected for analysis. Sterilized swabs were used to collect stool samples from hospital laboratory specimen into tubes of Cary Blaire transport media (Himedia laboratories Pvt. Limited Mumbai, India). Stool samples were transported to University of Eastern Africa-Baraton, microbiology laboratory for culture and isolation of *Salmonella*.

Culture and Isolation of *Salmonella*

Stool samples were cultured at 37^0C in Selenite F broth (Himedia laboratories Pvt Ltd Mumbai India) for 18 - 24hrs for enrichment after which the cultures were streaked onto plates of *Salmonella Shigella* agar (Himedia laboratories Pvt Mumbai India) selective medium for *shigella* and *Salmonella* and cultured for 18-24hrs at 37^0C. Red colonies with black centers were carefully selected and streaked onto plates of Xylose lysine DeoxycholateAgar (XLD) (Himedia laboratories Pvt Mumbai) selective medium for *Salmonella* then cultured for 18-24 hrs at 37^0C. Red colonies with black centers were selected and subjected to biochemical tests. Indole,Methyl Red,Voges Proskauers,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 -8^0C 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 gar plates, suspended in 150ul of sterile distilled water in a micro centrifuge tube, gently vortexed and boiled for 10min in a water bath at 100^0C. Micro tubes were then centrifuged at 10000rpm (Spectrafuge 16M, Labnut international, USA) for 5min at 4^0C.Top supernatant were carefully aliquoted by micro pipette and used as a source of DNA template.

Amplification of *invA* ,bla$_{TEM}$, 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^{0}C for 5 min followed by 30 cycles of 94^{0}C for 15 min , annealing at 57^{0}C for 1min and 72 ^{0}C for 30 sec final extension 72^{0}C for 7 min. For bla$_{TEM}$: Denaturation 95^0 C for 10 min followed by 30 cycles of 95^{0}C for 30 sec , 55^{0}Cfor 1 min and 72^{O}Cfor 1 min, final extension 72 ^{0}C for 7 min. For *sul2:* Denaturation 95^0 C for 8 min followed by 30 cycles of 95^{0}C for 30 secs , 56^0 C for 1 min and 72^{0}C for 1 min, final extension 72 ^{0}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 *inv*A 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://w.w.w.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 *Enterobacteriacea*, This was followed by 29% (n=48) *Salmonella spp*, 19% (n=32) *E.coli*, 10% (n=17%) *Citrobacter* and 6% (n=10) *Proteus.*

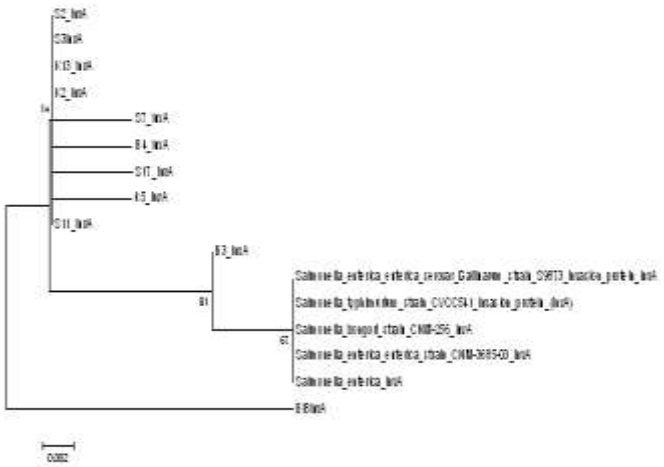

Figure.2 : Phylogenetic tree based on *invA* gene consensus nucleotide sequences.The tree was constructed in mega 5.1 software using maximum likelihood method. Numbers at the nodes indicates percentage of occurrence in 1000 bootstrapped trees

Antimicrobial susceptibility

100% (n=48) *Salmonella* strains were resistant to at least one antimicrobial agent. Resistance to amphicillin 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.

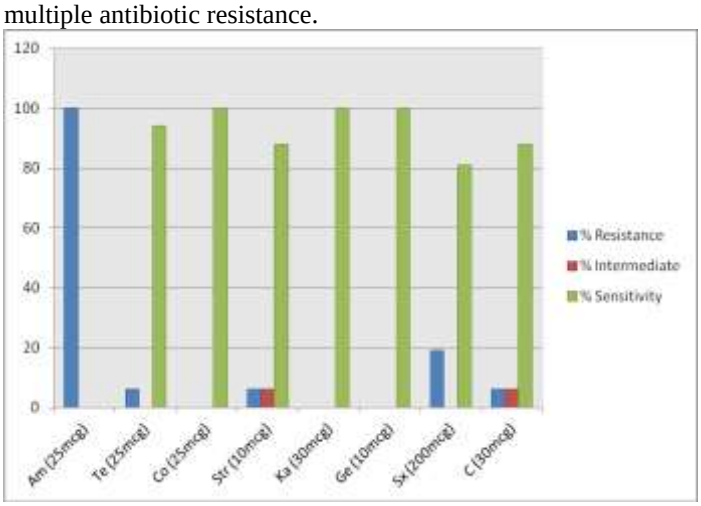

Figure 3: Antimicrobial susceptibility pattern of *Salmonella* isolates from Kapsabet hospital

Antimicrobial Resistance genes

PCR results were consistent with antimicrobial susceptibility results (Fig.3). All ampicillin resistant isolates gave positive amplicons for bla$_{TEM}$ 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 bla$_{TEM}$ and *sul2* genes.

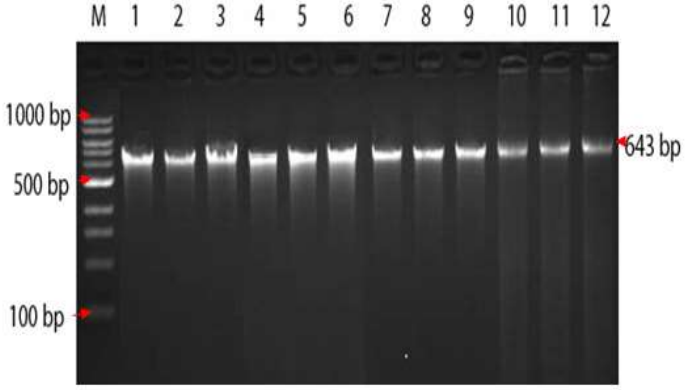

Figure 4: PCR gel showing bla$_{TEM}$ gene products for *Salmonella* isolates.M:100bp DNA ladder, lanes 1-12 positive isolates

Primer	Oligonucleotide sequence	(Inqaba biotechnology)
bla$_{TEM}$ f	ATGAGTATTCAACATTTCCG	
bla$_{TEM}$ r	ACCAATGCTTAATCAGTGAG	

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,

livestock and industrial use. However, there is limited access to safe and clean sources of water for domestic use.

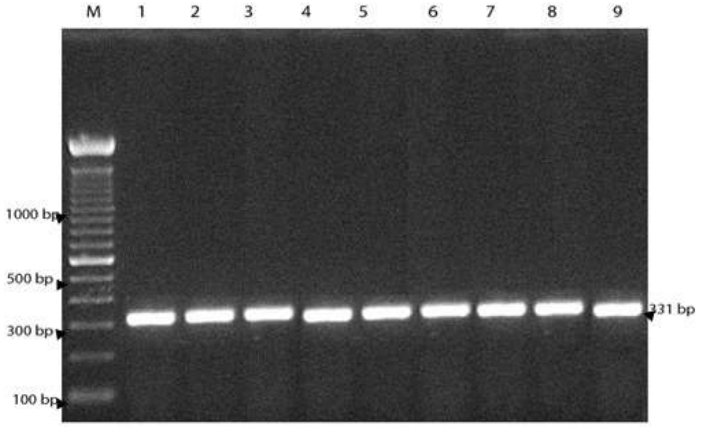

Figure 5: PCR gel showing *sul2* gene products for *Salmonella* isolates.M:100bp DNA ladder, lanes 1-9 positive isolates

Primer	Oligonucleotide sequence	(Inqaba biotechnology)
*s*ul 2 f	CACTGCCACAAGCCGTAA	
sul 2 r	GTCCGCCTCAGCAATATC	

Over 75% of households have restricted access to piped sources of water. The rest of the population utilizes untreated water from streams found all over the District [21]. Kapsabet division (District headquarter) is the densiest division in Nandi Central with 275 persons/km^2 [18].The rapidly expanding urban centres are in need of sanitation facilities to reduce pollution of water sources .81% of urban and 92% of rural house holds use pit latrines as main mode of human waste disposal [18]. Pit latrines are likely to over flow during rainy season forming part of the surface run off that ends into open water surface hence contributing to microbial load. Underground seepage from pit latrines is suspected to be another source of pollution. This could have accounted for the prevalence of Salmonellosis in study area. The ability of *Salmonella* to be transmitted by any of these routes depends on its resistance to environmental factors, which controls its survival and its capacity to be carried away by water, this survival capacity depends on species and pollution sources [20].

All *Salmonella* isolates in the present study expressed *invA* gene. Amplification of *invA* an international standard procedure for detection of *Salmonella* [22] confirmed the isolates are salmonellae. *invA* gene is located on *Salmonella* pathogenicity island 1(SPI-1) and is essential for full virulence in *Salmonella*. It is thought to trigger the internalization required for invasion of deeper tissues as documented by other authors [22]. Sequence analysis of *invA* gene of the isolated strains revealed 98% maximum identity upon blast in mega 5.1 with *Salmonella* strains from genbank Accession numbers; CP003386.1, CP002614.1,AP011957.1(http://w.w.w.ncbi.nlm.nih.gov/BLAST). Based on BLAST search ,the distribution of *Salmonella* isolates was as follows; *Salmonella* serovar *typhimurium* 56% (n=27), *Enteritidis* 29%(n=14),*Paratyphi C* 6%(n=3),*Paratyphi A* 4%(n=2),*Gallinarum* 2%(n=1),*S. Dublin* 2%(n=1). The most prevalent serotype was *Salmonella typhimurium* which attests to its capacity of adaptation and survival.Phylogenetic tree based on the blast search was constructed and the topological robustness of the trees was evaluated by a bootstrap analysis involving 1000 replications (fig 2).

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 amphicillin, chloramphenicol, cotrimoxazole and tetracycline. Studies in western Kenya [14], documented all *Salmonella* isolates to be resistant to all antibiotics tested except ciproflaxin. 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 pool 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, bla_{TEM} gene was amplified in ampicillin resistant isolates, while resistance to sulfamethoxazole was mediated by *sul2* gene. Both genes are documented to be associated with class1 integrons and other mobile genetic elements. bla_{TEM} genes are located on transposon *Tn3* [5,29]. The β -lactamases, coded for by the bla_{TEM} gene are the commonest mechanisms by which facultative anaerobic gut flora resist β-lactam antibiotics. The bla_{TEM} 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.

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