WELCOME TO
Research Proposal for Ph.D in Cell and Molecular Biology

Submitted to: Institute of Research and Postgraduate studies, Maseno University, Kenya.

TITLE: MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT HUMAN Salmonellae typhimurium STRAIN IN WESTERN KENYA.

SUBMITTED BY: David Onyango, B.Sc Hons (Moi), M.Sc (Maseno).
Department of Zoology, Maseno University, Kenya.

SUPERVISORS: Prof. Dr. Eliud N. Waindi PhD.
Department of Zoology
Maseno University.

Dr. Rose Kikai PhD.
ESPUDEC Maseno University.
Introduction

- Resistance to antibiotics is a problem that confronts:-
  - 1. Human medicine
  - 2. Veterinary medicine
  - 3. Agriculture.

- Antibiotic resistance: a) Thwarts treatment of inpatients and outpatients
  b) Compromises therapy for animals, fish, and agricultural crops.

- The frequency of resistance in bacteria and the numbers of antibiotic drugs to which they are resistance are increasing.

- Factors contributing to antibiotic resistance are:-
  1. The chemical structure of the antibiotic itself
  2. The type of resistance mechanisms being selected for:-
  - Change of membrane permeability and development of ultrastural target to the dug
  - Alter metabolic pathways leading to bypass to reaction inhibited by these drugs
  - Efflux of drugs out of the bacteria
  - Involvement of enzymatic reactions that cleave the drugs e.g β- lactamases, acetyltransferases
  - Genetic changes and subsequent selection processes.

- The resistance displayed by Salmonella reflects the environment in which the organism thrives; which can be illustrated by both sub-therapeutic and therapeutic uses that can increase the likelihood of multidrug-resistance.
• The global increase in resistance to antimicrobial drugs, including the emergence of bacteria strains that are resistant to all available antibacterial agents, has created a public health problem of potentially crisis proportions.

• One of the currently experienced drug resistance microbe is *Salmonella typhimurium*.

• *Salmonella* causes tens of millions of cases of food poisoning or typhoid each year resulting in hundreds of thousands of deaths despite the prophylactic measures being taken.

• *Salmonella typhimurium* is now resistant to 5 types of antibiotics, according to experts from CDC and prevention

• *Salmonella* is often resistant to Ampicillin, Chloramphenicol, Streptomycin, Sulfonamides and Tetracycline; Quinolones.

• Among the drug resistance mechanisms displayed by *Salmonella* is the genetic changes that may be transferred horizontally by spontaneous mutation in a locus that controls susceptibility to a given antimicrobial drugs.
Justification of the study

• Studies of microbial physiology help scientists understand the biological processes that pathogens use to resist drug treatment. This knowledge can lead to the development of novel strategies to overcome or reverse these processes.

• In this environment of multiple antimicrobial agents and diverse antimicrobial resistance mechanisms, selection of the appropriate antimicrobial agent for therapy of infection is challenging, both for empiric therapy or for infection with a defined pathogen.

• In addition to its adverse effect on public health, antimicrobial resistance contributes to higher health care costs. Treating resistant infections often requires the use of more expensive or more toxic drugs and can result in longer hospital stay for infected patient.

• Therefore, routine antimicrobial susceptibility testing must increasingly serve a dual purpose, to guide choice of antimicrobial agents for therapy in the individual patient and to form national policies in relation to control of antimicrobial resistance.
• However the lack of high quality, comparable data from different regions of the country is a significant impediment of forming a clear picture of the scale of the problem.

• Various antibiotics have been in the market purported to treat microbial infections (Malonza, 1997). However due to ease access to the antibiotics in the Kenya market, individuals have responded negatively to these antibiotics when infections set in; especially in Acquired immunodeficiency Syndrome patients (AIDS) (Hoeprich, 1983).

• In a cross-sectional study done prospectively for a four months period on “estimation of the prevalence of typhoid as a problem of public health in Kenya” Gakunyi et al., (2003), found that the overall prevalence of typhoid was 5.2%.

• The prevalence range in different province was between 1.25% and 9%, while the range in different districts was between 0.5% and 18.4%. Prevalence rate did not differ significantly with sex (p>0.05) but the prevalence rates had significant differences with age (p< 0.001).
• Conclusion was therefore made that the national prevalence rate of typhoid fever in Kenya was very low. However the prevalence rates were high and significant public health concern in specific regions of Rift Valley, Eastern and Nyanza Province (Gakunyi, 2003).

• Going by the figures obtained by Ng’wena et al., (2004)– mortality rates of the six leading diseases in four health centers Homa-Bay (Nyanza), Kericho, Koibatek, West-Pokot (Kapenguria) (Rift valley) was typhoid fever; the prevalence following rainfall patterns between 1999-2002 was 2.7%, 3.0%; 4.4%, 0.9%, 1.7%; 3.3%, 5.2%; respectively (Ng’wena, 2004).

• Study by Onyango (2003) in Western Kenya, the prevalence of S. typhimurium was found to be 23.3% and 60% among immunocompromised and immunocompetent children in Western Kenya. The aforementioned generated data indicates that typhoid is prevalent within the study areas.
• In a recent antimicrobial drug survey in Mukumu Mission Hospital (Kakamega) by Onyango August 2004, it was found that 42% of the patients suffering from *Salmonella typhimurium* were resistant to Ciprofloxacin, 87% to Chloramphenical, and 67% to Gentamycin.

• The cause of this resistance is not known by the health practitioners and this is posing a public health problem/concern.

• If molecular epidemiology is to aid in the understanding and control of Salmonella acquisition and spread of antibiotics resistance, then it could facilitate development of effective prevention and control strategies; the purpose of this study is therefore to determine “Molecular characterization of antibiotic resistant human *Salmonella typhimurium* strain in Western Kenya” using molecular techniques. If this is done it will help in intervention measures.
Main Objective
To determine molecular characterization of human *Salmonellae typhimurium* resistance to antibiotics in Western Kenya.

- **Specific Objectives**
- **1.** To isolate *Salmonellae species* in stool specimen collected from in and out patients departments in the selected health centers in Western Kenya.
- **2.** To determine drug resistance in the isolated *Salmonellae species* colonies in specimen collected from in and out patients in the sited health centers in Western Kenya using standard MIC, Disk Approximate or Double Disk, Disk Diffusion methods.
- **3.** To isolate pure *Salmonella typhimurium*, amplify and sequence the obtained pure *Salmonella typhimurium* DNA fragments using automated sequencer and then compare the obtained sequence with the standard *S. typhimurium* LT2 sequence (see standard reference for LT2 sequence).
• MATERIALS AND METHOD

• Study Site
• **Nyanza Province.**
  • New Nyanza Provincial General Hospital – Kisumu District
  • Kisumu District Hospital – Kisumu District
  • Sindo Health Center - Suba District
  • Homa-bay District Hospital* – Homa-bay District

• **Western Province**
  • Mukumu Mission Hospital – Kakamega District
  • Busia District Hospital- Busia District.

• **Rift Valley Province***
  • Koibatek Health Center*. 

**Study populations**

This study will involve male and female children aged one month and above and adults of age 18 years and above. The sample population will be 34 patients infected by *S. typhimurium* as the sample size from each health center.

Formulae for sample size determination:

\[ N = \frac{(P_1 q_1 + P_2 q_2) \cdot K}{(P_1 - P_2)^2} \]

Where \( N \) = Number of subjects to be used for study in each group.

\( P_1 \) = Frequency/ probability of outcome in group 1 \( q_1 = 1 - p_1 \)

\( P_2 \) = Frequency / probability of outcome in group 2 \( q_2 = 1 - p_2 \)

Level of significance \( P = 0.05 \)

\( K \) = Constant dependent on significance level and power = \( (Z_\alpha + Z_\beta)^2 \)

\( K = (Z_\alpha + Z_\beta) \) where \( Z_\alpha \) and \( Z_\beta \) are normal derivatives corresponding to significance level \( \alpha \) and power \( (1 - \beta) \).
**Collection, preservation and use of stool specimen**

- Issuing Health centers laboratory personnel with stool containers for collection of diarrhea from randomly selected patients.

- Recording of anthropometrics measurements namely, age, sex, weight before collecting the sample.

- Preservation of the stool samples in a cool box having ice packs at 8°C and then transported to Maseno University department of Zoology laboratory work area within 18 hour from collection time.

- In the laboratory, approximately $1 \times 10^{-6}$ grams of stool (standard inoculation wire loop volume) will be inoculated on bacterial culture media and incubated at 37 °C for 18-24 hours.

- Cleaning of working surface using 10% Jik solution and alcohol before and after inoculation work for safety of the laboratory users. Also, prohibition to the working area will be ensured for any unauthorized persons.
**Procedure for determination of Salmonella species**

**Inoculation:** While in the field, the collected diarrhoeal stool specimen will be inoculated into Selenite – F broth and incubated at 37ºC overnight for maximum recovery of organisms.

Using a inoculation wire loop, a 50μl (1 x 10⁻⁶ gram a standard inoculation wire loop volume) drop from Selenite – F and the remaining diarrhoeal stool will be inoculated on the culture media MacConkey and Desoxycholate Citrate Agar (DCA) by the streaking technique and incubated for 18 hours at 37 ºC for determination of the enteropathogens.

Colonies from MacConkey will then be subcultured in Nutrient broth (and preserved at 4°C in the refrigerator for future use), while a portion will be inoculated on Kliger Iron Agar (KIA), and SIM. Indole, Methyl red, Voges Proskauers, Citrate (IMViC) test will be done to confirm and identify the Enterobactericeae (standard procedure techniques).

**BACTERIAL SUSCEPTIBILITY TESTING**

**Methods for drug testing**

**Disk Diffusion Testing (ADD)**

In this study, a sensitivity disk will be impregnated using test drug of choice at various concentrations and then placed on the culture plate with pure bacteria isolates to determine minimum inhibition zones and zone diameter.
• Maximum Inhibition Concentration zone (MIC) method.
  The MICs of antimicrobial agents will be determined by E-test and broth microdilution technique where two folds serial dilutions of antimicrobial agents in 100μl of antibiotic medium 3 with an inoculation of $10^3$ to $10^4$ CFU of logarithmically grown cells will be prepared based on McFarland turbidity technique.

• The MIC will be determined as the lowest concentration of the antimicrobial agents that inhibits visible growth after 18 to 24 h of incubation at 37°C. The MIC report will be the means for quadruplicate experiments.

• Isolation, amplification and sequencing of resistant DNA fragment
  • DNA Isolation
    • 1.5ml of the pure isolates will be obtained and then spined for 2 min in micro-centrifuge. This will then be re-suspend in 56 μl TE buffer, 30 μl of 20 mg/μl protein K. Vortexed and incubate for 1 hour at 37°C. 100μl of 5M NaCl will then be added to the mixer and vortexed thoroughly.
To the mixer 80μl of Cetyltrimethylammonium bromide (CTAB)/NaCl solution will be added and vortexed then incubated at 65°C for 10 min. The DNA will be extracted with an equal volume of Chloroform / Isoamyl alcohol and spinning for 5 min in micro-centrifuge. The aqueous phase will then be transferred to a fresh tube and DNA extracted by adding phenol/chloroform / Isoamyl alcohol and spinning for 5 min. DNA will be precipitated with 0.6 vol. Isopropanal in a fresh tube. The precipitate will then be washed with 70% ethanol and the supernatant removed. The pellet will then be dried briefly in lyophilizer and then re-suspend in 100μl in TE buffer ready for Polymerase Chain Reaction.

**Amplification of DNA fragment using Restriction Fragment Length Polymorphism (RFLP).**

Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) will be used in multiplex PCR reaction to amplify DNA fragments obtained from resistant *Salmonellae* species (see appendix for protocol). The isolated multi drug resistant (MDR) Salmonella DNA will be mixed with Restriction fragment lengths genes predicted for various resistance drugs of study in a multiplex reaction independently.
The resultant mixer will then be electrophoresized to obtain RFLP electrophoretic patterns of controls strains and clinical isolates of *Salmonellae typhimurium*. This will then be compared to the stand gene sequence of *Salmonella typhimurium*

**Sequencing of the obtained *Salmonella typhimurium* DNA fragment.**

The obtained amplicon from PCR-RFLP above will then be subjected to Sangers enzymatic method of DNA sequencing according to protocol of Vistra DNA Labstation kits that use fluorescent dye (Amersham, 1996). The fluorescent dye will dye the different dNTPs and this will be visualized on a screen and the sequence noted and printed