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RESEARCH ARTICLE

ANTIBIOTIC SENSITIVITY PROFILE OF ENTERIC BACTERIA ISOLATED FROM SOIL SAMPLES IN NAIROBI KENYA

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ABSTRACT

Objective: Soil is a significant reservoir for enteric bacteria and other pathogenic bacteria, as it is a frequent recipient of waste materials. The study was aimed at determining the prevalence of enteric bacteria from various soil samples collected in Nairobi and environs and to compare their drug susceptibility profile with those from clinical samples.

Methods: Soil samples were collected from randomly selected GPS coordinates in Nairobi within a radius of 30km from Nairobi city center. Ten grams of each of the soil samples were serially diluted then plated on Mueller-Hinton agar and incubated at 30°Centigrade overnight, the colonies were Gram stained and the Gram-negative colonies inoculated on Analytic Profile Index kit for further identification. Antibiotic sensitivity testing was done and the results compared with that of clinical isolates.

Results: Out of the soil samples (n=236) inoculated onto Mueller-Hinton agar, 17 (7.2% prevalence) were positive for *Proteus salmonicida*. Other isolated Gram negative bacteria were *Myroides spp, Pseudomonas putida, Pseudomonas aeruginosa, Stenotrophomonas maltophila* and *Alcaligenes spp. Proteus salmonicida* showed a higher sensitivity to the antibiotics compared to the clinical *Proteus* except for Cefotaxime antibiotic which was resistant to it.

Conclusion: Soil may be a significant a reservoir of drug resistant enteric bacteria contributing to antibiotic resistance as indicated by *Proteus salmonicida* resistance to Cefotaxime antibiotic, compared to *Proteus* species from the clinical source.

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INTRODUCTION

Humans are in contact with soil constantly, either directly or indirectly via food, water and air and thus soil may act as a vector and source of important human disease causing agents. Although many of the diseases associated with soils have been well characterized and studied, enteric diseases and their link to soil have been understudied and possibly underestimated. In order to clarify this connection, diseases associated with soil have been classified depending on the origin of the etiological agent as follows : (a) soil-associated diseases which are caused by opportunistic or emerging pathogens that belong to the normal soil microbiota (e.g. *Aspergillus fumigatus* is a very common fungus occurring in soils and can infect the lungs via inhalation of spores), (b) soil-related diseases, which result in intoxication from the ingestion of food contaminated with entero- or neurotoxins (*Clostridium botulinum*, *C. perfrigens* and *Bacillus cereus*), (c) soil-based diseases caused by pathogens indigenous to soil (which include *C. tetani*, *B. anthracis*, and *C. perfringens*) and (d) soil-borne diseases caused by enteric pathogens which get into soil by means of human or animal excreta (Toze, 1997). Enteric pathogens transmitted by the fecal–oral route are bacteria, viruses, protozoa and helminths. Fruits and vegetables frequently come in contact with soil post-harvest and thus may become contaminated with soil enteric bacteria present in sewage sludge or manure spread. One of the first cases of infection with *E. coli* O157:H7 linked to the use of animal excreta as manure was with an ovo-vegetarian woman. The woman consumed almost exclusively the food produced in her garden, in which she used the manure from her own cow as a fertilizer (OMRI, 2011). In 1970, an outbreak occurred as a result of the ingestion of vegetables irrigated with wastewater. Further studies indicated that *Vibrio cholerae* was present in the irrigated soils (Shuval *et al*., 1998). Fruit juice and cider may become contaminated as a result of the fruit falling to the ground and coming in contact with soil which may contain pathogens from animal excreta or sewage sludge used as fertilizer. Bacterial antibiotic resistance is a serious public health concern due to the reduced potency of antimicrobial agents used in the treatment of infectious diseases (Martínez and Baquero, 2002). Enteric bacteria are present in large numbers in the human and animal gut, are medically important as infectious agents and exhibit antibiotic resistance (Paterson, 2002). Antibiotics are extensively used in human and

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veterinary medicine, and in agriculture, for the treatment of infections, growth enhancement, and prophylaxis in food animals. This leads to selection of drug- and multidrug-resistant bacteria (Barbosa and Levy, 2000). Antibiotic-producing microorganisms are found naturally in soil. This suggests intrinsic chromosomal antibiotic resistance originated in the soil in response to harsh environments generated by such antibiotic-producing microorganisms (Randal and Woodward, 2001). Whether naturally occurring or commercially made, stable antibiotics accumulate in soil inhabited by food animals and where antibiotics are used. This leads to selection for multidrug resistance, which can be chromosomally (intrinsic) or plasmid-encoded acquired (Owens *et al*., 2001). It is thus important to compare the drug sensitivity profile of these enteric soil bacteria, with those of clinical isolates in order to assess the risk of antimicrobial resistance due to environmental pressure.

MATERIALS AND METHODS

Study site

The study was carried out at the Kenya Medical Research Institute at the Center for Microbiology Research (CMR) at the Mycology Opportunistic Infections laboratory in Nairobi. The soil samples were drawn from a 30 kilometer diameter radius centered within Nairobi city Centre, Kenya. The study was expected to provide samples that are representative of Nairobi metropolitan and its environs. The study site is a high altitude area of around 1660 meters above sea level and it has a moderate climate with maximum temperature of 28^{o} Centigrade and minimum temperature of 11^{o} Centigrade (World Travel, 2012). It receives rainfall ranging from 15 millimeters to 200 millimeters annually (World Travel, 2012). It is located in Latitude 1^{o} 17'S and Longitude 36°48'E (Maps of World, 2012).

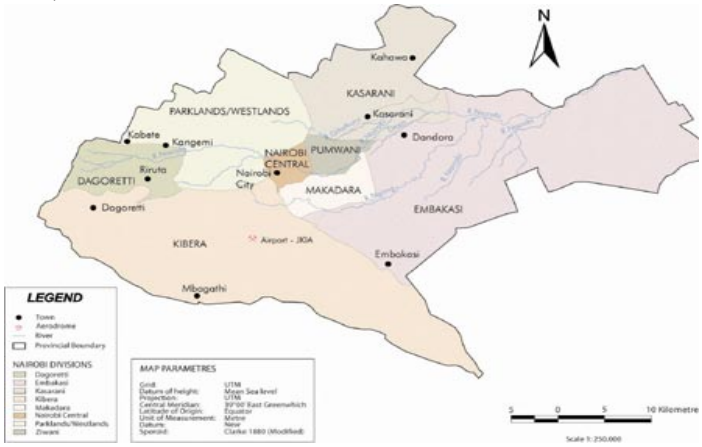

Figure 1. Map of Nairobi and its environs where the GPS locations were randomly selected (Maps of World, 2012)

Sampling

The GPS coordinates were awarded random numbers then simple random sampling method was used to select the random sites. In the event that the random site was unavailable due to access restrictions or lack of an appropriate site (example stones), the closest available site was used and the GPS coordinates recorded. The choice of the test antibiotics were selected from that fo recommended treatment of enteric bacteria that is, Ampicillin, Cefuroxime, Gentamycin, Ciprofloxacin, Gentamycin, Naldixic, Co-trimoxazole, Chloramphenical, Cefotaxime and Erythromcin. The four selected antibiotics were Ciprofloxacin, Gentamycin, Cefotaxime and Chloramphenical.

Sample collection

Once at the sample site, the GPS coordinates was recorded and sterile metal scoop was used to scoop some soil from the randomly selected site, and then placed in sterile paper bag and placed in a transportation box to the laboratory. The samples were then named starting with the zone (similar region) from which a sample was collected, followed by the sample number from that zone. For example sample 79.2/2 represents zone 79.2 and 2nd sample from that zone

Sample processing and identification of enteric bacteria

Ten grams of each of the soil samples was serially diluted in sterile distilled water in ratio 1:10, and then incubated for around two hours at 30^{0} Centigrade. After incubation, a loopful of the diluted soil sample was inoculated on Muller-Hinton agar (HiMedia Lab, India) plates, and then incubated overnight at 30^{0} Centigrade. After incubation, the colony characteristics were observed, and then the selected colonies were Gram stained and observed under the microscope under high power objective lens with aid of oil emulsion. The rod shaped Gram negative bacteria were sub-cultured on Muller-Hinton agar to get pure isolates. The Gram negative bacteria were most likely represent the enteric bacteria and their corresponding colonies were be inoculated onto Analytic Profile Index Kit (API 20E, BioMereiux, France), and then incubated overnight at 30^{0} Centigrade. After incubation, the AP1 20E was scored based on various biochemical reactions such as Hydrogen Sulphide production, various amino acid and carbohydrate reaction among others, that aided in identification of the enteric bacteria. The color reactions were observed (some with the aid of added reagents such as JAMES reagent), and the reactions (plus the oxidase reaction done separately) were converted to a seven-digit code which were read on the manufacturer's manual to show the corresponding enteric bacteria species.

Antibiotic susceptibility testing for the enteric bacteria

The identified enteric bacteria was be streaked onto a new Mueller-Hinton agar plate to get a purity plate. Approximately 4-5 isolated colonies were picked and inoculated into 5mL normal saline and emulsified uniformly. The turbidity was adjusted to that of 0.5 McFarland (Approximately 1.5 *10^{8} CFU/mL) and the inoculum swabbed on the entire surface of Muller-Hinton agar (HiMedia Lab, India) plates three times while rotating the plate 60^{0} between streaking to obtain uniform inoculation. The plates were then allowed to stand at room temperature for about three minutes to allow any surface moisture to be absorbed before application of the drug discs. The antibiotic discs were applied on the surface of the plates using a sterile forceps, and then incubated at 30^{0} Centigrade overnight. The zones of inhibition were then compared to those of enteric bacteria isolated from clinical samples using the data

from tests done in the laboratory, during the same duration of the study. Antibiotic susceptibility testing was done according Clinical and Laboratory Standards (2007). Sterile water with no soil sample was used as a method of negative control in the study. Also *Escherichia coli* ATCC 25922, was used as standard control strain to check for the efficacy of the antibiotics.

RESULTS

A total of 236 soil samples inoculated onto Mueller-Hinton agar plates yielded 52/236 [22%] of bacteria. The 52 samples that grew bacteria, 38/52 [73%] were Gram negative bacteria of which they were inoculated onto the Analytic Profile Index (API 20E) kit for identification. The 38 Gram negative bacteria isolated, 17/38 [44.7%] were identified as *Proteus salmonicida,* 11/38 [28.9%] were *Myroides spp,* 5/38 [17%] were *Pseudomonas putida, Pseudomonas aeruginosa* and *Stenotrophomonas maltophila* were two each [5.2% each], and one *Alcaligenes spp* [2%] was identified using the API 20E kit.). The other isolated Gram negative bacteria (*Myroides spp, Pseudomonas putida, Pseudomonas aeruginosa, Stenotrophomonas maltophila* and *Alcaligenes spp)* are not members of the Enterobacteriaceae family though some maybe isolated from the intestinal tract such as *Pseudomonas* and *Alcaligenes spp.*

Table I. Gram stain reactions of the isolated bacteria

Number of Isolates	Gram stain reaction	Cell shape
6	Negative	Cocci
4	Positive	Cocci
38	Negative	Rods
4	Positive	Rods

In the growth on Mueller- Hinton agar, 52 isolates were bacteria out of the 236 soil inoculations, representing 22% prevalence of bacteria in the soil. When the 52 isolates were Gram stained, most of them were Gram negative rods (n=38).

Distribution of the enteric soil isolates

Most of the isolates that yielded Proteus salmonicida bacteria were found to occur at the North Western and South Eastern part of Nairobi, as shown on figure 6 below.

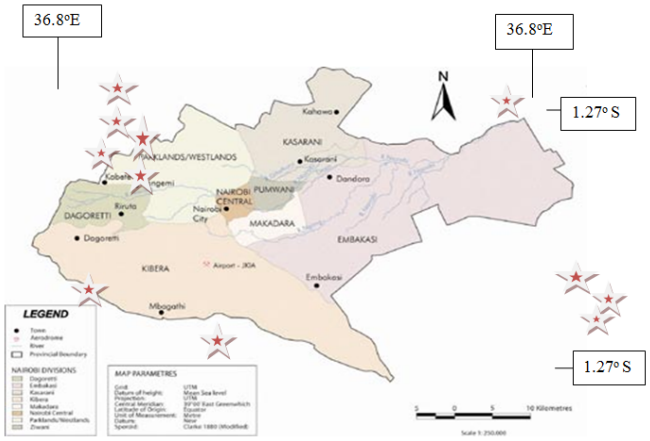

Figure 6. Distribution of enteric bacterial soil isolates in study area- The red stars indicate the soil sample zones that yielded *Proteus samlonicida*

Table 2. Antibiotic sensitivity profile of *Proteus salmonicida* from soil

Isolate	Zones of inhibition in millimeters			
	Ciprofloxacin	Gentamycin	Cefotaxime	Chloramphenicol
40.1/3	26	27	6	16
79.2	27	28	6	36
2.3/8	24	25	6	36
29.2/10	24	28	6	30
47.3/3	24	26	6	34
74B.2/2	23	25	6	34
74B.2/15	26	30	6	28
79.2/6	25	28	6	20
62.3/2	27	30	6	40
9.2/9	22	25	6	33
79.3/12	26	30	6	30
79.3/7	26	29	6	37
2.3/15	25	28	6	41
79.2/9	33	30	6	41
79.3/1	24	24	6	36
9.2/1	25	26	6	32
71.2/5	22	25	6	37

Most of the isolates from soil samples demonstrated the least resistance to Chloramphenicol, with zones of inhibition ranging from 16 mm to 41mm. High resistance levels were observed in Cefotaxime with zones if inhibition at 6mm. The zones of inhibition observed in Ciprofloxacin and Gentamycin, ranged from 22 mm to 27 and 24 mm to 30 mm respectively.

Table 3. Antibiotic sensitivity profile of *Proteus* species from clinical isolate from otitis

Drug	Zones of inhibition in mm
Ampicillin	13
Cefuroxime	24
Gentamycin	12
Ciprofloxacin	21
Co-trimoxazole	14
Chloramphenical	11
Cefotaxime	18
Erythromycin	18

The *Proteus* species from clinical isolate (otitis–ear infection) demonstrated the least resistance to Cefuroxime, while it was most resistant to Chloramphenicol antibiotic as shown in table three

Table 4. Antibiotic amounts impregnated on disc and breakpoints (CLSI) for enteric bacteria

Drug	Amount impregnated on disc (µg)	Breakpoints(mm)
Ampicillin	2	14-16 I
Cefuroxime	30	15-22 I
Gentamycin	10	13-14 I
Ciprofloxacin	5	16-20 I
Nalidixic	30	14-18 I
Co-trimoxazole	50	11-15 I
Chloramphenical	50	13-17 I
Cefotaxime	30	15-17 I
Erythromycin	15	>22 R

The zones of inhibition were compared to the Clinical Laboratory Standard Institute breakpoints of 2007.

Upon incubation overnight, zones of inhibition were observed on the cultured plates as shown in figure seven. Most of the isolates did not show any zones of inhibition (resistance) on Cefotaxime antibtiotic as shown in figure seven.

Figure 7. Antibiotic sensitivity-the figure above shows *P. salmonicida* isolate that was sensitive to Chloramphenicol (right) and resistant to Cefotaxime (left)

Proteus species isolated from otitis was used to compare the antibiotic sensitivity profile with the *Proteus* isolated from the soil samples. *Proteus salmonicida* showed to be generally more sensitive to the antibiotics, compared to the *Proteus* from otitis as shown on the graph in figure eight. However *P. salmonicida* was resistant to Cefotaxime antibiotic while the *Proteus* from clinical specimen was sensitive to the same antibiotic.

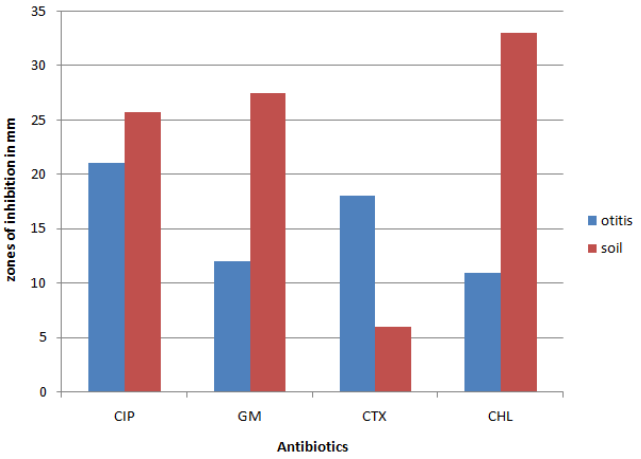

Figure 8. comparison of inhibition zones of *Proteus* species from the soil and clinical samples - the graph shows higher zones of inhibition of soil samples than of the clinical sample (otitis). NB: CIP (Ciprofloxacin), GM (Gentamycin), CTX (Cefotaxime), CHL (Chloramphenical)

Data analysis

One-way Analysis of Variance was used to test the effects of the antibiotics on *P. salmonicida.* Significant effect was observed overall ($F=2.729$, $d.f=3$ at $p\leq0.005$) hence the null hypothesis of was rejected. In Cefotaxime however, no effect was observed in since there was no variation of the zones of inhibition from the mean. Ciprofloxacin showed the most effect among the four antibiotics since it had the greatest variation of zones of inhibition from its mean from its mean.
Student's t-test was used to compare the zones of inhibition between the soil and clinical isolates. Significant results were also observed ($t=3.873$, $d.f=3$ at $p\leq0.05$). Chi-Square test was used to test association between the soil samples zones and number of enteric bacteria isolated: $X^2=2.364$, $df=2$, $p\leq0.05$. The results show a higher significance value at $p\leq0.05$, hence there was association between the number of enteric bacteria isolated and the soil sample zones.

DISCUSSION

Proteus salmonicida was the only enteric bacteria isolated out of the six Gram negative bacteria species identified via Analytical Profile Index (API 20E). The bacterium can be acquired from eating contaminated fish. It is pathogenic for fish, causing a disease known as furunculosis. Out of the soil samples (n=236) inoculated onto Mueller- Hinton agar, 17 grew *P. salmonicida* bacteria, which represents a prevalence of 7.2% of enteric bacteria in the soil. This low prevalence compares well with the study done by Ntabo *et al*., (2012) who were analyzed bacteria from soil samples in Juja and Kakamega forest. Out of the 137 pure isolates they got, two isolates of *Serratia marcescens* were the only enteric bacteria, which represents a prevalence of 1.5%. These results contrast with the study done by Burgos *et al*., (2004) investigating the presence of multidrug-resistant enteric bacteria in dairy farm topsoil. They isolated 102 enteric bacteria from 11 dairy farm top soils and 9 isolates were obtained from adjacent roadsides (non-dairy soil). The large number of enteric bacteria isolates could be attributed to the cow dung that was present in the dairy farms. The enteric bacteria they isolated were: *Citrobacter braakii, Citrobacter freundii, Citrobacter koseri, Enterobacter gergoviae, Enterobacter taylorae, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Shigella* spp, and *Serratia plymuthic.*

Analytic Profile Index kit (API 20E) provided a direct way of identifying enteric bacteria and other fastidious Gram negative bacteria. Of the 38 Gram negative isolates inoculated onto the kit, it provided identities as *Proteus salmonicida, Myroides spp Pseudomonas putida, Pseudomonas aeruginosa, Stenotrophomonas maltophila* and *Alcaligenes spp.* Of these Gram negative bacteria, *Proteus salmonicida* was the only enteric bacteria targeted. The enteric bacteria gave positive reactions on ADH (Arginine DiHydrolase), TDA (Tryptophane DiAminase), VP (Voges Proskauer) and GEL (Gelatinase). According to Willey *et al*., (2008) ADH is an enzyme present in the enteric bacteria that is responsible for releasing ammonium from Arginine during the urea cycle. TDA is an enzyme used by bacteria to produce Indole via deaminaton of Trypyophan. Positive VP test meant that the enteric bacterium was able to produce acetion in the culture (Willey *et al*., 2008). Gelatinase is an enzyme used by the enteric bacteria to break down protein gelatin from collagen (Ryan and Ray, 2004). However, it is noted that *Escherichia coli* a typical enteric bacteria, was not isolated in this study.

Antibiotic sensitivity profile was also performed on the enteric bacteria. Disc diffusion method was used according to Clinical and Laboratory Standards of 2007 (CSLI, 2007). Four randomly chosen antibiotics were used (Chloramphenicol, Cefotaxime, Ciprofloxacin and Gentamycin) and *P. salmonicida* was most sensitive to Chloramphenicol while resistant to Cefotaxime antibiotics. This could mean the bacteria had developed resistance to the antibiotic due to environmental exposure, as the Cefotaxime antibiotic showed

sensitivity to standard *Escherichia coli* (ATCC 25922) that was used as a method of control to show the efficacy of the four antibiotics used. Soil bacteria have been shown to develop antibiotic resistance to some of the antibiotics, as a means of survival since they are exposed to a myriad of other antibiotic producing bacteria e.g. Actinomycetes (Davies, 1994). Although the exact method of resistance could not be established in this study, resistant gene transfer through plasmids may be implicated considering the prevalence of plasmids in soil bacteria (Wilke *et al*., 2005). Other mechanisms of bacterial resistance such as efflux pumps and porin mutations could also have occurred. . In the study done by Burgos *et al*., (2004) they used 22 isolates for further study based on medical importance or high frequency of occurrence. They used Minimal Inhibitory Concentration method (MIC) to performed antibiotic sensitivity profile on the 22 isolates. The antibiotics they used were Chloramphenicol, Penicillin G, Nalidixic acid and Tetracycline. Most of their isolates showed higher resistant levels to Chloramphenicol which was contrary to this study, as the same antibiotic showed highest sensitivity. Most of the isolates demonstrated the least resistance to Nalidixic acid and Tetracycline. The isolates also showed highest resistance to Penicillin G.

In data analysis, one-way Analysis Of Variance (ANOVA) was used to test the effect of the treatment (the four antibiotics) on the replicates/subjects (isolates). Ciprofloxacin was found to have greatest an effect within and between groups. This means it was the most effective drug among the four antibiotics chosen. Chloramphenicol showed the highest zones of inhibition on paper but upon analysis it was not as effective as Ciprofloxacin, since its zones of inhibition were not that varying from the mean. Cefotaxime did not show any effect within and between the groups as the antibiotic gave the same zones of inhibition of 6 millimeters hence there was no difference from the mean. Thus the antibitotic was the least active among the four chosen. In summary, the most effective antibiotics were Ciprofloxacin followed by Chloramphenicol, Gentamycin and Cefotaxime was the least effective one. Burgos *et al*., (2004) used the non-parametric one-tail Wilcoxon-Paired sample test to test the effects of salicylate on the antibiotic resistance to Chloramphenicol, Nalidixic acid, Penicillin, and Tetracycline. The one-tailed test was used, as the priori hypothesis was that salicylate increased antibiotic resistance in the isolates. Results were considered significant at $P \leq 0.05$. In conclusion, soil may be a significant a reservoir for the enteric bacteria contributing to antibiotic resistance as indicated by *Proteus salmonicida* with resistance to Cefotaxime antibiotic, compared to *Proteus* species from the clinical sources which was sensitive. This study suggests that the soil serves as an under-recognized source of resistance with the potential to reach clinical isolates of the bacteria. The study however showed a low prevalence of enteric bacteria from the soil sampled.

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