

**ISOLATION SEROTYPING AND MOLECULAR CHARACTERIZATION
OF ENTERIC PATHOGENS FOR VALIDATION OF “PEEPOO”
SANITIZATION ALONG HANDLING CHAIN AT KIBERA, NAIROBI
KENYA**

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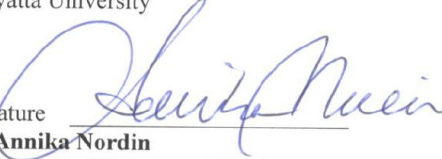
DECLARATION


This thesis is my original work and has not been presented for a degree or other awards in any other University

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DEDICATION

I dedicate this work to my dear loving wife, Mrs. Wangechi Nduhiu, my three wonderful daughters; Ms. Nyawira Nduhiu, Ms. Muthoni Nduhiu and Ms. Wanjiru Nduhiu for their prayers, understanding and support during the long working hours towards this work. To my loving mother who believed in me and encouraged me to pursue this study, she has gone through the most trying moments of her life and her trust for the almighty God has seen her through it all. “For it is God that works in me both to will and to do of His good pleasure”

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
AMREF	African medical research foundation
API	automated profile index
BLAST	Basic local alignment search tool
BST	Bacteria source tracking
BICMA	German company for hygiene technology
CBD	Central business district
CFU	Colony forming unit
DALY	Disability adjusted life year
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EC	European commission
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EUCAST	European committee on antimicrobial susceptibility testing
ERIC	Enterobacterial repetitive intergenic consensus
ECDC	European center for disease prevention and control
EFSA	European food safety authority.
EMEA	Europe Middle East and Africa
GBS	Guillain Barre syndrome
GTG₅	Guanine-Thymine-Guanine
HGP	Human Genome Project
IBS	Irritable bowel syndrome
ILP	Initial launch project
JMP	Joint monitoring programme
KSH	Kenya shilling

KM	Kilometers
MLVA	Multiple locus variable number tandem repeat analysis
MDGs	Millennium development goals
mm	millimeter
MIC	Minimum inhibition concentration
NACOSTI	National commission of science and technology
NGs	Next generation sequencing
SGDs	Second generation DNA sequencing
SNPs	Single nucleotide polymorphism
Peepoo	Single use sanitization toilet bag
PCR	Polymerase chain reaction
pH	Negative log of hydrogen ions
PFU	Plaque forming unit
QMRA	Quantitative microbial risk assessment
RFLP	Restriction fragment length polymorphism
REP-PCR	Repetitive extragenic palindromic-PCR
RAPD	Random amplification of polymorphic DNA
ReA	reactive arthritis
spp	Species
XLD	xylose lysine deoxycholate agar
UN	United Nations
UNDP	United Nation's development fund
UNICEF	United Nations children fund
WHO	World health organization

ABSTRACT

An estimated 1.8 billion people in the world use water coming from fecally contaminated sources. This is as a result of 80% of the human generated wastewater ending up in rivers and other reusable water bodies before treatment. The hygienic balance is locally complicated by the fact that 57% of people in informal settlements have no access to basic sanitation, including latrines and toilets. This study sought to evaluate alternative toileting in the name of “Peepoo”, as single use personal toilet for human excreta sanitization and reuse as safe fertilizer, thus preventing dissemination of pathogens to the environment. A cross sectional study was conducted to sample 11 schools enrolled in the project working site in Kibera. Using standard microbiological methods, baseline faecal pathogen concentrations were determined as prevalences at the start of sanitisation and used to validate the “Peepoo” bag along handling chain. Specific primers were used for molecular characterization of isolated pathogens and *detection* of resistant genes. The prevalence of pathogenic bacteria (*Salmonella enterica subspecies arizonae IIIa* (0.34%), pathogenic *Escherichia coli* (7%), *Campylobacter spp* (18.3%), protozoa (*Cryptosporidium spp* (2.4%), *Entamoeba histolytica* (28%), soil transmitted helminth (*Ascaris lumbricoides* (20%) and *Trichuris trichura* (13%) eggs and gastrointestinal viruses (*Rotavirus* (0%) and *Adenovirus* (1.1%)), were determined. There was marked difference in parasitism distribution among schools that was attributed to contamination of school compound with raw sewage. The identified bacteria and parasitic pathogens (*Salmonella enterica*, *Escherichia coli*, *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter coli* and *Ascaris lumbricoides*) in relation to pH, temperature and ammonia concentration were used to validate sanitation ability of the “Peepoo” bag along handling and reuse chain. Pathogenic *Escherichia coli* (69%) including EHEC (6.0%), EPEC (3.5%), ETEC (2.0%), DEAC (2.0%), EAEC (0.7%), EIEC (0.4%) and *Campylobacter jejuni* (92%), *Campylobacter coli* (88%), *Campylobacter lari* (76.9%) and *Campylobacter spp* (25%) were genetically identified and found to be resistant to 1st and 2nd generation antibiotics at different levels. Viral infection (Rotavirus and Adenovirus) was not identified as a health issue during the study period. Sequencing of PCR products from selected pathogenic *Escherichia coli* isolates confirmed their molecular identity. The results from this study demonstrated the ability of “Peepoo” bag to effectively inactivate pathogens found in human excreta when stored for 8 weeks at ambient temperature. This significantly reduced the risk of pathogens internalization by coriander grown in soil fertilized with human excreta. The peepoo bag should therefore be deliberately promoted as a method of human waste management in informal settlements and as alternative source of organic fertilizer. Mass deworming of children is also recommended after every three months in informal settlements.

CHAPTER ONE: INTRODUCTION

1.1 Background information

In developing countries, available facilities in the urban set ups have increasingly been strained, partly due to a trend in urban/pre-urban population movement (Reis *et al.*, 2008). One of the outstanding facilities under immense strain is sanitation, negatively affecting both environment and public health. Human excreta has been shown to contain high amounts of plant nutrients that can be used to boost crop production (Winker *et al.*, 2009). However, the excreta also contains infectious organisms that must be destroyed or reduced to acceptable levels, before the excreta is put to agricultural use (Esrey, 2001). These micro-organisms, particularly bacteria, can be resistant to common antibiotics and may have multidrug resistant genes (Christabel *et al.*, 2012). On-site sanitation technologies have been a challenge in slums due to congestion and poor water drainage. For this reason, other innovative off-site sanitation technologies have been sought, to allow safe handling and disposal of human excreta in slums (Genser *et al.*, 2008).

To solve the challenge of effective pathogen destruction and thereby prevent infectious disease transmission, sanitisation technologies need to be diversified as well as refined to offer methods applicable in all different contexts. The hygienic quality of fertiliser produced from excreta ought to be high, to avoid health risks to both the handlers and end product consumers. This would be achieved through

establishment of disease transmission pathways when treated excreta is used on arable land, and to achieve high hygienic quality of the products.

Excreta disposal systems are defined as improved by the WHO/UNICEF Joint Monitoring Program (JMP) for water supply and sanitation, when privately owned and if they prevent human contact with the excreta (WHO, 2006). This definition has not incorporated systems involved in excreta treatment as well as the treated sewer-transported excreta (Baum *et al.*, 2013). However, going by Baum *et al.* (2013) definition of improved sanitation, only sewerage connections that are treated before release to the environment are included.

The number seven UN millennium development goal (MDG) that came to close at the end of 2015, had targeted to halve the proportion of people lacking basic sanitation by the year 2015 (WHO, 2015). According to WHO/UNICEF JMP (2015) report, this target was not achieved since 2.4 billion people still lack access to improved sanitation facilities (WHO, 2015). Going forward, the JMP identified open defecation as one of the challenges to be addressed within the sustainable development goal (SDG). Open defecation is used as a marker of extreme poverty and is targeted to be eliminated by the year 2030. Improved sanitation for every human being will result in clean environment and good health thus restoring human dignity (WHO, 2015).

Disease dissemination is prevented by providing clean unshared toilet, more so in a single defecation enclosure and when sanitisation of faeces starts immediately upon use, independent of further handling (Vinnerås *et al.*, 2009). The single unit principle and the collection system enable use of convenient collection and transport of human excreta on a daily basis. Crop production can be increased by supplying a locally available fertilizer, which improves yields by supplying limiting nutrients to soil as well as improving water holding capacity (Heinonen-Tanski and Wijk-Sijbesma, 2005). The aim of the present study was to assess the sanitization effectiveness of “Peepoo” as a single use personal toilet for human excreta, and reuse as a fertilizer. The risk involved in reuse of untreated human excreta was determined by testing the drug resistant patterns of characterized bacterial pathogens. The same was sequenced for an in-depth understanding of the environmental contamination with human enteric pathogens in the study area.

The process of validation required the bag to be subjected to a field application, with known starting point of fecal pathogens and establish conditions necessary for the WHO guidelines on fecal reuse as fertilizer. A baseline analysis was therefore undertaken to establish levels of pathogens in human excreta in the study area. In order to establish the risk involved with the flying toilets, pathogens were isolated from human excreta, domestic water, and soil. These were then characterized in terms of pathogenicity and antimicrobial resistance. A part from being self-

sustained, the program is expected to improve livelihoods in terms of good health and increased crop production.

1.2 Problem statement

Kibera, the biggest urban slum in Kenya, is faced with several sanitary challenges due to overcrowding and poverty including lack of toileting facilities, which leads to high prevalence of infectious diseases (UNDP, 2005; Feikin *et al.*, 2011). The few available pit latrines are shared by up to a hundred individuals and they are either too far (15 meters) from the houses or worse still, too near (1 meter). Women and children feel insecure when using the latrines located 15 meters from their houses, which is a challenge at night (Karanja, 2008).

The toilets in Kibera are inhabited by rodents and flies that visit human dwellings contaminating foods and utensils (Kagiri, 2007). The situation is even more desperate in wet rainy seasons as raw human excreta are swept into living rooms (Unger, 2007). As a result, prevalence of sanitation and hygiene related diseases have remained all time high, although little about it has been documented (AMREF, 2007).

The provision of “Peepoo”, as a single-use, self-sanitising, biodegradable toilet bag should be a solution to the sanitation crisis, matching the immediate and changing need of the user. Though Peepoo has been used in emergency responses

elsewhere, it has however not been validated as a hygienic toileting facility and for reuse as fertilizer, especially in an African urban informal settlements.

1.3 Justification

An estimated 1.8 billion people use water coming from fecally contaminated sources. This is as a result of 80% of human generated wastewater ending up in rivers and other reusable water bodies before treatment (UN, 2015). The hygienic balance is complicated by the fact that 1.8 billion people have no access to basic sanitation, including latrines and toilets. As a result, close to 1000 children die daily due to water and sanitation related diarrhoeal diseases (UN, 2015). The human excreta ending up in wastewater contains tons of plant nutrients that could account for up to 30% of the annual global conventional fertiliser production (Kone *et al.*, 2010). Use of treated human excreta as a fertiliser and soil conditioner will address both the basic human hygiene and crop production needs.

Having a well-managed sanitation program in place will not only improve the populations' health and restore human dignity but will also go a long way in the appropriate management of human waste. Human waste management has for a long time been a nightmare in the study area, occasioned by inadequate or complete lack of methods for sanitization and disposal. Peepoo, a single use self-sanitization bag, can be used at the convenience of households or learning facilities. It is a cheap and hygienic method of human waste management. The idea of using the peepoo as a toilet bag was conceived to give a more hygienic

alternative to the infamous flying toilets in Kibera slum. The peepoo bag has been tested for hygiene in Sweden (Vinneras *et al.*, 2009) and requires field validation in the country before being recommended to the National Environmental Management Authority (NEMA).

1.4 Research questions

- a) What is the prevalence of enteric pathogens in school going children at Kibera?
- b) What is the effect of Peepoo sanitization on the pathogens and indicator organisms in human excreta for reuse as fertilizer?
- c) What are the molecular characteristics and antimicrobial resistance patterns of selected bacterial pathogens along Peepoo handling chain?

1.5 Hypothesis

Use of Peepoo as a single use individual toilet bag is not an effective sanitization system and attendants at the handling chain are predisposed to health risks.

1.6 Objectives

1.6.1 General objective

To undertake isolation, serotyping and molecular characterization of enteric pathogens for the validation of “peepoo” sanitization along handling chain at Kibera

1.6.2 Specific objectives

- i) To determine prevalence of bacterial pathogens, gastroenteric parasites and selected enteric viruses in school going children at Kibera.
- ii) To determine viability of thermo-tolerant coliforms, bacterial pathogens, gastroenteric parasites and selected enteric viruses along Peepoo handling chain.
- iii) To phenotypically and genetically characterize selected bacterial pathogens isolated along the Peepoo handling chain for antimicrobial resistance and molecular identification.

1.7 Significance of the study

The project evaluated the Peepoo sanitation and reuse system regarding risk of enteric bacterial infection with the purpose of optimizing sanitisation within the Peepoo. This is in a context with high prevalence of persistent pathogens and develop management strategies to minimize identified risks in the system, a successes of which will provide access to hygienic toileting facility, particularly to the most vulnerable population, including women and children. It is also expected that enteric pathogens circulating in the population will be contained. As a result, a healthier more enabled population, with reduced child morbidity, mortality and financial strain occasioned by frequent hospitalization and treatment. The environment in the study area will improve as a result of proper human waste management through Peepoo treatment and end use as a plant nutrient. On the

other hand, Peepoo will provide cheaper and more nutritive soil fertilizer, with expected increased crop production. Use of Peepoo is expected to improve public health by preventing disease dissemination as well as increase crop production through reuse of hygienically safe human excreta as fertilizer.

CHAPTER TWO: LITERATURE REVIEW

2.1 Human excreta

Human excreta is defined as the waste generated after metabolism, which is generally proportional to the food consumed by adults (Jonsson *et al.*, 2004). The urine part of waste is composed of metabolized waste, while feces is part that form the indigestible food component (Petersen, 2007). High vegetable diet means that a large amount of fiber is undigested and contains a high percentage of soil nutrient. These nutrients need to find their way back to the soil for a sustainable crop production cycle (Vinneras *et al.*, 2008).

One way to replace the soil nutrients that are removed by crops is through recovery and use of human urine and faeces. This is not a new practice since it has found its use in crop production since time in memorial (Muskolus, 2008). For instance, human excreta were used as a source of plant nutrients in Europe until the mid-19th century (Bracken *et al.*, 2007).

2.2 Challenges in using human waste as fertilizer

2.2.1 Residuals

Heavy Metals will be found concentrated mostly in faeces as a result of consuming contaminated crops. However, the levels are much lower than those found in chemical fertilizers (Winker *et al.*, 2009), and comparable to levels in animal manure.

Hormones and pharmaceuticals will be found as residuals in human excreta and they pose a challenge in the use of urine and faeces as fertilizer (Richert *et al.*, 2010). However when the same is discharged to the waterbody which normally is the practice, they have a direct negative effect to the aquaculture. The amount of microorganism in a cubic meter of soil is equated to those found in a cubic kilometer of water (Lypygina *et al.*, 2002). Microorganisms have a way of breaking down hormones and pharmaceuticals. Due to this ability, they have been used in water purification (Astrazeneca, 2006). This is contrary to what happens with aquatic environment, where sex disruptions and antibiotic resistance build – up has been reported (Kim and Aga, 2007).

2.2.2 Pathogens

Having taken care of the other factors, disease causing micro-organisms are continuously discharged through human excreta. Faeces can contain large amounts of pathogenic bacteria, viruses, protozoa and helminths (WHO, 2006). This is a major constrain in the use of human waste to condition soil. Human faeces are thus considered as contagious and must be handled as such, until effective sanitization has taken place (WHO, 2006). Otherwise faecal contamination of the environment will continue to be a major cause of intestinal parasitic infection (Moubarrad and Assobhei, 2007) as well as other zoonotic infections (Albihn, 2002).

Human faeces have a high concentration of enteric bacteria some of which are pathogenic. During systemic infections urine as well is known to excrete *Leptospira interrogans* and *Schistoma haematobium* and in very rare occasions *Salmonella typhi* or *paratyphi* and *Mycobacterium* (Wilson and Gaido, 2004). Bacterial pathogens excreted in faeces include *Salmonella spp*, *Escherichia coli*, *Campylobacter spp*, *Yersinia spp*, *Vibrio cholera* and *Shigella spp* (WHO, 2006). Enteropathogenic viruses shed in faeces include rotaviruses, astroviruses and caliciviruses. Others occurring in faeces but not necessary gastroenteric, include adenoviruses, hepatitis A and E viruses (Guardabassi *et al.*, 2003). Some of these like Hepatitis E also infect pigs and are hence zoonotic (Vinneras *et al.*, 2008).

Parasites are predominantly shed in faeces and in urine for those infecting the urinary system, helminths like *Ascaris lumbricoides* and *Trichuris spp* being most common in low income settings (Venglovsky *et al.*, 2006). Others, including *Hymenolepsis nana*, *Ancylostoma duodenale*, and *Necator americanus* (WHO, 2006). Protozoa of interest include *Entamoeba histolytica*, *Cryptosporidium spp* and *Giardia spp*, which are associated with waterborne out breaks even in the high-income countries (WHO, 2006).

2.3 Treatment of excreta

Health protection measures must be put in place with the aim of reducing health risks to involved persons. Some of the recommended measures include; treatment

of human excreta to remove pathogens, restricted use of human excreta as fertilizers, and personal and food hygiene practices (WHO, 2006). To make use of soil nutrients that are readily available in human waste, the waste requires to be taken through the process of pathogen reduction to non-infectious levels or better still complete destruction for others (WHO, 2006).

There exists established excreta sanitization procedures based on pathogens inactivation factors including temperature, pH, moisture and ammonia (Pecson *et al.*, 2007). Different government authorities have come up with regulations on the hygienic qualities of sewage sludge or wastewater for re-use. Those without well established regulation borrow from global acceptable regulation, like the WHO Guidelines for the safe use of wastewater, excreta and greywater in agriculture (WHO, 2006). The WHO guidelines recommends that sanitized excreta should have no *Salmonella* in 25 grams wet/weight, fecal coliforms counts should be less than 1000 per gram of total solids and helminthes eggs (viable *Ascaris lumbricoides*) should be less than 1 in one gram of total solids. *Enterococcus* have also been used as an indicator, requiring a reduction of 5 log₁₀ (EC, 2006; WHO, 2006).

2.4 Peepoo

The Peepoo toilet bag is 150 by 380 mm and 25 µm thick, with an inner hand cover funnel (upper circumference of 580 mm; length of 220 mm, thickness of 12

µm). The bag is made of a biodegradable plastic based on polyethylene, pigments and a starch-based pro-oxidant additive. The inner layer is designed for personal hygiene, to cover the users hand when the bag is used without a seat (kiti) or better still wrapped over the kiti. The total weight of each Peepoo is 10 grams of which 6 grams is urea contained in a filtering pouch made of cellulose. Each used Peepoo can contain up to 800 ml of faeces and urine, and remains odourless for 24 hours (Vinneras *et al.*, 2009) (Figure 2.1).

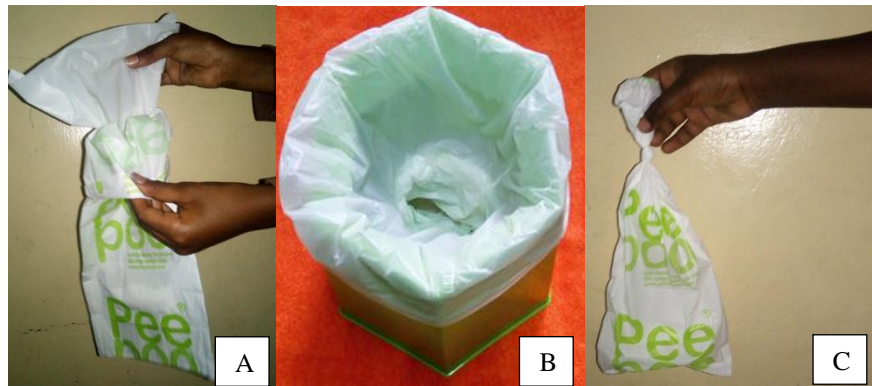


Figure 2.1: Peepoo bag with the outer layer held down (A), outer layer wrapped over kiti (B) and a used peepoo bag secured with a knot (C).

Peepoo is an alternative for the ‘flying toilet’ in Kenyan slums that enables immediate sanitation of human excreta upon defecation and turns the same into valuable fertilizer. The bag meets European Union standard EN13432 on plastics, since it is bio-degradable breaking down to carbon dioxide, water and biomass. Peepoo is an innovation of Professor Anders Wilhelmson and since 2016 production and management of peepoo is by International Aids Service (IAS company Ltd), with a daily production of 5000 units (Peepoople, 2016).

In an impact assessment Ondieki and Mbegera, (2009) reported that 90% of Peepoo users in Kibera had a strong acceptance. It was an absolute sanitation solution within Kibera since the bag is safe and clean to handle. During the assessment period 50% of the respondents admitted disposing their waste through the flying toilet approach and hence the need for Peepoo use. Due to the limited number of toilet facilities people queue to use the toilets. Use of Peepoo was reported as time saving and convenient to users. Human waste disposal is a major problem in every slum setup. The Peepoo bag was welcome as an alternative sanitization method for reuse of the waste as fertilizer; this would not only take care of waste menace but earn an income to the Peepoo users (Ondieki and Mbegera, 2009).

2.5 Peepoo use in Kenya

The distribution and training on how to use peepoo bag in Kenya is under Pepeople Kenya, a non-governmental organisation (NGO) registered in 2010, working in Kibera, Nairobi. The NGO was started with an objective of building a sustainable sanitation system, to improve the health and livelihood of Kibera residents, provides entrepreneur opportunities and contribute towards fertilizer production. This is being pursued through provision of personal toilet to slum dwellers and schools in the study area. With the success of the current use, the

same could be scaled up to humanitarian responses including refugees and internally displaced individuals (Peepoople, 2013).

Peepoos are sold to the community for 3 Kenya Shilling (Ksh) each by women micro-entrepreneurs, whereas the use in schools is subsidised by donor money. Used Peepoos are brought by users to two drop-points, staffed by service operators and opened daily. At the drop-point, a refund of 1 Ksh is paid for each used Peepoo that is delivered. The Peepoos are collected in Jumbo sanitisation bags (Appendix V) (woven polypropylene bags with a tube liner) held in place by a movable rack. From drop-points, used Peepoos are transported daily to a storage area where the used Peepoos are sanitised for 4 weeks. Some Peepoo bags never reach the drop point and are used by Peepoo users as fertiliser in their bag gardens, a compact system for urban cultivation. The collected and sanitised Peepoos are used in agricultural projects to assess the fertilizing and soil improving potential in the Country (Peepoople, 2016).

2.6 Urea sanitization

Urea is the sanitizing agent in the peepoo bags. The urea degrades upon contact with bacterial enzymes in the faeces to form ammonia and carbonates, both of which contribute to pathogen inactivation (Park and Diez-Gonzalez, 2003). Toxic effects from unionized ammonia (NH_3) on several types of organisms have long been known and identified as a factor for pathogen inactivation in waste material

(Ward and Ashley; 1977; Mendez *et al.*, 2002). The biocidal effects have been confirmed on various bacterial (Park and Diez-Gonzalez, 2003; Mendez *et al.*, 2004), viral (Ward, 1978; Cramer *et al.*, 1983; Emmoth *et al.*, 2011), and parasitic (Jenkins *et al.*, 1998; Pecson *et al.*, 2007; Nordin *et al.*, 2009) pathogens. Inactivation rates are related mainly to NH_3 concentration and temperature (Pecson and Nelson, 2005; Nordin, 2010), whereas an alkaline pH is crucial attaining higher ammonia equilibrium (Emerson *et al.*, 1975).

Exploited strengths with this sanitisation method is that it functions within the ambient temperature range, it is non-harmful to users, and it increases the nitrogen concentrations in waste materials destined as fertilizer to improve soil nutrients. Ambient temperature combined with urea has been used in excreta treatment with encouraging outcome; increased temperature as a factor of sanitisation improves the efficiency of ammonia by increasing both the formation and action of NH_3 (Nordin *et al.*, 2008; Vinnerås *et al.*, 2008).

2.7 Abiotic factors influencing urea sanitation

In urea treatment controls, some enteric bacteria have been shown to be highly inactivated at elevated pH (basic) but remained active when temperature was increased keeping pH constant (Ottoson, 2007; Nordin, 2010). A pH of 6.8-7.5 is taken as the normal range for faeces (www.medicinenet.com) depending on the consumed diet. An alkaline pH has been shown to positively affect the amount of

ammonia in form of NH_3 in faeces, with an increased effect on sanitisation (Nordia *et al.*, 2009). Treatment of faecal material with urea increases its pH. The amount of urea added is of importance particularly when the end product is to be used to fertilize soil for agriculture since this increases the nitrogen value of the end product for crop nutrient (Fidjeland, 2010). Time and the prevailing conditions are overall features that will affect the survival of micro-organisms in the environment. However, the withholding or storage time to achieve recommended reduction or inactivation levels is a dependent factor to temperature, pH and type of treatment if any (Jonsson *et al.*, 2000; Hoglund, 2001). Variables such as pathogen load, volume of faecal material, moisture content, pH and storage temperature in individual Peepoo affects the overall treatment outcome and needs individual monitoring to come up with an effective sanitization model.

2.8 Biocides

The European parliament and council directive 98/8/EC defines biocides as active substances and preparations containing one or more active substances, that have been put up in the form in which they are supplied to the user intended to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect, on any harmful organism by chemical or biological means. These include quaternary ammonium compound used at health care, household, products surface, preservation food industry and pharmaceutical cosmetics preservation. They have

a general mode of action by membrane destabilizer, producing cytoplasmic protein aggregation (EU, 2009).

There are possibilities that development of resistance to antibiotics can as well be as a result of the use of compounds with bacterial activities. These include biocides like preservatives, sterilants, antiseptics, disinfectants and metals with antimicrobial activities like silver, zinc and copper (EFSA, 2009). Biocides are widely used in animal husbandry, food manufacturing plants and processes to either sanitize the foods or and the machinery. They are also used in treatment of food animals and maintenance of hygiene handling environment in the medical field and as food preservatives and for general hygiene. Laboratory experiment on use of biocides has been shown to activate the expression of structural and regulatory genes in *Salmonella spp* and *E.coli* (EU, 2009). Several publications have shown *in vitro* linkage when bacteria are exposed to bisphenol and triclosan (Braoudaki and Hilton 2004), biguanide chlorhexidine (Koljalg *et al.*, 2002).

The use of biocides, unlike antibiotics, are unregulated in terms of choice of use and dosages. They however, form a major role in infection prevention processes in health care. The process of decontamination using disinfectants is carried on equipment's, surfaces and intact skin whereas open skins and mucosa surfaces are treated with antiseptics. Biocides find their way to the environment through household waste water as well as through solid waste. Chlorine is largely used for

water treatment but its by-products are toxic to the marine life. Antimicrobial impregnated surfaces are gaining popularity in the food industry. Whereas this is meant to protect environmental spoilage by slow release of biocides in the surrounding, it would also result to localized selective pressure and ultimately biocide and or antibiotic resistant micro-organisms (Ahlbom *et al.*, 2009).

2.9 Indicators and pathogens for sanitisation

2.9.1 *Salmonella* as a pathogen

Salmonella spp. are straight rods 0.7-15 by 2-5µm which are Gram negative motile facultative anaerobic. They have both respiratory and fermentative type of metabolism with an optimal growth temperature of 37°C. They Catabolize carbohydrates with production of gas and acid. They are oxidase negative, catalase positive, indole and Voges Proskauer negative, methyl red and Simmons citrate positive. *Salmonella* occur in humans and other warm blooded animals as well as in cold – blooded animals, the environment and foods. These are the causative agents of several conditions including gastroenteritis, septicemia, typhoid fever and enteric fevers (Holt *et al.*, 1996). *Salmonella* belong to the family Enterobacteriaceae. The *Salmonella* genus is made up of two species namely, *Salmonella enterica* and *Salmonella bongori* (Grimont and weill, 2007). *Salmonella enterica* is divided into six sub-species, namely; *S. enterica subsp enterica*, *S. enterica subsp salamae*, *S. enterica subsp arizonae*, *S. enterica subsp*

diazonae, *S. enterica subsp houtenace*, *S. enterica subsp. indica* , and all have distinguishable characteristics.

The subspecies enterica serovars, accounts for more than 99.5% of the isolated *Salmonella* strains. According to the Kauffman and White scheme the *Salmonella* genus has over 2500 serovars. Not all *Salmonella* are host specific, some have a generic host range. Non- pathogenic animal serotypes do cause infections in human with *S. Typhimurium* and *S. Enteritidis* accounting for highest *Salmonella* foodborne outbreaks internationally at 24.1% and 6.6% respectively (Greig and Ravel, 2009). Foodborne salmonellosis is estimated to constitute 80% of *Salmonella* cases, other routes including waterborne, personal contact, nosocomial, contact with pets and pet foods as well as contaminated drugs accounting for the rest (Greig and Ravel, 2009).

Infection with *Salmonella spp* has a strong association with foods of vegetable origin including fresh juices, fruits and most importantly those eaten uncooked. These could be contaminated at points along the production chain or even during growth of the vegetables. Contamination could take place if soil used to grow the vegetables is not free of contaminated animal or human excreta (Wood *et al.*, 1991; Hanning *et al.*, 2009). Food animals may harbor *Salmonella spp* without symptoms but pass the pathogen to food consumers, notable among them being through eggs, poultry, pork and raw dairy products (Freitas *et al.*, 2010).

Salmonella serotype Enteritidis has been shown to strongly associate with bakery, eggs and layers (Gormley *et al.*, 2011). The high infection rate of foodborne salmonellosis is as a result of; cross – contamination of different foods, when food products are inadequately cooled or when the cold – chain is broken by lack of refrigeration. Also when products are consumed undercooked or worse still when food handlers are infected and end up contaminating the food they are handling along the production or processing or resale chain and upon sale (Gormley *et al.*, 2011; King *et al.*, 2011).

Salmonella spp cause intestinal inflammation without toxin production. Individuals at risk will include; infants, elderly, immunocompromised and those with gastric hypoacidity. Depending on inoculum and host status, *Salmonella* has an incubation period of 6-72 hours; presenting as fever, nausea, diarrhea, cramping sometime with vomit chills, headache and myalgia. Recovery within 7 days without treatment is in most cases observed. After acute infection, organisms are present for up to 7 weeks particularly in children, hence becoming chronic carriers in about 0.5% cases. These carriers have the ability to disseminate the pathogen to the environment contaminating drinking water as well as causing re-infection (WHO, 2005). The organism has the ability to enter the blood stream via draining mesenteric lymph nodes resulting to fatal bacteremia in 5% of the cases, mostly observed with immunocompromised patients (Hohmann, 2001).

Gastroenteritis due to salmonellosis is usually self-limiting and treatment with antibiotics is not advised, except in case of being at risk of developing bacteremia. Antibiotics may destruct the normal endogenous flora which is protective resulting in prolonged *Salmonella spp* excretion (Murase *et al.*, 2000).

2.9.2 Molecular identification of *Salmonella*

Polymerase chain reaction (PCR) methods have been developed to characterize *Salmonella* using the invasive gene (*invA*) able to identify the genus. Other genes; *sefA* and *fliC* have been used to identify serotypes Enteritidis and Typhimurium respectively (Stone *et al.*, 1994, Oliveira *et al.*, 2002).

2.9.3 *Salmonella spp* inactivation with ammonia

Salmonella spp inactivation in faecal material using NH_3 and temperature at varying concentrations has been modelled, allowing for prediction of inactivation using the combinations of the two variables (Nordin, 2010). Nordin *et al.* (2009) demonstrated inactivation of *Salmonella spp* in 27 days at 14°C without urea treatment. This time was reduced to 5 days by treating faeces with 2% urea at the same temperature. The relationship between the rate of inactivation, temperature and ammonia concentration achieved from breakdown of urea, as well as the pH of the extractants has been identified as a robust efficient option for recycling human excreta (Nordin *et al.*, 2009). However, this process of *Salmonella* inactivation has not been applied in a field setup with high levels of parasitic and

bacterial infestation. The uniqueness of individual Peepoos in terms of the users' levels of infection and diet will expose the treatment process to a wide range of interaction.

2.10 Faecal indicator and pathogenic organisms

Faecal coliforms test is considered a good indicator of pathogens ability to re-grow in excreta. This is a characteristic that makes them preferred to virus and parasites that lack the ability to replicate outside living tissues (WHO, 2006). Faecal coliforms including *E. coli* are always present in large numbers in faecal material, as opposed to other pathogenic enterobacteriaceae most notable *Salmonella spp*, *Shigella* and *Yersinia spp*. The survival characteristics of *E. coli* is considered similar to others belonging to enterobacteriaceae and can thus be used to indicate inactivation of these enteric pathogens occurring in low densities in similar environments (Nordin, 2010), when only a few individuals are infected.

Enterococcus has been used as an indicator for faecal pollution. It has been shown to persist longer in urea treated faecal material with a 10-fold longer time than that observed with enteric pathogens like *Salmonella* and *E. coli*; however it is considered not a good indicator of urea faecal inactivation, the reason being that the bacteria has low sensitivity to ammonia and has the ability to re-grow through the sanitisation process (Ottoson *et al.*, 2008).

2.10.1 Coliform bacteria

Coliforms as indicator organism in fecal sanitization are used as surrogate for other enteric pathogen. They indicate fecal contamination and the possibility of isolating *Salmonella spp* which is more elaborate and involving. Coliforms include genus *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella*. The genus *Serratia* and *Hafnia* are also considered as coliforms. They do not belong to any taxonomic group though they have common characteristics as being rod shaped, facultative anaerobic, non-spore formers, are gram negative and oxidase negative, they also ferment carbohydrate lactose producing acid and gas. Coliforms are normally found in the human intestines but also in animal. Some will also be found in water, soil and plants. *Escherichia coli* are the pre-dominant coliform in the gastrointestinal tract of human and animals.

Enterobacteriaceae is a family of approximately 20 genera. Coliforms belong to this family and the only group that ferments lactose. Fecal coliform or thermo-tolerant coliform have the ability to ferment lactose at 44.5°C which distinguishes them from other environmental coliforms. They are therefore a better indicator of fecal contamination than coliform bacteria in general (Tortorello, 2003).

2.10.2 Escherichia coli

Escherichia coli (*E. coli*) is a motile Gram negative bacilli in the Enterobacteriaceae family of *Escherichia* genus and *coli* type species *E. coli*

forms part of the normal flora of human and other mammals as the gut microbiota (Kaper *et al.*, 2004; Fratamico *et al.*, 2016). *Escherichia coli* colonizes the gastrointestinal tract of infants within hours of life, establishing mutual benefits with the host (Kaper *et al.*, 2004). On colonization, commensal *E. coli* will remain harmless to the host, only causing infection when the mutual environment is disturbed and in immune suppressed hosts. In such instances *E. coli* becomes infectious and is thus termed as pathogenic *E. coli*. Pathogenic *E. coli* are of different types (currently grouped into six groups) depending on the pathogenic gene (s) they possess. Pathogenic *E. coli* can be limited to the intestinal mucosa as well as be disseminated to other extra-intestinal body parts.

Clinical syndromes resulting from infectious *E. coli* can be classified as; enteric or diarrheal; urinary tract infection and sepsis or meningitis, the last two being extra-intestinal infections (Kaper *et al.*, 2004). Differentiating pathogenic strains has largely depended on serotyping methods. In 1933 Adams was able to implicate *E. coli* strains to pediatric diarrhea outbreak. Later in 1944 Kauffman proposed a serological classification of *E. coli* scheme. The same has been modified and according to the modified Kauffman scheme, surface antigens including the O-polysaccharide antigens, flagellar H-antigens and Capsular K-antigens have been used to serotype approximately 186 O-group *Escherichia coli* and 53 H-type serotypes (Fratamico *et al.*, 2016).

Though serotyping is useful in *E. coli* identification, it cannot be used to characterize the clinical syndromes of the strain. It is also a time consuming and expensive method, requiring well-trained work force. There remains the need to identify the specific virulence factor(s) in the pathogenic bacteria. This has been achieved through phenotypic assays for instance the HEp-2 adherence assay for diarrheagenic *E. coli* and the most recent genotypic methods that detects specific genes encoding the pathogenic characteristics of the pathogenic *E. coli* (Kaper *et al.*, 2004).

By use of specific set of virulence genes carried by *E. coli* and disease caused, it has been possible to classify them into pathotypes including; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) shiga toxin – producing *E. coli* (STEC), diffusely adherent *E. coli* (DAEC) and adherent invasive *E. coli* (AIEC). Hybrid pathotypes have also been described including the enteroaggregative hemorrhagic *E. coli* (EAHEC) carrying both the STEC and EAEC associated genes (Fratamico *et al.*, 2016), such hybrid was reported by Frank *et al.* (2011) as EAHEC serotype 0104:H4 causing 54 deaths and over 3800 illness in an outbreak, when an EAEC was demonstrated to have acquired a phage carrying the shiga toxin gene of the STEC.

2.10.3 Pathogenic factors in diarrheal *E. coli*

ETEC has one or more enterotoxins; heat labile (LT) LT1 and LT2 or the heat stable (ST) as STa and STb. EPEC has proteins involved in attaching and effacement of the host cells microvilli. It has the EPEC adherence factor (EAF) plasmid and gene cluster encoding the bundle-forming pili (bfp) gene. Typical EPEC are those isolates with the EAF plasmid, whereas isolates with the bfp gene but lacking the EAF plasmid are classified as atypical EPEC. STEC, produces two shiga like toxins 1 and 2 encoded for by gene stx1 and stx2 which are potent cytotoxic (Vidal *et al.*, 2005).

EIEC possess a gene located in a virulence plasmid (PInv) 140 MDa encoding type III secretion system. EaggEC, adherence to HEP-2 cells has a self-agglutination pattern that is aggregative adherence (AA) fimbriae I and II (AAFI and II). AAFII is encoded in the PAA plasmid, gene aafII. DAEC, adhere to HEp-2 cells in a non-localized pattern, by detection of gene daa required to express the F1845 fimbriae (Vidal *et al.*, 2005).

2.10.4 Pathogenesis of *E. coli*

EPEC was first described by Bray in 1945 after an infant diarrhea outbreak in United Kingdom. The EPEC has the attaching and effacing (A/E) characteristic that enables the bacterial to intimately attach to the host's intestinal epithelial cells. This characteristic is encoded for in the locus of enterocyte effacement

(LEE). A 94 kDa protein, intimin is encoded by LEE, as an outer-membrane that enables the intimate attachment to the epithelial cells by EPEC (Kaper *et al.*, 2004).

There are other virulence factors that are encoded outside of the LEE. These include a 385 kDa lymphostatin (LifA), able to inhibit lymphocyte activation. The EPEC adherence factor (EAF) plasmid, found in typical EPEC, encode for the bundle-forming pilus (BFP). BFP causes interbacterial adherence and thus transfer of plasmids as well as adherence on epithelial cells. It is within this plasmid that the plasmid-encoded regulator is located, with the ability to regulate the *bfp* operon and other genes in the LEE. A typical EPEC have the LEE that does not contain the EAF plasmid (Trabulsi *et al.*, 2002).

Bacterial induced diarrhoea may be as a result of several concurrent mechanisms including active ion secretion and increased intestinal permeability. Intestinal inflammation and microvilli effacement, result to poor absorptive intestinal surface area (Kaper *et al.*, 2004). Enterohaemolytic *Escherichia coli* (EHEC) was first identified as a human pathogen in 1982. This strain causes haemorrhagic colitis, non-bloody diarrhea and haemolytic uremic syndrome (HUS). The bacteria has it's main reservoir in bovine intestine and outbreaks have been associated with consumption of undercooked hamburgers. Other food types have also been linked to EHEC, including beef sausages, unpasteurized milk, melon, apple juice, lettuce

and radish sprouts. The strain has very low infection dose of less than 100 cells and has been linked to untreated drinking water, recreational activities, farm and zoo visits. There are reports linking it to possible air-borne transmission (Varma *et al.*, 2003). The EHEC, has several strains of pathological importance, the 01570:H7 serotype being of chief importance. Other important serotypes include 026 and 0111 serogroups which happen to be more prominent than the former.

Key virulence factor in EHEC is called the verocytotoxin (VT), also Stx. It is made up of 5 identical B sub-units. The B sub-unit enables the holotoxin to attach to the host target cells. Stx also has a single A subunit, with the ability to cleave ribosomal RNA, stopping protein synthesis (Melton and O'Brien, 1998). The Stx contains Stx1 and Stx2 sub-groups with 55% homology. Stx has the ability to travel in the blood stream from the colony to the kidneys and destroy renal endothelial cells by direct toxicity and triggering inflammatory reactions through induction of cytokines and chemokines (Andreoli *et al.*, 2002). As a result, the patient suffers from HUS characterized by haemolytic anaemia, thrombocytopenia and possibilities of fatal acute renal failure. Stx has also been shown to trigger intestinal epithelial cells apoptosis (Jones, 2002). For strains to qualify to be EHEC and not shiga toxin-producing *E. coli* (STEC), they possess the LEE pathogenicity island encoding for type III secretion system plus effector proteins. These are homologous for those of the EPEC.

ETEC is known to be the cause of watery diarrhea and is incriminated with travelers' diarrhea (Nataro and Kaper, 1998). The type is of pathological importance in animals as well, but have colonization factors K88 and K99 that lack in human strains of ETEC. Colonization by ETEC is enabled by more than one proteinaceous fimbrial colonization factors (CFs) as colonization Factor Antigen (CFA), Coli surface antigen (CSA) or putative colonization factor (PCF). Most human ETEC have been demonstrated to have the CFA/I, CFA/II and CFA/IV (Wolf, 2002). ETEC have two groups of enterotoxins—namely, heat labile enterotoxins (LTs) and heat-stable enterotoxins (STs). The two may be expressed together by a single strain or separately. The LTs are functionally and structurally closely related to the cholera enterotoxin (CT). LTs are able to stimulate prostaglandin synthesis as well as the enteric nervous system, both of which results in stimulation of secretion and inhibition of absorption, manifesting as diarrhea (Kaper *et al.*, 2004).

LTs have also found use as mucosa adjuvant, triggering increased antibody response when incorporated in vaccines administered orally, nasally and transdermally (Kaper *et al.*, 2004). On the other hand the STs are made up of small single-peptide toxins of 2 unrelated sub-classes namely, STa and STb. The sub-classes are different from others in structure and mechanism of action. Sub-class STa is the only one so far associated with human disease (Nataro and Kaper, 1998). STa has been reported to suppress proliferation of colon cancer cells

through a guanylyl cyclase C-mediated signaling cascade. Population with high prevalence of ETEC has reported very low occurrence of colon cancer, a scenario of developing countries (Pitari *et al.*, 2003).

EAEC cause persistent diarrhoea in both adults and children. These are *E. coli* strains lacking the heat-labile enterotoxins as well as the heat-stable enterotoxin but are able to adhere to HEP-2 cells in an auto-aggregative pattern (Kaper *et al.*, 2004). EAEC infection is by colonization of intestinal mucosa of the colon, secretion of enterotoxins and cytotoxins. An EAEC flagellin protein is involved in inflammation reactions by including release of IL-8. Interleukine 8 will intern stimulate release of neutrophils and migration across epithelium, tissue damage and secretion of fluids hence persistent diarrhea (Steiner *et al.*, 2000).

2.10.5 Entero-invasive *E. coli* (EIEC)

The EIEC strain has been defined as one that is closely similar to *Shigella spp.* in their biochemical, genetical and pathological characteristics (Pupo *et al.*, 2000). Though pathogenic species of *Shigella* cause dysentery characterized by fever, abdominal cramps, diarrhea with blood and mucous, EIEC has been shown to cause invasive inflammatory colitis with occasional dysentery. The two bacteria however share the essential virulence factors. The organisms have been reported to cause macrophage apoptosis after infecting them. This pathogenicity is carried by the 213kb virulence plasmid (Sansonettti, 2002).

2.10.6 Diffusely adherent *E. coli* (DAEC)

The DAEC pattern of adherence to HEP-2 cells monolayers is used to characterize them. They also cause diarrhea in less than one year old children (Scaletsky *et al.*, 2002). Most of the DAEC (75%) produce a protein, fimbrial adhesion F1845 that protects them from the hosts complement system. DAEC strains develop a long cellular extension that is used to wrap around adherent bacterial cell (Bernet-Camard, 1996).

2.10.7 Uropathogenic *E. Coli* (UPEC)

Escherichia coli are one of the infectious agents affecting the urinary tract. Uropathogenic strains of *E. coli* show large and small pathogenicity islands with genes absent in the chromosome of faecal strains (Welch *et al.*, 2002). The strain has ability to infect immune competent hosts, starting with colonization of the bowel, then the peri-urethral ascending the urethra to the bladder. In the bladder, urinary tract infectious (UTI) *E. coli* expresses type1 fimbriae. This enables attachment to the epithermal cells via mannose moieties of uroplakin receptors (Kaper *et al.*, 2004). This results to apoptosis and exfoliation. Cystitis cause strains to continually express type 1 fimbriae and are confined to the bladder (Kaper *et al.*, 2004). Pyelonephritis strains have less expressed type 1 fimbriae thus releasing the bacteria from the bladder epithelium. The bacteria then ascends the ureters to the kidneys. The kidney epithermal cells expresses digalactoside

receptors which allow bacteria attachment via the P-fimbriae (Korhonen *et al.*, 1986). Through hemolysis renal epithelium is destroyed.

2.10.8 Meningitis sepsis associated *E. coli* (MNEC)

The MNEC pathotype is incriminated for the Gram-negative neonatal meningitis. Infections with MNEC result in up to 40% deaths or severe neurological defects to survivors (Dawson *et al.*, 1999). Meningitis causing strains are haematogenously spread with a bacteraemia of more than 10^3 colony-forming units per ml of blood required to cause disease. The translocation from blood to the central nervous system is a transcytosis process causing no damage to the blood-brain barrier (Stins *et al.*, 2001).

2.11 *Campylobacter* spp

Campylobacter spp are microaerophilic and appear under light microscopy as very small, curved, thin, Gram-negative rods, 1.5-5µm, with corkscrew motility joining to form zigzag shapes. Colonies on agar are round to irregular with smooth edges. They can show thick translucent white growth to spreading, film-like transparent growth (Nachamkin *et al.*, 1992; Holt *et al.*, 1994).

Campylobacters are established in the intestinal tract of wild and domestic animals, particularly in birds, asymptotically as temporal carriers but causing illness in humans (Nachamkin *et al.*, 1992). The bacteria can survive up to five

months at -20°C but dies off in a few days at room temperature (Blaser *et al.*, 1980; Fricker and Park, 1989; Castillo and Escartin, 1994). They are vulnerable to air exposure, drying, low pH and heating (Nachamkin *et al.*, 1992). Three species namely; *C. jejuni*, *C. coli* and *C. lari* account for 99% of human *Campylobacter spp* isolates, with *C. jejuni* contributing 90% of the isolates. *Campylobacter fetus* and *C. upsaliensis* have also been isolated in humans (Klein, *et al.*, 1986; Patton, *et al.*, 1989; Linton, *et al.*, 1996).

Campylobacter spp are a leading cause of enteric illness (Scallan *et al.*, 2011; Havelaar *et al.*, 2012) manifesting as mild to severe diarrhea with watery loose stool that is most of the times followed by bloody diarrhea (Nachamkin, *et al.*, 1992). Infections also manifest as meningitis, pneumonia, miscarriage, severe form of Guillain Barre syndrome (GBS) and reactive arthritis (ReA) (Blaser, *et al.*, 1986; Nachamkin, *et al.*, 1992).

The major sequelae for *Campylobacter spp* are, Guillain-Barre syndrome (GBS), reactive arthritis (ReA) and irritable bowel syndrome (IBS) (WHO, 2013). This has been reported in high income countries, incriminating *Campylobacter* infections compared to other enteropathogenic bacteria including *Escherichia coli* and *Salmonella spp*. The situation is however different in the low income countries where the actual cause of gastroenteritis diarrhea is either underreported or unknown (WHO, 2013). The disability adjusted life year (DALYs) for *Campylobacter* in

certain parts of the world has been estimated as 9.3 per 1000 person-years in the united Kingdom between the years 2008-2009, 4.4 per 1000 in the USA and 5.8 per 1000 person-years in the Netherlands for the year 2009 (Scallan *et al.*, 2011; Havelaar *et al.*, 2013).

It has not been very effective to distinguish between *Campylobacter* species using phenotypic methods, however, genotypic methods have been developed capable of distinguishing the *Campylobacter* species. This has enabled more elaborate epidemiological understanding of *Campylobacteriosis*, thus identification of their source and route of infection (Wassenaar and Newell, 2000; Frost, 2001). Several writers have reported on the description of oligonucleotide primer pairs capable of distinguishing between *Campylobacter spp.* The use of multiplex PCR methods have resulted to cheap, rapid and sensitive genetically identification procedures (Klena *et al.*, 2004).

2.11.1 Antimicrobial resistance in *Campylobacter*

It was not until recently that *Campylobacter spp.* was shown to be resistance to various antibiotics, before that the bacteria was considered susceptible (Abdittachesoo *et al.*, 2014). Tetracycline among others is one of the antimicrobial agents which *Campylobacter spp.* have showed resistance to. Tetracycline being broad-spectrum has activities against both Grams positive and negative organisms, thus acting by protein inhibition. Resistance against its activity is well documented

and transferable both vertically and horizontally across same or dissimilar bacterial species. This is made possible through mobile components, including plasmids and transposons by conjugation (Roberts, 2005). It is via these components that tetracycline resistance (*tet*) genes are transferred to previously non-resistance bacteria recipients. The transfer takes place even between human and animal bacteria isolates (Robert, 2005).

In *Campylobacter spp*, tetracycline resistance has been reported to have been mediated by more than one (*tet*) genes. The *tet* (O) gene is the ribosomal protection protein, and plays the primary part in tetracycline resistance (Chopra, 2001) in *C. jejuni* and *C. coli* (Mazi *et al*, 2008). This is transferred as a plasmid encoded gene (Gibreel, 2002) or better still in a non-self-mobile form when transferred through the chromosome (Chopra, 2001). Similarly, the *tet* (S) gene is a ribosomal protein gene that is transferable through the same channels like the *tet* (O) gene.

The *tet* (A) and *tet* (B) are the efflux genes encoding for the 46kDa membrane bound efflux protein. This carries tetracycline from the cell membrane and its first known resistance role in *Campylobacter* was reported by Abdi-Hachesso *et al*. (2014).

2.11.2 Fluoroquinolone resistant *Campylobacter*

Campylobacter quinolone resistance is mainly due to single point mutation in the quinolone resistance determination region of *gyrA* gene (QRDF) (EFSA, 2009; Nguyen *et al.*, 2016), at amino acid 86 by replacement of threonine (Thr) by isoleucine (Ile) (Piddock *et al.*, 2003). Occasionally, mutation in topoisomerase IV (ParC) results to resistance against quinolones. Other amino acids substitutions have been reported by Paddock *et al.* (2003) and others as pro-104 + Ser, Thr-86 to Lys, Ala 70 to Thr, Asp 90 to Asn and transitions codon 119 though the role of fluoroquinolone resistance has not been shown in the mutations (Ruiz *et al.*, 1998; Zirnstein *et al.*, 1999; Bachoural, 2001; Hakanen *et al.*, 2002). In *Campylobacter*, there has been no documented change in relation to quinolones resistances of the *gyrB* sub unit gene (Changkwanyeeun, 2016). Piddock *et al.* (2003) noted that *Campylobacter* resistance to Ciprofloxacin is not only mediated by mutations on the *gyrA* gene though it remains the most frequent.

2.12 Viral pathogens

Viruses are obligate intracellular parasites that multiply only when in their host cells. They are minute, measuring 20-300nm. They consist of nucleic acid covered by a protein coat. Enveloped viruses have an additional lipoprotein outer layer. When outside host cell they remain dormant but some retain infectious ability for a period of time in the environment. The ability of a viral particle to survive outside host cells and remain infectious is of public health importance. Such an infectious

particle can be transmitted through direct contact, indirectly by a vector or through vehicle transmission (by droplets, aerosol, fecal-oral route, food, water amongst others).

Persistence of virus in the environment depends on biological, chemical and physical factors. The viral particle composition and type also play a role in its survival. In different environmental matrices, the survival of viruses is influenced by specific factors. In liquid environmental matrices, factors important include the microbial antagonism, chemicals in the liquid, ionic strength and temperature. When found on surface, virus survival is influenced by temperature, sunlight, moisture, relative humidity, antiviral chemicals and biological agents. In an experimental setup, Margri *et al.* (2015) demonstrated faster inactivation of Adenovirus at higher temperature (33°C) and higher ammonia concentration (250mM). A combination of ammonia and alkaline pH (9.5) gave a faster viral inactivation. Temperature range of 4-33°C was on the other hand shown to have no pronounced inactivation of adenovirus but increased by a factor 7 when 1.5% urea was incorporated in the treatment. This observation agreed with other writers who reported different heat stability levels with different viruses (Sobsey and Meschke, 2003).

2.12.1 Human Adenovirus

Human adenovirus was first isolated in 1953 in adenoidal tissue. Human Adenoviruses (HAdVs) belong to the genus Mastadenovirus, with seven known species (subgroups) HAdVs-A to HAdVs-G. They are non-enveloped double-standard DNA viruses infecting several human tissues. Different subgroups are capable of causing different human infections; with subgroups F and G, known for gastrointestinal infections, subgroup E, with Keratoconjunctivitis and respiratory infections. Other subgroups have multiple infections including, urinary, respiration and gastrointestinal. HAdVs B type 2, 14 have been associated with fatal respiratory outbreak in a military base (Ghebremedhis, 2014).

2.12.2 Immune response to adenovirus

Immune response depends on several factors including the immune status of the host and the virus serotype. The virus has developed immune evasion mechanisms, for instance, viral infected cells do not undergo apoptosis expression of Class I MHC is inhibited. The virus also inhibits cellular response to interferons and tumor necrosis factor (TNF) (Gooding, 1990; Mahr, 1999). The alpha component of the viral capsid is able to provoke antibody production against all mammalian adenoviruses. These antibodies are found in the upper respiratory tract in 3 days and in serum at the 7th day after infection. The specific antibodies linger in host circulatory system up to 10 years after the primary infection. For this reason, re-

infection with similar serotype has not been common (Ghebremechin, 2014).

2.12.3 Rotavirus

Rotavirus causes gastrointestinal disease, pre-dominantly in children under 5 years and other young mammals. It accounts for annual deaths of approximately 300 children globally (Parashar, 2003). The rotavirus diarrhea is not very well understood and several mechanisms have been put forth; encoded toxin, enteric destruction resulting to malabsorption, enteric nervous stimulation and ischemia of villus.

Rotavirus infection is not restricted to the intestines but do spread to extra-intestinal sites (Ramig, 2004). Rotavirus is in the family Reoviridae, they have no envelop and are made of a three layers of capsid surrounding 11 segments of double stranded RNA. The genus is divided into 5 serogroups namely: A, B, C, D and E. Human infections are by serogroups A, B, and C while serogroups D and E infect animals (Ramig, 2004).

2.12.4 Viral inactivation

Several viruses including polioviruses, coxsackie and reoviruses have been studied on ammonia viricidal effects at 21⁰C. Rapid reduction of less than 5log₁₀ in 24 hours at pH 9.5, 290 mM NH₃ is in record. The exception is the dsRNA reovirus,

which shows approximately 2 log₁₀ reduction in 24 hours. There is a need to carry out more studies on inactivation of virus as well as their presence in human excreta, including dsRNA viruses, like the rotavirus and dsDNA viruses, like adenoviruses which have shown more resistance to ammonia as opposed to the more sensitive single stranded viruses (Cramer *et al.*, 1983).

Rotavirus has been demonstrated in water contaminated with human faecal samples in the study area (Kiulia *et al.*, 2010). However, no work has been carried out to investigate the effects of urea treatment on the virus in the management of human excreta and fertilizer production.

2.13 Protozoa infections

Cryptosporidium spp are protozoa and their oocysts are considered to be more tolerant to disinfection. However, temperature and ammonia have been used to effectively inactivate *Cryptosporidium* in biowastes (Finstein, 2004). Jenkins *et al.* (1998) demonstrated an 80% reduction of viable oocysts using 148 mM NH₃ in 24 hours and achieved 5log₁₀ inactivation in approximate 6 days.

However, inactivation of *Cryptosporidium* oocysts in human excreta using urea treatment has not been reported in a field setup. Other protozoa including *Entamoeba histolytica* and *Entamoeba coli* have been shown to be well inactivated

by ammonia at 3% concentration (Gordeeva and Chefranova, 1977), but not in human waste management and fertilizer production process.

2.13.1 Cryptosporidium

Cryptosporidium is a common protozoa in human, that cause intestinal infections. Infection with *Cryptosporidium parvum* presents as watery diarrhea, abdominal pain and malabsorption for several months. Microscopy as a diagnostic or detection tool for protozoa infections has been the gold standard test. However, unlike with worm eggs, protozoa microscopy only gives 60% sensitivity (Koffi *et al.*, 2014). Cryptosporidiosis is a zoonotic infection affecting both man and animals. Oocysts are 4-6µm. It causes self-limiting diarrhea in immunocompetent individuals and intractable diarrhea in immunocompromised patients. A prevalence of 30% has been reported in low income countries. Outbreaks have been reported as waterborne infection and hospital or day-care acquired (Ungar, 1990).

2.13.2 Amoebiasis

Amongst the several species of the genus *Entamoeba* infecting humans only *Entamoeba histolytica* is pathogenic, causing amoebic dysentery. The name, *Entamoeba histolytica* is derived from the latin words, 'histo' meaning tissue and 'lytica' for destruction as they secrete histolysin able to digest the gut of infected

person (Stanley, 2003). It is transmitted through the fecal-oral route as well as through the housefly as a mechanical vector.

Lack of proper sanitation is a global problem and has been one of the risk factors in amoebiasis, most affected being the children. WHO (2013) report indicated that amoebiasis caused by *E. histolytica* accounts for a third of 100,000 parasite caused mortalities globally, hence being one of the major public health problems in the tropics. The number of people that were ill by intestinal parasites was estimated to be 450 million in 2001, amoebiasis being one of the major infections (WHO, 2001).

Kenya is a tropical country with a large urban population, most of who have poor access to clean sufficient water and sanitary facilities. The situation is made worse in informal settlements in major towns with only 43% of the population having access to clean water and proper sanitation (Kavili *et al.*, 2016). This situation is not any better in ASAL parts of rural Kenya, which has 15% latrine coverage with poor supply of clean portable water as reported by Kauli *et al.* (2016).

2.14 Geohelminths

Like other human intestinal parasites, soil transmitted helminths (STH) prevalence have a strong association with the social economic status of a population. They have been shown to cause under nutrition and cognitive impairment (Brokers,

2000). The common STH are the round worms (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*) and hookworm (*Ancylostoma duodenale* and *Necator americanus*) (Horton, 2003). The global burden of diseases due to STH is estimated at 39 million disability adjusted life years.

One or multiple STH will be found to infect more than a billion individuals worldwide. Infection with STH is as a result of ingesting eggs in contaminated soil or foods. The infective larval of hookworm penetrates the skin of human host when in contact with infected soil (Horton, 2003).

Preventive therapy has been endorsed (WHO, 2001) as a global strategy to control STH and Schistosomiasis diseases. It involves the regular administration of efficacious anthelmintic to the target population, with an aim of reaching 100% school-aged children and at least 75% of the population at risk. A drawback with such a strategy is that reinfections still occur at varying frequencies (Ziegelbauer *et al.*, 2012). To address this, integrated control approaches have shown lots of success in China (Wang, 2009), through interrupting transmission leading to local elimination of the parasitic infections. Ziegelbauer (2012) in a systematic review and meta-analysis to assess the effect of sanitation, concluded that sanitation was associated with reduced risk of transmission of helminthiasis in humans. Cure alone has been stated as of no use as one can almost immediately pick an infection. Emphasis must therefore be put on practices that prevent soil contamination with

STHs (Horton, 2003). This is achievable through the integrated control practices including preventive chemotherapy and education through campaigns, improved basic sanitation and access to safe clean water (Ziegelbauer, 2012). *Ascaris* eggs have a complex egg wall made up of four layers, each of these layers are resistant to inactivation and are amongst the most persistent infectious pathogens (Wharton, 1980; Ghiglietti *et al.*, 1995). Though inactivation patterns for *Ascaris* eggs have shown variations within studies, it is evident that a temperature combination with NH_3 plays a crucial role in ammonia sanitisation (Nordin, 2010). Inactivation of *Ascaris* eggs is regarded as a model that can be applied for parasite inactivation using the ammonia sanitisation technique, being resistant and of concern to human health in the study area. *Trichuris spp* causes an infection considered to be very persistent, though not as much as *Ascaris* eggs (Ghiglietti *et al.*, 1995).

2.15 Antimicrobial resistant

Christabel *et al.* (2012) reported 40% multiple antibiotic resistant environmental enterobacteria isolates from Kibera, Kenya suggesting possibilities of drug misuse among the population. The author also demonstrated ability of the isolates to transmit resistance to susceptible bacteria sharing the environment. Antibiotic resistance patterns is a bacteria source tracking (BST) tool (Mohapatra *et al.*, 2007), and its applicability in the proposed study will be used along other robust methods.

2.15.1 *Escherichia coli* antimicrobial resistance

Escherichia coli are one of the bacteria pathogens of international concern on antibacterial resistance according to WHO report (2014). It can develop resistance through different ways but in most of the times through mutation resulting in fluoroquinolone resistance. The bacteria can also acquire mobile genetic elements from plasmids, transposons, integrons and gene cassettes resulting in resistance to penicillin's (ampicillin or amoxicillin) as well as to third generation cephalosporin's.

Resistance to cephalosporins has been shown to be caused by extended spectrum beta-lactamases (ESBLs), enzymes which destroys beta – lactam antibiotics. Bacterial species have the ability to transfer and acquire ESBLs, causing them to be resistance to Beta-lactams and have demonstrated resistance to other several antibiotics. There have been reports of carbapenems resistance which is the treatment option available for ESBLs producing bacteria. In *E. coli*, this resistance is caused by metallo-beta lactamase enzymes making the bacteria resistance to all available beta-lactam antibiotics. Use of carbapenems, a broad spectrum therapy for severe *E. coli* infections is the alternative with third generation cephalosporins resistant bacteria. The implications are that the cost of treatment goes higher and possibilities of winding resistance to carbapenems cannot be taken for granted (Downie *et al.*, 2013).

Urinary tract infections is mostly caused by quinolone resistant *E. coli*, following treatment with the common oral quinolone. Use of the more expensive injectable drugs will continue to push the cost of treatment higher, thus consuming more health care incomes (WHO, 2014).

2.15.2 *Salmonella* antimicrobial resistance

Salmonella is also a pathogen of international concern with regard to antibacterial resistance as reported by WHO (2014). It is a zoonotic infection transmitted through contaminated water and foods of animal origin; partially undercooked meat, milk and eggs. Non-typhoidal *Salmonella* (NTS) are known to be one of the causative agents of food-transmitted diarrhea.

Antimicrobial resistance in NTS is on the increase globally with the emergence of multi-drug resistant (MRD) *Salmonella*. Horizontal transfer of resistance encoding genomic elements has enabled spread of MDR among different *Salmonella* Serotypes (WHO, 2014). There is a great similarity between antibiotics used by humans and food producing animals. This has contributed to the risk of human infection with antibiotic resistant pathogens most notable NTS, *E. coli* and *Campylobacter spp.* Animals happen to act as reservoirs of these human pathogens with high possibility of resistance transfer to humans of which the magnitude is unknown (EFSA/ ECDC, 2012).

Instead of antibiotics being used in food animals for treatment and prophylaxis they are used in mass, as growth promoters. These are applied in healthy animals and in large quantities. However, their use as growth promoters has been forbidden in some EU countries since 2006. Antimicrobials are also used for plant protection (EFSA, 2008). Development of resistance in human pathogens found in food animals or even companion animals need to be clearly monitored for clear points of intervention to be established (Nisi *et al.*, 2013).

2.16 Pathogens internalization through plants roots and colonization in tissues

Salmonella spp and *E. coli* 0157:H7 has been demonstrated in intra-tissue of 33 days old lettuce planted in growth medium, 7 days post inoculation with 10^6 CFU/g (Erickson *et al.*, 2010; Ongeng *et al.*, 2015). Soil irrigated with contaminated water with *Salmonella spp* and *E. coli* using hydroponic system had the pathogens detected in leaves 18 days post-inoculation, with *E. coli* giving higher counts than *Salmonella spp* (Franz *et al.*, 2007). Ongeng *et al.* (2015) reported the ability of plant rhizosphere to support metabolism of *E. coli* during lettuces seed germination. The nutrients provided by seedlings during germination support bacterial multiplication and thus the explanation of survival in growing plants. Regardless of the bacteria introduction route to the plant such as through irrigation water, soil or manure, the bacteria has the ability to establish and colonize the plants rhizosphere with the ability to migrate in the plant system (Warrier *et al.*, 2003; Ongeng *et al.*, 2011; Ongeng *et al.*, 2015).

Plant rhizosphere has been described as being highly nutritious and supports diverse microbial types; survival of human pathogen in this environment is thus faced by fierce competition (Berg *et al.*, 2005). Organisms with the ability to withstand antibiotics survive well in the plants rhizosphere (Berg *et al.*, 2005), also those with the ability to utilize roots exudates as source of carbon (Yang *et al.*, 2001; Ongeng *et al.*, 2015).

Plant rhizosphere has been shown to be a gene exchange hot spot (Knudsen *et al.*, 1988), however, more work is needed to investigate possibilities of enteric pathogens acquiring antibiotic resistant genes in the rhizosphere (Ongeng *et al.*, 2015). Bacteria internalization is dependent on the type, age and health condition of the plant as well as the bacteria concentration exposed. While some plants such as lettuce, cabbage and radish allowed internalization others like spinach subjected to the same conditions and bacteria concentration had no indication of bacteria internalization (Jablasone *et al.*, 2005; Ongeng *et al.*, 2011). The ability of enteric pathogens to survive in the plant tissue and adopt effectively needs more study (Ongeng *et al.*, 2015).

2.17 Molecular characterization

2.17.1 Bacterial typing

Typing of bacterial strains is used in diagnosis of bacterial infections in all scientific disciplines to enable treatment. It is also important in epidemiological investigation (Yildirim *et al.*, 2011). Phenotypic and genotypic methods are used for bacteria identification to strain level. Development of bacterial antibiotic resistance, possibly genetically manipulated, could be used in bioterrorism (Yildirim *et al.*, 2004). Phenotyping bacterial for identification, involves the use of colonial morphological characters depicted in different bacterial culture media, biochemical tests, staining methods and antibiotic resistance tests. Phenotyping methods are limited, takes days and are not species specific (Li and Fournier, 2009). As a result, more robust genetic methods have been developed that can be used for bacterial profiling and fingerprint identification. These are broadly divided into three: DNA band pattern, hybridization and sequencing (Li and Fournier, 2009).

2.17.2 DNA Sequencing

DNA sequencing allows a collection of high data throughput. Over time, the cost of sequencing has drastically reduced by up to 100 fold. This is credited to the Human Genome Project (HGP). Cost effective sequencing platforms are now available making sequencing possible to individual investigators. This have evolved with it unique challenges, due to the large data generation there is need to

rethink on robust protocols and effective data analysis methods as well as having good experimental designs. This end, next-generation sequencing would provide answers in higher data throughput, better genome analysis and availability to routine users at affordable cost. Short-read sequencing requires to be mapped against known references. This has been made easy as a result of completion of the whole human genome and major model organisms (Shendure and Hanlee, 2008).

2.17.3 Sanger sequencing

The Sanger sequencing method has been in use since early 1990s. Developed by Fred Sanger in 1977, it is known as dideoxy method. It is a capillary based semi-automated procedure involving random DNA fragmentation followed by cloning of the fragments into high copy number plasmids. *Escherichia coli* is then transformed and amplified with colonies bearing plasmid DNA insert. On the other hand, if the interest was targeted sequence, then a PCR is first done using specific primers flanking the target. The resulting PCR product has several amplicons and they are thus amplified. In both cases, these are then taken through a cycle sequencing reaction similar to a PCR, with template denaturation, prime annealing and extension cycles. During sequencing, the primers used flank the region of interest and is thus complementary to a known region.

The difference to a conventional PCR is that every primer extension is terminated by fluorescent labeled dideoxynucleotides (ddNTPs). The extension products will

thus be labelled with a terminating ddNTP identical to the terminal position of its corresponding nucleotide. The resulting sequencing product is then resolved in a high-resolution capillary polymer gel able to separate a single stranded fragment with a fluorescent label. As the four colours fluorescent (the labeled fragments) migrate out of the electrophoresis capillary they cause a laser excitation that is detected as readout in the Sanger sequences trace. These are software translated to DNA sequences. The Sanger sequencing is able to achieve approximately 1000bp read-lengths with a per-base accuracies of 99.999% (Shendure and Hanlee, 2008).

Second generation DNA sequencing (SGDs) that use cyclic-array strategies is a commercial products that including, the 454 genome sequencers by Roche applied science, Basel solexa technology in the illumine (San Diego), Genome Analyzer under the Solid platform (Applied Biosystems), the Polonator (Dover/Harvard) and the Heliscope single molecular sequencer technology (Helico-Cambridge, MA, USA) (Shendure and Hanlee, 2008).

Like the Sanger method, first DNA is randomly fragmented to prepare DNA library, this is achieved by use of restriction enzymes or by mechanical methods. Common adapter sequences are then ligated *in vitro*. Polony array is then generated by use of bridge PCR or emulsion PCR, where PCR amplicon are clustered on a planar singly, or onto the micro-scale beads surface in bridge and emulsion PCR respectively. Sequencing is enzyme driven by polymerase or ligase

of primed templates (Shendure, 2005; Turcati *et al.*, 2008). At each synthesis cycle, data is acquired by imaging of fluorescently labelled polymerase incorporated nucleotide.

The Next generation sequencing (NGs) has advantages over the Sanger technologies in that libraries are prepared *in vitro* and clonal expansion is also *in vitro* circumventing the use of *in vivo Escherichia coli* transformation and colony picking. It results to higher degree of parallelism and compared to capillary based sequencing resulting to millions of sequence reads. The next generation sequencing (NGs) also use very little volume of reactants and thus translating to low DNA sequencing costs. However, SGDs also known as NGs has the disadvantage of resulting to very short-reads with a 10 fold less accuracy compared to Sanger sequencing (Shendure and Hanler, 2008)

Illumina sequencing happens to be the most popular among the NGs platform. It is a synthetic sequencing with fluorescent reversible terminator technology. In the reverse termination, all four bases are labelled with a different fluorescent dye. After every sequencing cycle every base has a terminator group cleaved off. A flow cell is coated with a lawn of oligo pairs and single DNA libraries hybridized to primer lawn and the bound libraries extended by polymerase. These results to double stranded DNA molecules which are denatured and the original DNA template washed away leaving the newly synthesized strand attached covalently to

the oligo on the lawn. The single stranded molecules, then flips over to the adjacent complementary primer making a bridge by hybridizing. The extension by polymerases forms double stranded bridge. The double stranded bridge is then denatured resulting into two single strands templates covalently bound to the flow cell. The flip over, extension and denaturation cycle is repeated as the bridge amplification cycle forming multiple bridges. The dsDNA is finally denatured and the reverse strands cleaved and washed away. Forward DNA strands are left attached to the flow-cell; these are blocked to prevent unwanted DNA priming. Sequencing primers are then hybridized to adaptor sequence, and sequencing is achieved by synthesis, forming tens of millions clusters per flow-cell lane (Shendure and Hanlel, 2008). Detection sequences is real time during elongation and will thus not require the use of electrophoresis (Ahmedian and Hober, 2006).

Advantage of Sanger sequencing over next generation sequencing (NGs) is that it gives longer reads of 1000bp as opposed to NGs that results to 250bp. The conventional, Sanger method is also accurate at 99.99% which NGs having an accuracy of 85%. The cost of sequencing a mega-base, ranges from 60 US dollars to 1 US dollar, depending on which NGs platform one is using. The 454 emulsion PCR being the highest at \$60 while Heliscope single molecule and polonator emulsion PCR costing the least (Wood *et al.*, 2007).

2.17.4 Basic local alignment search tool

Basic local alignment search tool (BLAST) is a sequencing algorithm, a self-contained step by step procedure. It is to be performed in bioinformatics to compare information obtained from primary biological sequences or in nucleotides sequences. When a BLAST search is undertaken one is able to compare a query sequence with a known sequence. Known sequences are found in a sequences database allowing one to make determination on the level of resemblance. The BLAST algorithm and its program were designed in 1990 at the National Institute of Health (Altschul *at el.*, 1990).

2.17.5 Genotyping protocol

The typical genotyping protocol involves three broad steps starting with target amplification method, most popular being PCR amongst others like the strand displacement amplification. This is then followed by primer extension meant to discriminate alleles. This step can also be achieved by pyrosequencing, structure specific cleavage or hybridization. Lastly, the allele specific products are identified by fluorescence mass spectrophotometry, electrophoresis or microarray (Chen and Sullivan, 2003).

2.18 Gaps in knowledge addressed in this study

The driving factors for this study are informed by the sustainable development goal (SDG) number six of the United Nations (2015) that is seeking for ways to

address the current sanitation and hygiene inadequacy in the next 15 years. Most notable being the course of open defecation and with particular emphasis to the most vulnerable situations involving women and children. To this end, this study sought to evaluate alternative toileting in the name of Peepoo.

Peepoo bag as off-site human excreta handling and disposal technology is aimed at replacing the 'flying toilet'. The flying toilet is a mode of handling human excreta common in the slums, where one places his/her excreta in a plastic bag which is then closed and thrown the furthest one could (UNDP, 2006). The bags end up on roof tops, on the paths or roads as well as on the drainage systems. This results in accumulation of human waste in the environment, contaminating the same with enteric pathogens which end up in the food-chain and drinking water systems in the slums. Enteric infections remain high throughout the year, with the vulnerable groups being the most affected (Davis *et al.*, 2014).

This study sought to address a number of knowledge gaps including; the ability of the Peepoo bag to contain and effectively destroy enteric pathogens found in human excreta in the study area, the possibility of using the Peepoo treated human excreta as a fertilizer without exposing both the user and the consumer to human transmitted enteric infections. In addressing the mentioned gaps the study also aimed at understanding the current risks faced by the population due to lack of adequate sanitation. These were addressed by investigating the presence of

intestinal parasites infecting asymptomatic school going children. Also under investigation are pathogenic bacteria and viruses including; pathogenic *Escherichia coli*, *Salmonella spp*, *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, Rotavirus and Adenovirus. The prevalence of each of these has been established in the current study, their drug resistant patterns, and presence of antibiotic resistance encoding genes established. This information formed a baseline upon which the sanitization effectiveness of Peepoo bag was validated over a period of 10 weeks. The physical chemical conditions necessary for the Peepoo bag to effectively sanitize human excreta to the WHO (2006) recommendation for reuse as a fertilizer was established.

It is therefore the hope of the study that use of the Peepoo bag will immediately be implemented in the study area and in other similar situations as an off-set toilet that will allow reuse of excreta as fertilizer. This will address issues of human waste management, reduced environmental contamination, reduction in disease transmission, improved human dignity as we bring to an end open defecation and improve the public health.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The study was carried out in primary schools located at Kibera informal settlement, Nairobi County, Kenya. Kibera is located at an altitude of 1670m above sea level, between longitude 36⁰, 46' 59.999" East and latitude 1⁰ 19' 0.0001" South and it is about 140 Km South of Equator. It is 5 Km South of Nairobi Central Business District (CBD), the Capital of Kenya. Kibera is divided into 9 official villages namely; Mashimoni, Silanga, Laini Saba, Makini, Kianda, Soweto, Kisumu Ndogo, Gatwekera and Lindi. The average living place is 3 m², with an average of 5 persons per place. This is the Peepoople Kenya project site and the project has a working relationship with the community. Limited use of Peepoo has been going on since 2010 at Silanga village. The study site presents a population with diverse enteric infections (Karanja, 2002) thus ideal for testing Peepoo effective treatment of human faeces for reuse as fertilizer.



Figure 3.1: A map of the study area showing the map of Kenya, Nairobi city and extrapolated Kibera slum. The administrative villages where the sampled private primary schools are located

Source: <https://maxks-squatter-settlement.wikispaces.com/Case+Study-Kibera>
<http://mapkiberaproject.yolasite.com/maps-and-statistics.php>

3.2 Study population

The current study was conducted in private primary schools in Kibera slum in Nairobi, Kenya. This study formed part of a bigger study working with schools allocated to one of the four arms using stratified randomization technique. Three out of the four arms, had different interventions (peepoo, deworming,

peepoo+deworming) with the fourth being the control schools. This study was undertaken with the peepoo arm schools, made up of 11 primary school each with an average of 118 pupils. Samples were randomly obtained, with relative numbers proportionate to school population. The schools were distributed in 5 administrative villages namely; Lindi, Silanga, Laini Saba, Gatwekera and Mashimoni (Figure 3.1; Table 3.1).

Table 3.1: Details of sampled primary schools

School ID	GPS coordinates	Village	School population	Samples collected
A	-1.311110807, 36.79168948	Mashimoni	189	59
B	-1.311242166, 36.79197589	Laini Saba	120	38
C	-1.316991284, 36.79943976	Silanga	143	62
D	-1.309617466, 36.79378727	Laini Saba	170	44
E	-1.319225821, 36.7954909	Silanga	175	79
F	-1.314113432, 36.78119856	Gatwekera	35	19
G	-1.311645845, 36.79295297	Laini Saba	150	80
H	-1.314686804, 36.79094518	Lindi	128	53
I	-1.311645845, 36.79295297	Lindi	79	30
J	-1.314934698, 36.79072262	Lindi	181	69
K	-1.31681813, 36.80007032	Silanga	111	47

3.2.1 Schools distribution on Google map and topography of sampled schools

The sampled schools were identified with alphabetical letters A to J. Their true identities were confidentially kept to avoid negative publicity resulting from the outcome of this study. Using a mobile pad (AUIS Zenpad 7.0, China), GPS coordinates and photography of sampled school were obtained during sample collection. The GPS coordinates were used to generate a google map of the

sampled schools (Figure 3.2). In addition, photos were taken to document differences in school location in terms of drainage (Figure 3.3).

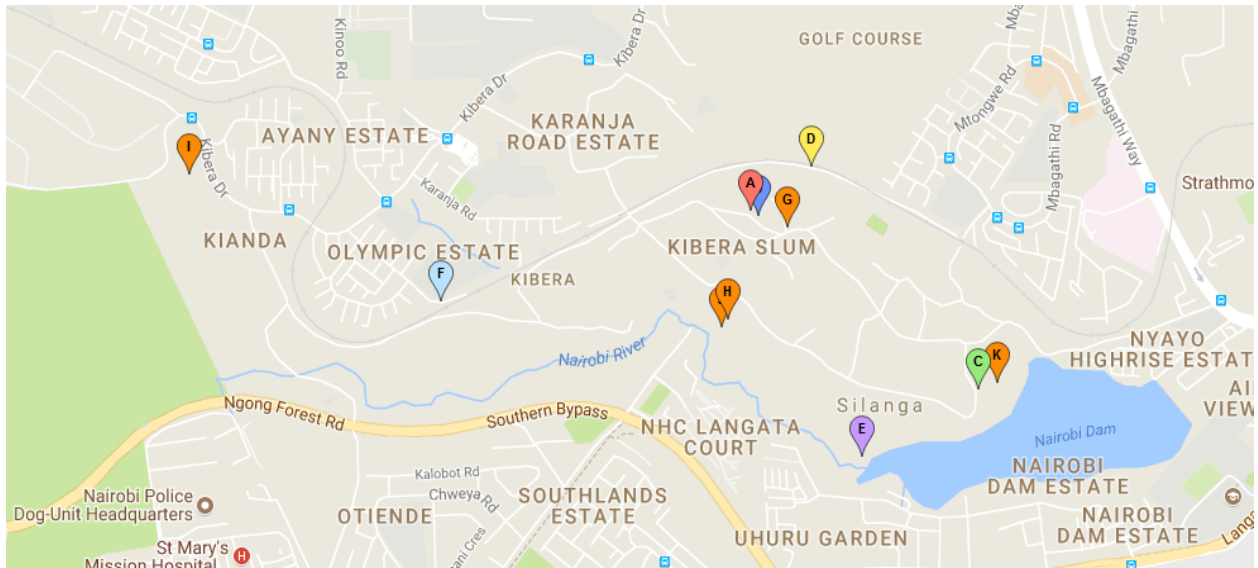


Figure 3.2: Google map showing positions of sampled schools.

The school positions were generated on Google Earth using GPS coordinated obtained during sampling.



Figure 3.3: School E, located on a poor drainage near Nairobi dam, exposing children to hazardous environmental contaminate as opposed to school B located on high ground with good drainage and clean environment

3.3 Sample size determination for the baseline study

Kibera is home to approximately 270,000 people with a population density of 77,000 persons/Km² (Olack *et al.*, 2014). Peepoo is used mostly in schools by 20,000 people as a sanitation solution (Peepoo Kenya, 2016). With known prevalence (40.7%) of soil transmitted helminths in school going children at urban informal settlement in Kenya (Davis *et al.*, 2014). The following formula was used to determine the desired minimum sample size (Martin *et al.*, 1998):

$$N = Z\alpha^2 pq / L^2,$$

N = desired minimum sample size,

Z α = 1.96 at 95%,

P = probability of occurrence 0.407,

q = 1-p,

d² = Desired confidence of 0.05

Where;

$N = 1.96^2 \times 0.407 \times 0.593 / (0.05)^2 = 370$ samples, adjusted to 580 samples to fit within the project requirement of the purpose of peepoo intervention program which was above the requirements of this study.

3.4 Study design

A cross sectional study was conducted to sample schools randomly allocated to the “peepoo intervention arm” by the intervention project that funded this study. This study was carried out in Kibera during the month of July 2015. Eleven schools

allocated to this arm were provided with Peepoo bags without urea on the first day of the sampling. The peepoo bags from study schools were collected, placed in cool boxes and transported to University of Nairobi for laboratory analysis to establish the baseline information for all the test parameters. Depending with the size of the school, 40 to 80 samples were analyzed and peepoo bags with urine were omitted. Five hundred and eighty samples were randomly collected and analyzed from both gender and different ages determined by class (pre-primary to class 6).

Four schools with the highest helminth infections were selected based on the baseline results. Peepoo bags with urea were either marked 'G' or 'B' for girl and boy respectively and distributed to the selected schools on the second week of sampling. The used peepoo bags were collected on daily basis for five days. One hundred used peepoos from each of the four selected schools were collected in plastic bags. In total, four hundred used peepoos from the four schools were placed into one sanitization storage bag (big bag) each day. The storage bags were marked week 2 storage, week 4 storage, week 6 storage, week 8 and week 10 and kept at ambient temperature. As a control, one plastic bag containing 100 untreated samples used in establishing the baseline was included and kept with the urea treated Peepoos. Automatic temperature loggers (Tinytag aquatic2, TG-4100, Chichester, UK) with internal storage were placed in sanitization storage bags.

A soil sample was collected in a sterile plastic bag from each school near the toilets or in a nearby wastewater dish. Water used for drinking in the participating schools was also sampled in a sterile container and transported to the laboratory in a cool box. Baseline analysis of all the samples collected (faeces, soil and water) was done on gastroenteric parasites including *Ascaris lumbricoides*, *Trichuri trichura*, *Hymoleptis nana*, *balantidium coli*, *Entamoeba histolytica* and *Entamoeba coli*, *Cryptosporidium spp*; Enterobacteriaceae including thermo-tolerant coliforms, *Escherichia coli*, *Salmonella spp*, *Proteus spp*; Campylobacter; including *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and enteroviruses; *Adenovirus* and *Rotavirus*. At the specified storage period, eight samples were randomly picked from each school comprising of four girls and four boys for viability test. Ten Control samples were analyzed alongside test samples, at all the testing periods (Figure 3.4).

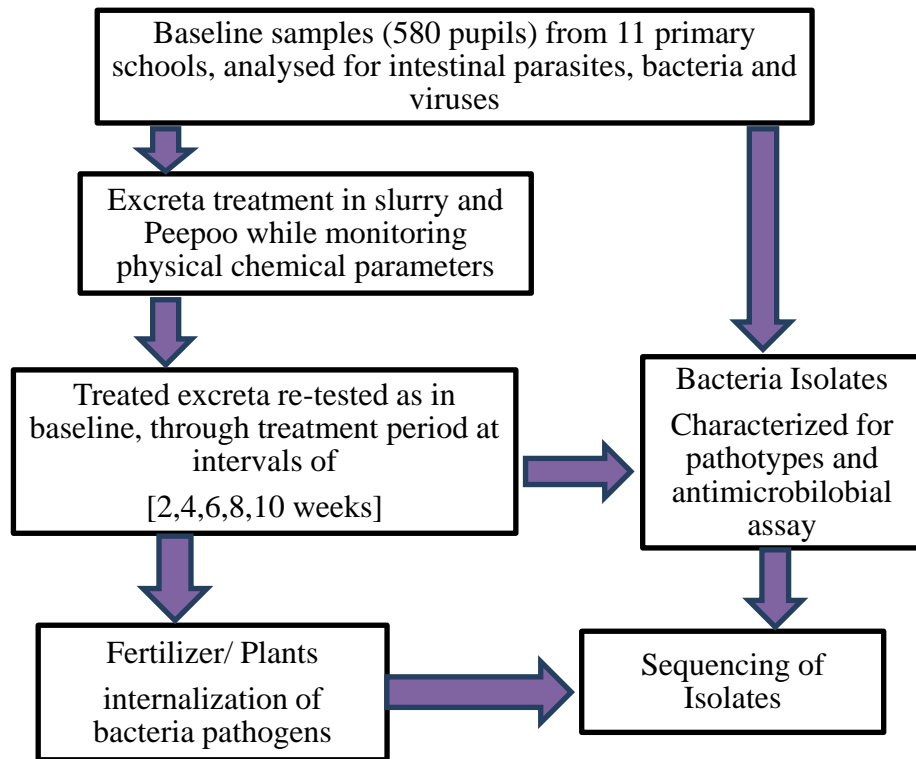


Figure 3.4: Flow chart showing the sequential study activities from sample collection to internalization and sequencing of bacteria isolates

3.5 Inclusion and exclusion criteria

Schools with both lower and upper primary sections, with a population of more than 30 pupils and not participating in any other related study, qualified for this study. Those schools with pupil-latrines ratio of (or lower) 30:1 for boys and 25:1 for girls and involved in an intervention program during the last 18 month prior to this study were excluded.

3.6 Modified McMaster methods for baseline analysis of fresh stool samples for enteroparasite identification and enumeration

Two grams of stool samples were weighed and 10 ml sodium acetate buffered formalin solution added to preserve the samples before analysis and increase sensitivity for protozoa identification. This was followed by either concentration for better eggs and cyst recovery according to Utzinger *et al.* (2010) with modifications. Briefly, using a tough depressor, a homogenous faecal suspension was made by mixing the faeces with the fluid thoroughly. The homogenous faecal suspension was filtered through a strainer [0.15 mm aperture] into a clean container. The filtrate was mixed and 15ml transferred into a centrifuge tube.

The suspension was then centrifuged at 1000 rpm for 2 minutes and the supernatant poured off. To the deposit, 5mls of 10% formal saline was added and mixed with swab stick or glass rod, after which 3ml of ether were added and shaken vigorously. The mixture was then centrifuged again, regulating acceleration so that 1000 rpm was attained in 2min. The fatty debris were loosened at interface with swab applicator stick and the supernatant poured off with debris, leaving a small deposit at the bottom of the tube. The deposit was suspended with the last drop of fluid and mounted as a sub-sample on a microscope slide, stained with Lugos iodine and a cover slip placed on. The slide was examined under X10 objective, particularly for protozoa identification and enumeration.

The remaining deposit was re-suspended in 5 volumes pellet saturated sodium chloride and vortexed (Vortex, G560E, NY, USA). The suspension was centrifuged at 1000rpm for 2min. Both sides of the McMaster counting chamber were filled with the supernatant and allowed to stand for 2mins. The stained subsample of the filtrate was examined under a microscope at X10 magnification. All eggs within the engraved area of both chambers of the MacMaster chambers were counted. The number of eggs per gram of faeces was calculated by adding the viable egg counts of the two chambers together and multiplying the total by 50. These gave the viable eggs per gram (v. e. p. g) of faeces.

3.6.1 Faecal slurry preparation

Two methods were used to test for bacteria and parasites viability in the presence of urea. Faecal slurry was made by thoroughly mixing 60 peepoos previously selected from the baseline samples and stored on the basis of infection levels at 4°C without urea. This slurry was then divided into two equal portions of 2668 grams and placed in air tight plastic containers. In one of the containers, 110g of urea was added and thoroughly mixed giving 4.12% urea addition. In the second portion, nothing was added. Temperature loggers (Tinytag, Tg-4080) were placed into the slurry and the containers were air tight sealed and kept at room temperature.

3.6.2 Culture and enumeration of helminths eggs for viability test after urea treatment

Culture was done according to the Manual of veterinary parasitological laboratory techniques (1986) with modifications. Briefly, 2 grams of ova and cysts positive faeces were weighed into a 50ml beaker and 28ml of water added into the beaker. Using a tough depressor, a homogenous faecal suspension was made by mixing the faeces with the fluid thoroughly. The homogenous faecal suspension was filtered through a strainer of 0.15 mm aperture into a clean container. The filtrate was mixed and 15ml transferred into a centrifuge tube. The suspension was centrifuged at 1000 rpm for 2 minutes and 5ml of the supernatant poured into a petri dish and then 20ml of the 2% potassium dichromate added. The sample was incubated at 27°C for 14 – 21 days, being stirred daily and moistened with water as necessary.

After incubation, infective eggs were recovered from the cultures by decanting off the potassium dichromate to remain with the sediments. The sediments were transferred into a centrifuge tube and equal volumes of saturated salt solution added before centrifuging at 1000 g for 2 min. Both sides of the McMaster counting chamber were filled with the supernatant and allowed to stand for 2min. All the viable eggs within the engraved area of both chambers were counted at X10 magnification and the number of viable eggs per gram of faeces determined by adding the viable egg counts of the two chambers together and multiplying the total by 50.

3.7 Procedure for coliform enumeration and *Escherichia coli* culture from water, soil and stool samples

In the laboratory, five grams/ml of sample was weighed and suspended in 45ml of buffered peptone water to make a 1:9 (10^{-1}) dilution. Further, 10-fold serial dilutions were made; 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} in sterile physiological saline. Chromocult coliform agar (Merck Millipore Corporation, Germany) was reconstituted according to the manufacturer's instructions and kept at 50°C in water bath prior to use.

One ml of the diluted and vortexed samples was transferred into well labeled sterile petri dishes and 15ml of the cooled medium added. The plates were thoroughly mixed. The media was allowed to solidify at room temperature before being incubated at 44.5°C for 18 hours.

After incubation, characteristic colonies were examined, counted and recorded as total thermo-tolerant coliforms (appeared as salmon red) or total *Escherichia coli* (appearing as dark-blue to violet colonies). *Escherichia coli* were confirmed by overlaying the dark-blue to violet colonies with Kovacs reagent and observing for a cherry-red color change. Four characteristic colonies of *Escherichia coli* were sub-cultured on clean Chromocult agar to obtain distinct colonies. The pure cultures were stored in skimmed milk and a pool of all the four different isolates from a single sample stored in a separate vial at -80 °C for further characterization.

3.8 Procedure for *Campylobacter* culture from water, soil and human faecal samples

In the laboratory, five gram/ml of sample was weighed and suspended in 45ml buffered peptone water (BPW) (Oxoid, Hampshire, England); the suspension was incubated at 42°C for 18 hours in a 50ml closed culture tubes. Modified *Campylobacter* charcoal-cefoperazone deoxycholate agar (mCCDA) culture media supplemented with polymyxin B (2500IU), rifampincin (5mg), trimethoprim (5mg) and cycloheximide (50mg) was prepared according to manufacturer's instructions and stored at 4°C. After samples were pre-enrichment for 18hrs at 42°C incubation, the mCCDA plates were inoculated using a sterile swab and the plates incubated at 45°C for up to 48 hours under anaerobic conditions. Anaerobic conditions were achieved by enclosing a sachet of CampyGen (Oxoid, Hampshire, England) in an anaerobic jar with the cultures. At 24 hours of incubation, the plates were checked for characteristic growth and plates without growth were incubated for an additional 24 hours. Characteristic colonies (grey/white or creamy grey in color with moist appearance) were examined and counted. Several colonies were harvested and stored in skimmed milk at -80°C for further characterization.

3.9 Procedure for *Salmonella spp* culture from water, soil and human faecal samples

In the laboratory, five grams/mls of sample was weighed and suspended in 45ml buffered peptone water; the suspension was incubated at 37°C for 18 hours in a 50ml closed culture for pre-enrichmed. After 18hrs, 1ml of the pre-enriched

culture was inoculated into sterile 10mls tetrathione broth (Oxoid, Hampshire, England) and incubated aerobically at 37°C for 18hrs. The enriched broth cultures were subcultured on xylose-lysine-deoxycholate (XLD) agar (Oxoid, Hampshire, England) plates and further incubated for 18hrs at 37°C. At least 4 characteristic *salmonella spp* colonies per plate/sample were sub-cultured on another XLD agar media to obtain a pure culture. Pure cultures of suspect *Salmonella* were subjected to biochemical tests using the automated profile index (API) 20E system (Biomerieux, France). Colonies giving characteristic reactions were stored in skimmed milk at -80°C separately and as a pool per sample for further characterization

3.10 *Salmonella spp* confirmation with API 20E

Characteristic colonies were sub-cultured onto nutrient broth (Oxoid, Hampshire, England) slants and incubated at 37°C for 18 hours. A bacteria suspension was made by adding 3mls sterile distilled water onto each culture slant and an emulsification made with a sterile loop. The suspension was then inoculated into the different biochemical prolife of the API system and incubated in humid box at 37°C for 18hrs. Profile reactions were recorded and entered into the API 20E computer software (Epiweb software, Biomerieux, France) to generate interpreted identification profiles. Colonies interpreted as *Salmonella* were further confirmed using serological typing with both polyclonal and monoclonal *Salmonella* typing antisera.

3.11 Serological typing for *Salmonella* spp

The bacteria suspension used for API profiling was subjected to serological testing. Slide agglutination method was used for serological characterization, 20µl of homogenous bacteria suspension was placed on a clean microscope slide and equal volume of typing Prolab diagnostic antisera (Merseyside, UK) mixed. Agglutination was observed within 3 minutes for positive isolates. Those that gave a positive reaction with polyclonal antisera were tested with specific monoclonal antisera.

3.12 Bacteria uptake by experimental plants and persistence in peepoo conditioned soil

Human excreta prepared into slurry for viability test was also tested for applicability as a fertilizer. This was to establish possibility of crop contamination (internally and on the surface) with human pathogens (from the excreta fertilizer) at harvest. This was done by first testing slurry for *Ascaris* viability and using these findings to condition potted soil with the tested untreated excreta (control slurry) and with treated slurry at different treatment periods (2, 4, 6, 8, 10 and 12 weeks). Seedlings timed to mature at 2 weeks intervals were then transplanted into the conditioned soil in semi-controlled greenhouse, watered and monitored until harvest. At harvest, the mature crops were tested for the contaminating pathogens.

Twenty five grams of the slurry treated with urea were mixed with 1kg of subsoil in sodium hypochlorite disinfected planting pots, and 9 grams of Di-ammonium

phosphate (DAP) fertilizer were added and lettuce or/and Coriander (*Dhania*) planted in four replicates. Test control pots were included at every testing and planting period; Test controls were made up of four pots with soil, DAP plus untreated slurry and one with soil plus DAP without slurry.

Positive controls were also set up by planting coriander in 5 separate pots with 1000g pathogen free soil; three suspensions of *Escherichia coli* (isolates numbers 91, 90, and 521) and two suspensions of *Campylobacter* (isolates 592 and 525) at a concentration of 10^{10} cfu/ml were prepared. The potted plants were then inoculated with the bacteria suspensions respectively by pouring each suspension at the base of a different seedling.

Lettuces (*Lactuca sativa*) and coriander (*Coriandrum sativum*) seeds were disinfected with 3.85% sodium hypochlorite for 15 minutes and sown on sterile vermiculite in a 66 wells seed planter. They were kept in a closed dust-free green house and watered with distilled water. Upon germination, the seedlings were fed with sterile nutrient solution twice daily (Broughton and Dillworth, 1970) till ready for transplanting. Two weeks after germination, the seedlings were transplanted in the just prepared pots (urea treated slurry, untreated slurry, soil inoculated with bacteria isolates solution and soil alone) in the closed green house and closely monitored. A portion of vermiculite together with seedlings were submitted to the laboratory and analyzed for all the test parameters.

At day zero, both the slurry and mixed potted soils were tested for viability of *Ascaris lumbricoides* eggs, concentration of *Escherichia coli* and presence of *Campylobacter spp.* and *Salmonella spp.* Upon maturity of the plants (at six weeks), analyses were carried out on soil, roots, stems and leaves to establish presences of viable *Ascaris lumbricoides* (eggs) and presence of bacteria pathogens including *Escherichia coli*, *Campylobacter spp.* and *Salmonella spp.* on the surface and intracellular. The pH and ammonia concentration of soil were also determined at harvest.

At harvest, leaves were cut aseptically with sterile blade and placed in a sterile bag; the same was done for plants stems. The remaining part was then uprooted and also put in a sterile bag. About thirty grams of soil was obtained from each pot after mixing thoroughly with a sterile spatula and placed in a sterile container. This was repeated for all the plant replicates. At the laboratory, the weight of each sample was determined using a digital balance (OHAUS CORP, Av8108, USA) and the equivalent amount of sterile buffered peptone water (Oxoid) poured into the bag to make a 10-fold dilution. The samples were then stomached using a laboratory blender (Stomacher, Seward Medical London, SEI IPP UK) at moderate speed for 3 minutes to clean off surface contaminants.

The surface cleanup suspension from all the different plant parts as well as the soil suspension were separately placed in clean 15ml tube and tested for all organisms as stated in section 3.3. The samples, except soil were further cleaned in plenty of running tap water and rinsed with sterile distilled water. These were then soaked for 30 minutes in 3.85% sodium hypochlorite. Excess hypochlorite was thoroughly removed with sterile distilled water. The samples were then ground separately on sterile frozen pestle and motor. Ground preparations were suspended with equal volume of sterile buffered peptone water and after transfer to a sterile 15 mls tube, it was further diluted to make a final 10 fold dilution. This was repeated for all the plant samples and tested for the above described organisms.

3.13 Molecular characterization of bacteria isolates

3.13.1 DNA preparation from bacteria colonies

Only pure bacteria cultures were used for DNA preparation. Three distinct colonies were lifted from pure culture with a sterile wire loop and suspended in 0.5mls sterile distilled water. The suspension was then placed in a water bath and heated to boil for 30 minutes. After cooling to room temperature, the preparation was centrifuged at 15000 rpm and the supernatant harvested and stored at -20 °C as DNA preparation.

3.13.2 Polymerase chain reaction (PCR) for determination of pathogenic *E.coli*

Genomic DNA was prepared from pure cultures obtained from the stored vials as described in section 3.12.1. Due to the large number of *Escherichia coli* isolates, first the pools were sub-cultured and tested for the presence of pathogenic *Escherichia coli* using multiplex PCR as described by Kaper *et al.* (2004). The PCR was carried out using a Veriti 96 wells thermocycler (Applied Biosystems, model 9902, Singapore) in 0.2ml PCR tubes. A 25ul reaction mix was prepared by mixing 12.5ul of PCR master-mix (Taq polymerase 0.05U/ μ l, 0.4mM of each dNTP, 4mMMgCl₂) (Biolabs Inc., New England) with ten pairs of specific primers (10pmol) at 0.5ul each (Vidal *et al.*, 2005) and 2.5 μ l DNA template. Primers used for amplification of products encoding for pathogenic genes in *E. coli* are shown in Table 3.2. This was amplified with a program of initial heating at 94°C for 5 minutes followed by 94°C, 1.5 minutes of denaturation, 1.5 min at 60°C primer annealing and at 72°C for 1.5 min elongation for 35cycles, with a final extension of 72°C for 7 minutes.

The PCR products were visualized following electrophoresis in a 1.5% agarose (Genetics analysis grade, Fisher Scientific, New Jersey) gel stained with 0.02% ethidium bromide and amplicons identified against molecular marker (50 bp DNA ladder, England Biolab) run long side the samples. For confirmation the positively identified PCR products were submitted for sequencing (ABI 3500XL Genetic Analyzer).

Table 3.2: Primers used for patho-typing *Escherichia coli* isolates

Primer sequence 5'-3'	PCR product (pb)	Encoded gene	Reference Source of primers
F-CTCGGCACGTTTTAATAGTCTGG R-GTGGAGAGCTGAAGTTTCTCTGC	933	<i>ipaH</i>	Vidal <i>et al.</i> 2005
F-AGCTCAGGCAATGAAACTTTGAC R-TGGGCTTGATATTCCGATAAGTC	618	<i>virF</i>	Vidal <i>et al.</i> 2005
F-ATCCTATTCCCAGGAGTTTACG R-GCGTCATCGTATACACAGGAGC	584	<i>stx2</i>	Berlutti <i>et al.</i> 1998
F-GAACGTTGGTTAATGTGGGGTAA R-TATTCACCGGTCGGTTATCAGT	542	<i>daaE</i>	Vidal <i>et al.</i> 2005
F-TCAATGCAGTTCCGTTATCAGTT R-GTAAAGTCCGTTACCCCAACCTG	482	<i>eae</i>	Stacy-Phipps <i>et al.</i> 1995
F-CACAGGCAACTGAAATAAGTCTGG R-ATTCCCATGATGTCAAGCACTTC	378	<i>aafII</i>	Vidal <i>et al.</i> 2005
F- CAGTTAATGTGGTGGCGAAGG R- CACGCAACAATGTAACCGCTG	348	<i>stx1</i>	Berlutti <i>et al.</i> 1998
F-GGAAGTCAAATTCATGGGGGTAT R- GGAATCAGACGCAGACTGGTA GT	300	<i>bfp</i>	Stacy-Phipps <i>et al.</i> 1995
R-TCCTTCATCCTTTCAATGGCTTT F-GCACACGGAGCTCCTCAGTC	218	<i>lt</i>	Stacy-Phipps <i>et al.</i> 1995
R-AAAGGAGAGCTTCGTACATTTT F-AATGTCCGTGCGTTAGGAC	129	<i>stII</i>	Stacy-Phipps <i>et al.</i> 1995

3.13.3 Polymerase Chain Reaction to determine *Campylobacter coli*, *Campylobacter jejuni* and *Campylobacter lari*

Polymerase Chain Reaction (PCR) to determine the genus of the culture identified *Campylobacter* isolates was first done before identifying the three specific species.

The Suspect *Campylobacter* DNA preparation was amplified in a 25µl reaction mix by mixing 2.5µl 10X PCR buffer (Coraload), 0.5µl dNTPs, 0.125µl Taq DNA polymerase (Qiagen Inc., Mississauga, Ontario, Canada) and 0.1µl of each specific primer (10pmol), 2µl DNA template and 18.657µl DNase, RNase free distilled water. This was amplified using a program of initial heating at 94°C for 5 minutes

followed by 30 cycles of denaturation at 94°C for 1 minutes, annealing at 56°C for 1 min, extension at 72°C for 1min with a final extension of 72°C for 10 minutes using a Veriti 96 wells thermocycler, (Applied Biosystems, model 9902, Singapore) in 0.2ml PCR tubes. The PCR products were kept at -20°C until gel electrophoresis was done.

The *Campylobacter* genus-specific primers, C412F and C1228 R, described by Linton *et al.* (1996) were used to amplify 812bp fragment within the 16S rRNA gene of *Campylobacter* species. Forward primer; C412F 5'- F-GGATGACACTTTTCGGAGC; Reverse primer; C1228R 5'- R-CATTGTAGCACGTGTGTC. The *Campylobacter jejuni* and *coli*-specific primers *CjejlpxAF*, *CjejipxAR* and *CcollpxAF*, described by Klena *et al.* (2004) were used to amplify 331bp and 391bp fragment flanking the *C. jejuni*, and *C. coli lpxA* gene respectively in a multiplex PCR. Forward primer; *CjejlpxAF* 5'- F-ACAACTTGGTGACGATGTTGTA; Reverse primer; *CjejipxAR* 5'- R-CAATCATGDGCDATATGASAATAHGCCAT. Forward primer; *CcollpxAF* 5'- F-AGACAAATAAGAGAGAATCAG. The *Campylobacter lari* - specific primers; Forward primer; *lpxAC*, F-AGACAAATAAGAGAGAATCAG; Reverse primer; *lpxARKK2M*, R-CAATCATGDGCDATATGASAATAHGCCAT.

3.13.4 Polymerase Chain Reaction to determine genus *Salmonella*

Genomic DNA was prepared from pure cultures as described in section 3.12.1. PCR was carried out using a Veriti 96 wells thermocycler, (Applied Biosystems, model 9902, Singapore) in 0.2ml PCR tube. A 12.5µl reaction mix was prepared by mixing 6.25µl of master-mix with 0.25µl each specific primer (10pmol), 2µl DNA template and 3.75µl DNase, RNase free distilled water. This was amplified with a program of initial heating at 94°C for 5 minutes followed by 94°C, 4 minutes denaturation, 60°C, 1 min annealing, 72°C, 1 min extension for 30 cycles, with a final extension of 72°C 7 minutes. The PCR products were kept at -20°C until gel electrophoresis was done. Primer sequences for genus *Salmonella*; *invA* gene (Rahn *et al.*, 1992). Forward primer; 5'-TATCGCCACGTTCTGGGCAA-3' and reverse primer; 5'-TCGCACCGTCAAAGGAACC-3'

3.13.5 Gel Electrophoresis for PCR products

A 1.5% agarose gel in 0.5X tris boric ethylene-diamine-tetra-acetic sodium (TBE) containing 0.5µg/ml ethidium bromide was prepared. Agarose was melted in 0.5X TBE using a microwave. The gel was cast and allowed to solidify. Ten micro-liter portions was mixed with 2µl of 6X gel loading dye and loaded onto electrophoresis gel wells submerged in 1X TBE running buffer. Molecular weight maker 50bp DNA ladder (England biolabs), was loaded in one of the wells, also included was a known positive (*Salmonella* Typhimurium) DNA and a negative control (DNase free distilled water). A constant voltage of 10V/cm was applied

and amplified fragments were allowed to migrate until appropriate band separation was achieved. A UV transilluminator digital camera (Gelmax 125 imager , Cambridge UK) with a UVP software interphase computer (Upland CA, USA) was used to visualize DNA bands relative to the molecular weight marker and take photographs.

3.14 Phenotypic characterization of bacteria isolates for antimicrobial resistance along the peepoo handling chain

3.14.1 Antimicrobial sensitivity test (AST) for confirmed isolates of *Campylobacter*, *Escherichia coli* and *Salmonella spp*

Pure bacterial cultures were used for the AST, as explained in sections 3.7, 3.8 and 3.9. Inoculum for the AST was prepared by touching the top of at least 2 to 3 well isolated colonies with a sterile wire loop and then transferred into sterile normal saline solution (0.85% NaCl). The inoculum was emulsified on the inside of the tube to avoid clumping of the cells and turbidity adjusted to a 0.5 McFarland. A standard bacterium, *Escherichia coli* ATCC 25922 was included in every test as antibiotic breakpoint control.

The Mueller Hinton agar plates used with *Escherichia coli* and *Salmonella spp* were free of visible contamination, of a uniform depth of approximately 4mm and were not excessively wet, cracked or dry. A sterile cotton swab was dipped into the adjusted inoculum for 1 minute and rotated several times. The swab was then firmly pressed on the tube wall above the fluid to release excess inoculum. The

swab was then used to streak over the entire surface of the Mueller Hinton agar plate, by rotating the plate approximately 60° and repeating the streaking motion. This was repeated twice to cover the entire plate and finally by running the swab around the rim of the agar. The inoculated plates were allowed to absorb all excess moisture on the agar surface before the antimicrobial disks were applied. The disks were dispensed on the agar surface with a sterile multidisc dispenser. Six antimicrobial discs were placed on a single plate for ease of interpretation

Campylobacter spp isolates were phenotypically tested for resistance using selected antimicrobial agents according to the EUCAST method. Mueller-Hinton agar plus 5% de-fibrinated horse blood with 20 mg/L β-NAD (MH-F) were prepared and dried at 35°C, with the lid removed, for 15 min prior to inoculation to reduce swarming. Inoculum turbidity was adjusted to McFarland 0.5 prior to inoculation. The inoculated plates were incubated in micro aerobic environment at 41±1°C for 24 hours. Isolates with insufficient growth after 24 hours incubation were re-incubated immediately and inhibition zones read after a total of 40-48 hours incubation. The inhibition zones were read at edges as the point showing no growth viewed from the front of the plate with the lid removed and with reflected light. Only those antibiotics with EUCAST established breakpoints were tested namely; tetracyclines (Tetracycline; 30mg), quinolones (Ciprofloxacin; 5mg, Naladixic acid; 30mg) and macrolides (erythromycin; 15mg).

One hundred and sixty five *Escherichia coli* isolates were phenotypically subjected to twelve antimicrobial agents namely; Te; Tetracycline (30mg), CIP; Ciprofloxacin (5mg), NA; Naladixic acid (30mg), SXT; Trimethoprim-Sulfamethaxone (25mg), CRO; Ceftriaxone, AMC; Amoxycillin-clucianic acid (10mg) , CAZ: Ceflazime (30mg), AMP; Ampicillin (10mg), C; Chlonamphenical (30mg), CN; Gentamycin (10mg), S; Streptomycin (10mg), CXM; Cefuroxicin (30mg) according to the CLSI (2012) guidelines and breakpoint obtained. The 12 antimicrobial agents are classified into 9 subclasses CLSI (2012).

Four *Salmonella spp* isolates were phenotypically subjected to twelve antimicrobial agents namely; Tetracycline (30mg), Ciprofloxacin (5mg), Naladixic acid (30mg), Trimethoprim-Sulfamethaxone (25mg), Ceftriaxone , Amoxycillin-clucianic acid (10mg) , Ceflazime (30mg), Ampicillin (10mg), Chlonamphenical (30mg), Gentamycin (10mg), Streptomycin (10mg), Cefuroxicin (30mg) according to the CLSI (2011) guidelines and breakpoint obtained. One hundred and sixty five *Escherichia coli* isolates were phenotypically subjected to twelve antimicrobial agents namely; Tetracycline (30mg), Ciprofloxacin (5mg), Naladixic acid (30mg), Trimethoprim-Sulfamethaxone (25mg), Ceftriaxone, Amoxycillin-clucianic acid (10mg) , Ceflazime (30mg), Ampicillin (10mg), Chlonamphenical (30mg), Gentamycin (10mg), Streptomycin (10mg), Cefuroxicin (30mg) according to the CLSI (2012) guidelines and breakpoint obtained. The 12 antimicrobial agents can be classified into 9 subclasses CLSI (2012).

3.14.2 Antibiotic sensitivity test quality control and categorization

The growth was confirmed to be confluent lawn. Individual colonies of resistant organisms were observed around some antimicrobial disks. In instances when individual colonies were dispersed across the plate, the inoculum was too light and the sample was retested. The result of the control strain, *E. coli* ATCC 25922, was first compared to the disk diffusion quality control ranges for non-fastidious organism given in the CLSI guidelines M100. The zones were checked to be round and not oval, deformed or have jagged edges. The diameter of inhibition zones was measured considered as the area showing no obvious visible growth detected with unaided eye. A haze was disregarded. For antibiotic trimethoprim and sulphonamides, they allow growth of the bacteria for some generations before inhibition occurs. Therefore, the slight growth of 20% or less of the lawn of growth was disregarded, and the more obvious margin was measured to determine the zone diameter. When confluent growth was present up to the border of the disk, this was recorded as no inhibition and the diameter of the disk recorded as 6mm.

Clinical and Laboratory Standards Institute (CLSI) Guideline M100, Table 2A (Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae) (Appendix VI), was the interpretation guideline for the categorization of the test strain as sensitive (S), intermediate (I) or resistant (R). *Campylobacter spp* isolates were phenotypically

tested for resistance using selected antimicrobial agents according to the EUCAST method and interpretations done using disk diffusion (EUCAST standardized disk diffusion method, 2016), (Appendix VII).

3.15 Genotypic characterization of bacteria isolates for antimicrobial resistance along the peepoo handling chain

Ninety four phenotypically characterized *Campylobacter* isolates; 11 *C. lari*, 30 *C. coli*, 49 *C. jejuni* and 4 *Campylobacter spp* isolates were genotypically tested for the presence of genes encoding for resistance to three antimicrobial agents, namely; tetracyclines, quinolones and sulfonamides. Presence of four tetracycline resistance genes; tet A, tet B, tet C and tet O, two of quinolones resistance genes; gyr A and gyr B and two sulfonamides resistance genes namely sul1 and sul2 were tested. Similar tests were done for all the four *Salmonella enterica* and 75 *Escherichia coli* selected based on their unique phenotypic profiles out of the 165 isolates tested for antimicrobial sensitivity by the disc test. Multiplex PCR was carried out as described in section 3.12.3. Primers used for amplification of products encoding the resistant genes to the three antibiotic classes are shown in Table 3.3.

Table 3.3: Primers used for identifying tetracyclines, quinolones and sulphonamides encoding genes in selected bacterial isolates

Primer sequence 5'-3'	PCR product, pb	genes	Reference source of primers
F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	577	<i>Tet A</i>	Randall <i>et al.</i> , 2004
F-CCTCAGCTTCTCAACGCGTG R-GCACCTTGCTGAGACTCTT	635	<i>Tet B</i>	Randall <i>et al.</i> , 2004
F-ACTTGGAGCCACTATCGAC R-CTACAATCCATGCCAACCC	880	<i>Tet C</i>	Van <i>et al.</i> , 2008
R-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	<i>Tet O</i>	Abddi <i>et al.</i> , 2014
F-GCCTGACGCAAGAGATGGTT R-TCAGTATAACGCATCGCAGC	270	<i>Gry A</i>	Piddock <i>et al.</i> , 2003
F-ATGGCAGCTAGAGGAAGAGA R-GTGATCCATCAACATCCGCA	382	<i>gryB</i>	Piddock <i>et al.</i> , 2003
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCCTCGGTCTC	822	<i>Sul1</i>	Van <i>et al.</i> , 2008
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCCTCGGTCTC	285	<i>Sul2</i>	Van <i>et al.</i> , 2008

3.16 Presence of Rotavirus and Adenovirus in stool from pupils under five years old and soil samples

3.16.1 Prospect Rotavirus and Adenovirus microplate immunoassay

The immunoassay kits (ProSpect, Oxoid Ltd, Hants, UK) were used to separately test for presence of Adenovirus and Rotavirus antigens, in stool samples from school going children below five years. One milliliter sample diluent provided in the immunoassay kit was added to a 2ml container. This was used to prepare a 10% suspension or dilution of faecal specimen by adding 0.1g of solid faeces (approximately a small pea-sized portion). If the faeces were in liquid form, the 100µl was transferred into 1ml sample diluent and mixed thoroughly. This stool suspension was analysed by ELISA. In addition to the specimen, negative and positive controls were studied, at least one of each in every test set up.

Conjugate (ProSpect, Oxoid Ltd, Hants, UK) was added into each well at 100µl, the plate covered and incubated at 30°C for 60 minutes. The wells were then cleaned by shaking and aspirating the content of the wells followed by completely filling each well with dilute wash buffer (ProSpect, Oxoid Ltd, Hants, UK) for 5 times. After the last wash, the wells were dried by tapping them on clean paper towels to remove last traces of wash buffer. A 100µl volume of substrate (ProSpect, Oxoid Ltd, Hants, UK) was added to each microwell, covered and then incubated at 30°C for 10 minutes. After completion, the reaction was stopped by adding 100µl of stop reagent provided in the kit to each microwell and the wells thoroughly mixed before reading the results using an ELISA reader (Multiskan EX, Thermo Fisher Sci. Oy. Finland) at 450nm. The reader was blanked with air before the readings of the samples.

3.16.2 Quality control for the Rotavirus and Adenovirus ELISA test

The mean of the negative control values was less than 0.150 absorbance units, while the mean positive control value was above 0.500 absorbance units. A cut-off point was calculated by adding 0.100 (constant value provided by the kit) to the mean negative control value. The wells were interpreted as positive when an absorbance value was greater than the cut-off value and as negative when absorbance value was less than the cut-off value. Equivocal samples, i.e. those with absorbance value within 0.010 the cut-off point were to be repeated.

3.17 Direct immunofluorescence detection procedure for *Cryptosporidium*

A direct immunofluorescence kit (MeriFluor C/G, Meridian Bioscience, Inc. USA) for the simultaneous detection of *Cryptosporidium* and *Giardia* in fecal material was used. The available kit for *Cryptosporidium* is designed to detect *Giardia* as well but gives distinctive features differentiating the two. The kit uses the principle of direct immune-fluorescence. Detection reagent had a mixture of FITC labeled monoclonal antibodies directed against cell wall antigens of *Cryptosporidium* or *Giardia* antigens present in the specimen. Processed slides, were examined, for a dull orange to red counterstained background.

3.17.1 Preparation of specimen

Stool specimens were preserved in sodium acetate-acetic acid formalin (SAF). The samples were concentrated by first treating with ethyl acetate to dilipidate the samples hence easy adherence to treated microscopic slides. This was followed with sedimentation of oocytes in saline.

3.17.2 Immunofluorescence assay for *Cryptosporidium*

The entire kit reagents were kept at room temperature for 30 minutes before use. The wash solution was diluted to 1X concentration from the provided 20X concentration by mixing 2.5mls of 20X with 47.5 mls of distilled water. Using the provided transfer loop a drop of the fecal deposit was transferred onto the treated slide well and spread over the entire well. Care was taken not to scratch the treated

surface of the slide. A new transfer loop was used to prepare negative and positive wells using controls provided in the kit. The slides were air dried for 30 minutes at room temperature after which a drop of the detection reagent was added into each well followed by a drop of the counterstain. The reagents were then mixed using an applicator stick and spread over the entire well. This was done taking care not to scratch the treated surface of the slide. The slide was then incubated in a humid box at room temperature, away from light, for 30 minutes.

Using the diluted, 1X wash buffer the wells were rinsed to remove all the stain without disturbing the specimen and cross contamination between wells was avoided. Excess buffer was removed from the wells by tapping the long side of the slide on clean paper towel, without allowing the slide to dry. A drop of mounting media was added to each well and coverslip applied. The wells were examined using a X10 objective and *Cryptosporidium* oocytes confirmed with X100 objective.

3.17.3 Quality control of the direct immunofluorescence test

The negative well gave the expected negative results and the positive well was positive for *Cryptosporidium* oocytes, observed as round to slightly oval shaped, with varying diameter of between 2-6µm. Oocyst wall stained bright apple green with a visible suture line. *Giardia* cysts were also observed with similar staining characteristics but larger in diameter of 8-12µm.

3.18 Physical and chemical parameters

3.18.1 pH determination

The pH of individual Peepoos and faecal slurry was measured using a standard pH meter and electrode (pH mobile 826, Metrohm, Switzerland). The electrode was calibrated before measurements. Three pH standard buffer solutions; pH 4.01, 7.00 and 10.00 (Merck KGaA, Millipore, Germany) were used to do a three point calibration at a sensitivity of ± 0.5 . A 1:4 proportion of faeces to distilled water were made by weighing 7 grams faeces and adding distilled water to 35grams. For urea treated faeces (in peepoo and slurry), 10 ml distilled water was added immediately after weighing 7 grams of the sample to avoid ammonia loss and the 15 ml centrifuge tubes with the solutions were kept closed when not actively used. More distilled water was later added for final adjustment to 1:4 proportions. The mixture was vortexed to obtain a homogenous mix and allowed to stand at room temperature for 30 minutes. pH measurements were taken by placing the calibrated electrode in the faecal mixture and allowing time for the displayed reading to stabilize.

3.18.3 Ammonia measurement

Using an ion selective electrode for ammonia (NH₃-ISE High, Metrohm, Switzerland) and an 826 meter (Metrohm, Switzerland), the concentration of ammonia in the samples was determined from a non-integrated calibration curve

prepared each day measurements were performed. Three standard solutions of ammonium chloride; 0.001M, 0.01M and 0.1M were made by preparing 1 molar solution and making serial ten fold dilutions and corresponding mV measurements giving a linear relation against \log_{10} concentration (M).

For the ammonia measurements, a 100 mls of the standard solutions or sample was put in a plastic bottle with a magnetic bar and placed on a magnetic stirrer. To be in the measuring range urea treated faeces were diluted 500 times and untreated stool samples 100 times. One millilitre of 1Molar sodium hydroxide (1M NaOH) was added and the electrode immediately immersed. While stirring, the mV reading on the meter was taken after 30 seconds upon reaching equilibrium. The electrode was flushed in distilled water and dried on paper towel before taking another reading.

3.18.4 Temperature measurement

The incubation temperature was monitored using automatic temperature recording device (Tinytag TG-4100 temperature logger) placed in every set of storage bag to record temperature as an average over every 10 minutes. At the end of incubation, the temperature loggers were removed and stored data downloaded into a computer using a Tinytag USB Inductive Pad (ACS-3030, Switzerland).

3.19 DNA sequencing of selected pathogens isolated along the Peepoo handling chain

The PCR products obtained from the five types of pathogenic *Escherichia coli* (ETEC, EPEC, EAaggEC, EHEC and DAEC); encoding for genes *lt*, *eae*, *bfp*, *aafii*, *stx1*, *stx2*, *stII*, and *daaE* respectively, were purified using exonuclease1, shrimp alkaline phosphatase mixture (ExoSAP mix) according to the manufacturer's instructions. Briefly, this was done by adding 2.5µl of ExoSAP mix to 10µl PCR product. The mixture was then incubated at 37°C for 30 minutes and reaction stopped by heating at 95°C for 5 minutes. The clean PCR product was then quantified using a fluorimeter (Qubit 2.0, Invitrogen, USA).

The clean DNA was first labeled with BigDye terminator v3.1kit (Applied Biosystem, CA, USA) according to the manufacturer's instructions and loaded into Genetic analyzer (ABI 3730 capillary analyser; Applied Biosystems, Foster City, CA, USA) for sequencing. Sequences were obtained in ABI files that were opened and edited to remove unspecific ends using Bioedit version 7.0.4 (Hall, CA, USA) software. Clean sequences were then submitted to NCBI Genbank database (<http://blast.ncbi.nlm.nih.govblast.cgi/>) and BLASTn program used to test for homology and genetic identity of bacteria isolates. Seaview software (Guindon and Gascuel, 2010) was used for sequence alignment and phylogenetic to demonstrate relatedness among isolates obtained at different points of the handling chain. Rooted trees were constructed by neighbor-joining algorithm for three

enteropathogens encoding genes; *eae*, *Lt* and *bfp* together with 3 reference isolates from the GenBank U59502.2, AB647523.1 and EF540941.1.

The same was carried out on selected isolates of the two identified *Campylobacter* species (*C. coli* and *C. jejuni*) from this study. In addition genes encoding for antimicrobial resistance against quinolones (*gyrA* and *gyrB*), tetracycline (*tetA*, *tetB*, *tetC*, *tetO*) and sulphonamides (*sul1*, *sul2*) demonstrated in some of the bacteria pathogen types in this study were selected for sequencing.

3.20 Data Analysis

The data collected was subjected to multivariate analysis to establish sanitisation conditions in Peepoo bag and slurry in response to treatment factors such as pH, temperature, total ammonia and storage time. Post hoc analysis using bonferoni method was used to determine the withholding or storage time required for effective excreta sanitization in both peepoo and slurry system. Regression analysis was done to test the effect of temperature, pH and urea on the viability of *Ascaris* and coliforms on both slurry and peepoo systems. Logistic regression was done to test the effect of temperature and pH on *campylobacter spp* and *E.coli* in urea treated and untreated peepoo bags. Analysis of Variance was used to determine if there were significant differences in the viability of *Ascaris* and coliforms between the treated and untreated peepoo bags and slurry systems over time. Basic local alignment search tool (BLAST) was used to calculate statistical

significance on regions of similarity between the current study bacteria isolates sequences and the national center of biotechnology information (NCBI) sequence database.

3.21 Ethical consideration

Research clearance was sought from National Commission for Science, Technology and Innovation (NCOSTI) (Appendix IV), and Ethical clearance through the Kenyatta University Ethical Review Committees (Appendix II).

CHAPTER FOUR: RESULTS

4.1 Prevalence baseline data

4.1.2 Geo-helminths and protozoa in faecal samples, schools' soil and water

A total of 191(33%) faecal samples had soil transmitted helminths (STH) of these, 117 (20%) were *Ascaris lumbricoides* while 74 (13%) were *Trichuris trichiura*. Protozoa including *Entamoeba histolytica* was isolated in 163 (28%) samples. Other infections were by *Entamoeba coli* (34%), *Balantidium coli* (3.4%) and *Hymenolepsis nana* (1.9%). Out of the tested samples, 14 (2.4%) tested positive for *Cryptosporidium spp* by both acid fast staining microscopy and direct immunofluorescence (Figure 4.1a and 4.1b).

Out of the 10 soil samples (schools F closed in course of the study before water and soil were sampled) obtained from the study schools, only 4 (40%) had STH, three schools had *Ascaris lumbricoides* while one school had *H. nana*. Multiple infections with geo-helminths and protozoa were observed in children from all the schools under this study. The highest *Ascaris lumbricoides* infection was at 43 (54%) at one of the schools, followed by 13 (24.5%) while the least infection was at 3 (6.0%).

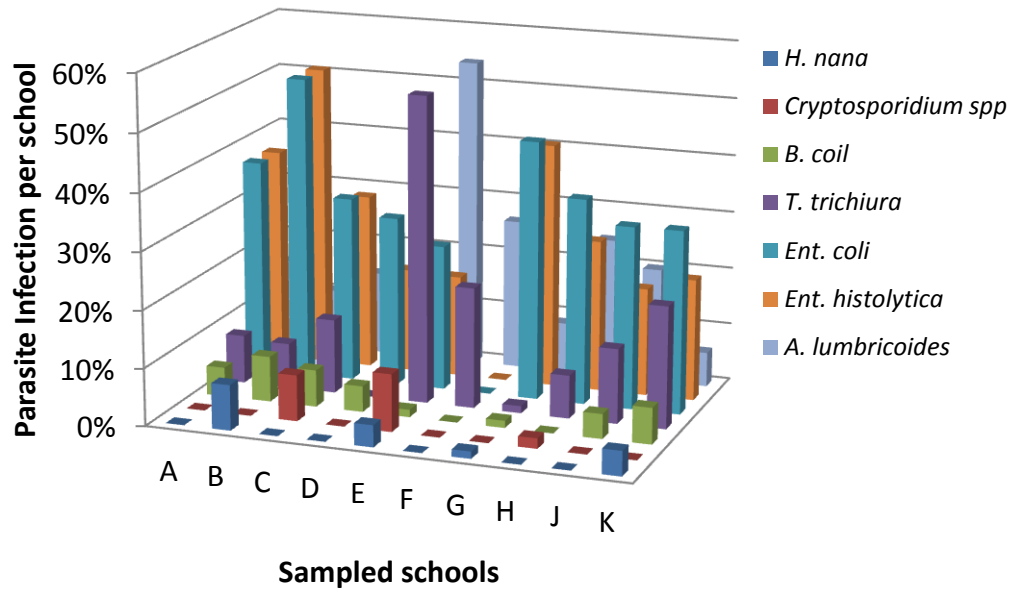


Figure 4.1a: Prevalence of gastroenteric parasites in the study schools, labeled A to K

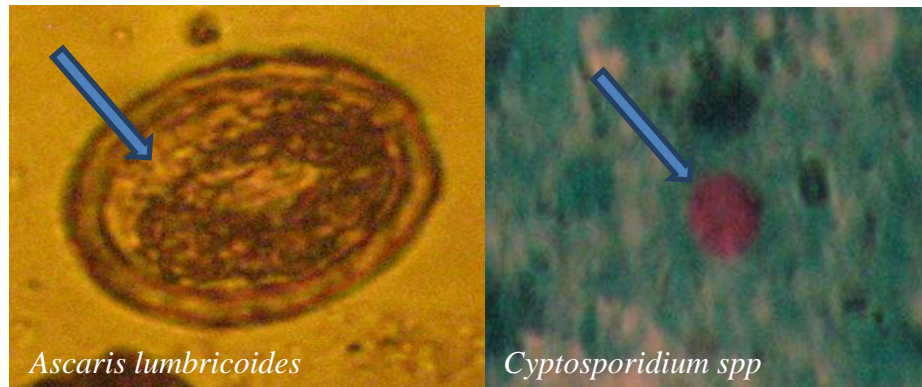


Figure 4.1b: Embryonated *Ascaris lumbricoides* ova under 400 times magnification of a light microscope, demonstrated after 21 days incubation viability test. Immuno-fluorescence stained *Cryptosporidium spp* recovered from fecal sample under a 1000 times magnification fluorescence microscopy (→)

4.1.3 Prevalence of bacterial and selected viral pathogens from faecal peepoo samples, schools' soil and water

Salmonella enterica subsp *arizonae* was isolated in 2 (0.34%) samples with nine out of the eleven schools having no isolate. Twenty four samples (4.14%) had *Proteus spp* isolated while 294 (50.7%) had *Campylobacter spp* by culture, of which 106 (18.3%) were confirmed as campylobacter by PCR amongst which 28 (9.5%) were *C. coli*, 44 (14.9%) as *C. jejuni* and 11 (3.7%) *C. lari*. Twenty three isolates (7.8%) were grouped in genera, since they did not fit in any of the three identified species. Only one school had no *Proteus spp* isolate while *Campylobacter spp* was isolated in all the 11 schools. A total of 244 (17%) samples had pathogenic *Escherichia coli*. Ninety samples from pupils aged 5 years and below were tested for rotavirus and only 1 (1.11%) was positive for Adenovirus (Table 4.1).

Table 4.1: Prevalence of selected bacteria pathogens in school going children (N=580), and selected viral pathogens from under 5 years pupils (N=90) in the study area

Schools n=11	No. of Samples	pathogenic <i>Escherichia</i> <i>coli</i> n(%)	<i>Salmonella</i> spp n (%)	<i>Proteus spp</i> n (%)	<i>Campylobact</i> <i>er spp</i> n (%)	<i>Rotavirus</i> n (%)	<i>Adenovirus</i> n (%)
A	59	6 (2.67)	0	0	24 (4.13)	0	0
B	38	0	0	2 (0.34)	10 (1.72)	0	0
C	62	3 (1.33)	1(0.17)	1 (0.17)	25 (4.31)	0	1(1.11)
D	44	0	0	2 (0.34)	19 (3.27)	0	0
E	79	10 (4.46)	0	1 (0.17)	49 (8.44)	0	0
F	19	3 (1.33)	0	2 (0.34)	14 (2.41)	0	0
G	80	14(6.25)	0	2 (0.34)	44 (7.58)	0	0
H	53	0	0	3 (0.51)	21 (3.62)	0	0
I	30	0	0	1 (0.17)	18 (3.1)	0	0
J	69	1 (0.44)	1 (0.17)	9 (1.55)	43 (7.41)	0	0
K	47	1 (0.44)	0	1 (0.17)	27 (4.65)	0	0
% Prevalence		7% (41/580)	0.34% (2/580)	4.14% (24/580)	50.69% (294/580)	0 %	1.11% (1/90)

Thermo-tolerant coliform (TTC) bacteria were present in 5 (50%) of the water samples tested from the 10 schools. Two (20%) had thermo-tolerant *Escherichia coli*. Four water samples (40%) had *Campylobacter spp*. All the 10 (100%) soil samples had both *Escherichia coli* and *Campylobacter spp*. None of the water and soil samples had *Salmonella*, *Rotavirus* or *adenovirus* isolated (Table 4.2).

Table 4.2: Prevalence of bacteria pathogens in soil and water from study area, N=10, Key: w = one water sample per school, s = one soil sample from each school, TT = thermos tolerant *E. coli* present in samples, 1= present, 0= absent

Schools	<i>Coliforms (TTC)</i> cfu/ml	<i>Escherichia coli</i> (TT)	<i>Campylobacter</i> spp
A	0 (w)	0 1 (s)	0 1 (s)
B	0 (w)	0 1 (s)	0 1 (s)
C	0 (w)	0 1 (s)	0 1 (s)
D	154 (w)	0 1 (s)	1(w) 1 (s)
E	300 (w)	0 1 (s)	1 (w) 1 (s)
G	5 (w)	0 1 (s)	0 1 (s)
H	0 (w)	0 1 (s)	0 1 (s)
I	28 (w)	1(w) 1 (s)	1(w) 1 (s)
J	300 (w)	1(w) 1 (s)	1(w) 1 (s)
K	0 (w)	0 1 (s)	0 1 (s)
% Prevalence	(5/10)50% (w)	(2/10)20% (w) (10/10)100% (s)	(4/10)40 % (w) (10/10)100% (s)

4.2 Inactivation of *Ascaris* using Peepoo bag and slurry systems at 2,4,6,8 and 10 weeks of storage

Viability of *Ascaris lumbricoides* eggs that were in peepoo bags with urea (treated) reduced to 31.1 % at the 6th week of storage and thereafter none of the identified eggs were viable to the 10th week. However, the controls (peepoo bags without urea) had 100% viability throughout the test period of 10 weeks. The mean pH for

treated peepoos remained almost constant between 8.2-8.8 during the 10 weeks of storage. However, the pH for the untreated peepoo had a gradual increase (5.9 – 7.1) over the storage time. The mean temperature (day 0 to sampling day) remained relatively the same for both treatments over the 10 weeks storage period with a low of 14°C and a high of 35.7°C (Table 4.3).

Table 4.3: Viability of *Ascaris lumbricoides* in peepoo bags over incubation period of 2 to 10 weeks. The average total ammonia nitrogen (TAN) concentration for peepoos with urea was 120mg

Storage in Week	Frequency of egg contamination (%)		Mean % viable, Peepoo treated (A)	Mean % viable, Peepoo untreated (B)	pH (Mean)		Temp °C (Mean)	
	n=32 with urea (A)	n=10 without (B)			A	B	A	B
2	40.6% (13)	40% (4)	100 %	100%	8.8	5.9	21	21
4	28.1% (9)	10% (1)	91.75%	100%	8.6	5.7	25.6	25.6
6	46.8% (15)	40% (4)	31.13%	100%	8.8	6.1	35.7	35.7
8	50% (16)	40% (4)	0%	100%	8.2	6.9	21.1	21.1
10	21.8% (7)	10% (1)	0%	100%	8.2	7.1	19.5	14

In the slurry, *Ascaris lumbricoides* were shown to be viable in the urea treated slurry until the 6th week of treatment while the untreated slurry had viable eggs at the 8th week. The pH of slurry with urea remained constant at 8.9 for the 10 weeks of storage, while that of untreated slurry had a slight gradual increment from 5.4 to 5.9 at week 10. The storage temperature was relatively similar for both treatments with a low of 10.5°C and a high of 27°C (Table 4.4).

Table 4.4: Viability of STH (*Ascaris lumbricoides*) in slurry over incubation period of 0 days to 10 weeks, N=4, The average ammonia (NH₃) concentration for slurry with urea was 124mg

Storage in Weeks	Mean % viable, slurry with urea (A)	Mean % viable, slurry without urea (B)	pH (mean)		Temp °C (mean)	
			A	B	A	B
0	100	100	8.9	5.4	10.5	11.0
2	100	100	8.9	5.4	20.1	23.1
4	93.6	94.2	8.9	5.5	15.3	15.6
6	19.8	16	8.9	5.7	21.6	22.7
8	0	20	8.8	5.7	27.0	25.6
10	0	0	8.8	5.9	23.5	22.7

4.2.1 Model for lag phase inactivation Kinetics for *Ascaris* at constant temperature, urea and pH

Peepoo treatment with urea and slurry treatments (with urea and without urea) gave an inactivation pattern that started with a lag phase. This was from day zero to day 36 where hardly no inactivation was observed with the three treatments. From day 36 to day 56, a log-linear inactivation was observed with both treatments that had urea, while slurry without urea took until day 70 to get complete inactivation of egg viability. It was therefore, observed that inactivation of more than 99% of *Ascaris* eggs was achieved in 56 days of treated human excreta in both systems, at an average ambient temperature of 23.8 °C (keeping NH₃ at 33mg/L for slurry and 34mg/L for peepoo bags). The Peepoo bags without any urea had no inactivation observed during the 100 days storage period (not plotted) (Table 4.2).

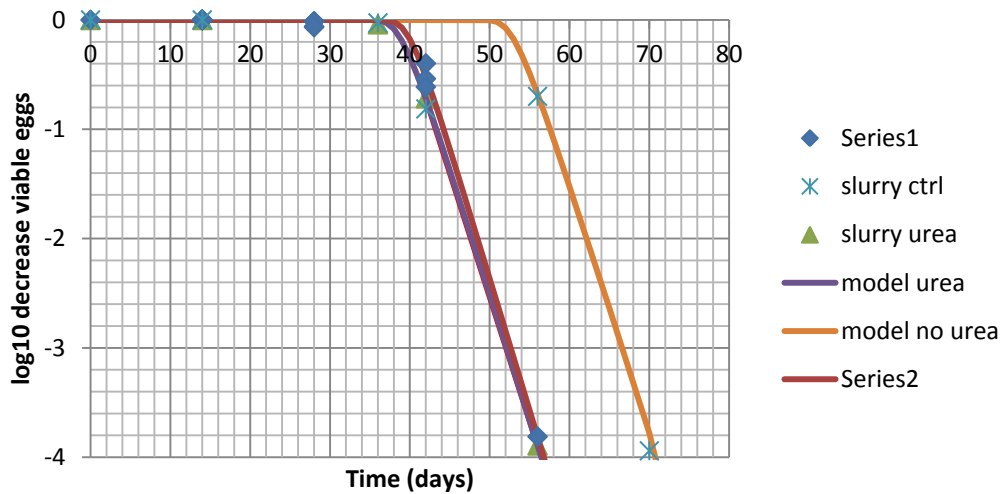


Figure 4.2: The log₁₀ decrease in *Ascaris* egg viability in faecal slurry with urea (green triangle), Peepoo bags with urea (blue square) and slurry without urea (blue star) showing a lag phase model for inactivation.

A two-way analysis of variance yielded a main effect for the treatment, $F(1, 14) = 709.51$, $p < 0.05$, such that the average viability of *Ascaris* eggs in the treated peepoo was significantly lower ($M = 100\%$, $SD = 0$) than for untreated peepoo ($M = 45.2\%$, $SD = 45.6$). The main effect of time was significant ($F(4, 14) = 110$, $P < 0.05$). The interaction effect was also significant ($F(4, 14) = 110.8$, $p < 0.05$), indicating that urea treatment had an effect on the viability of the *Ascaris* over time. Similarly, with the slurry, a two-way analysis of variance yielded a main effect for the treatment, ($F(1, 10) = 12.36$, $p < 0.05$), such that the average viability of *Ascaris* in the treated slurry was significantly lower ($M = 35\%$, $SD = 45.5$) than for untreated ($M = 38\%$, $SD = 44.2$). The main effect of time was also significant, ($F(6, 10) = 2863.5$, $p < 0.05$) and the interaction effect was also

significant, ($F(6, 10) = 25.1, p < 0.05$), indicating that the urea treatment had an effect on the viability of the *Ascaris* over time.

With the treatment peepoo bags, post hoc comparisons using bonferroni test indicated that the *Ascaris* viability in week 2 (M=100, SD=0) was significantly different from week 8 (M=20, SD=44.7) and week 10 (M=20, SD=44.7). Week 4 was significantly different from week 8 (M=20, SD=44.7) and week 10 (M=20, SD=44.7). However, viability in week 6 (M=48.4, SD=35) was not significantly different from viability in all the other weeks. The same was observed with the slurry since the Post hoc comparisons using bonferroni test indicated that the *Ascaris* viability for week 1 (M=100, SD=0), week 2 (M=100, SD=0) and Week 4 (M=92.6, SD=2.1) was significantly different from week 6 (M=16, SD=3.8), week 8 (M=10, SD=11.54), week10 (M=0, SD=0) and week12 (M=0, SD=0). This gave a cut off or a breaking point at week 6 for both systems, which is in agreement with the lag-phase modeling method (Figure 4.2).

Using regression analysis for peepoo bag, both pH and temperature had a negative effect on *Ascaris* viability though this effect was not significant, $p > 0.05$. The same was observed with the slurry's pH with $p > 0.05$. However the slurry's temperature had a significant prediction with $p < 0.05$.

4.3 Inactivation of *Escherichia coli*, *Salmonella* and *Campylobacter* using slurry systems at 2,4,6,8 and 10 weeks storage

Thermo-tolerant coliforms and *E. coli* were observed up to the second week of storage with the treated slurry and at the fourth week of incubation at 2.45 log₁₀ cfu/g for the untreated slurry. *Salmonella spp* was isolated from slurry with urea at the second week of storage but none later. *Campylobacter spp* was only isolated in urea treated slurry before treatment at day zero. However, with the untreated slurry, *Campylobacter spp* was isolated at every test interval, up to the tenth week (Table 4.5).

Table 4.5: Viability of bacteria pathogens from peepoo in big-bag over incubation period of 0 days to 10 weeks, Average storage temperature; 24.2°C, Average ammonia (NH₃); 34mg

Slurry with urea (A) & without urea (B)	Incubation time (weeks)	(\bar{x}) TTC Log ₁₀ Cfu/g	(\bar{x}) Escherichia coli Cfu/g	Salmonella spp +/- (1/0)	Campylobacter spp +/- (1/0)
A	0	9.38	9.28	0	1
B	0	9.17	9.01	0	1
A	2	0	0	0	0
B	2	6.92	6.83	1	1
A	4	0	0	0	0
B	4	6.07	6.07	0	0
A	6	0	0	0	0
B	6	0	0	0	1
A	8	0	0	0	0
B	8	0	0	0	0
A	10	0	0	0	0
B	10	0	0	0	1

A two way analysis of variance yielded a main effect for the urea treatment ($F(1, 200) = 245.76, p < 0.05$), such that the average count of coliforms in the urea

treated bags was significantly lower ($M= 0.166$, $SD = 0.72$) than in the untreated peepoo bags ($M=2.5$, $SD =1.88$). Similarly a two-way analysis of variance yielded a main effect for the urea treatment ($F (1, 16) = 5.15$, $p < 0.05$), such that the average viability of coliforms in the urea treated slurry was significantly lower ($M= 0.78$, $SD = 2.7$) than in the untreated slurry ($M= 2.35$, $SD = 3.55$). The main effect for time in the peepoo bags was highly significant ($F (4, 200) = 23.03$, $p < 0.05$). The interaction effect was also highly significant ($F (4, 200) = 12.76$, $p < 0.05$), indicating that the Urea treated peepoo bags had an effect on the count of the coliform over time. With the slurry system the main effect of time was also significant ($F (6, 16) = 10.08$, $p < 0.05$).

A regression analysis of the urea treated peepoo bags showed that pH significantly predicted coliform count with $b = -.255$, $t (39) = - 2.14$, $p<0.05$. Temperature also had a significant prediction with $b = 0.167$, $t (2.17)$, $p<0.05$. Similarly, a regression analysis of urea treated slurry showed that temperature had significant prediction of coliform count with $b = -0.41$, $t (24) = -4.33$, $p<0.05$. However, pH had no significant prediction of coliform count with $b = -0.58$, $t (22) = -2.07$, $p>0.05$.

The post hoc comparisons using bonferroni test indicated that the coliform count in the treated peepoo bags for the 2nd weeks ($M=1.41$, $SD =1.93$) was significantly different from counts in the 8th week ($M = 0.38$, $SD = 1.2$) and coliform counts in the 10th week ($M = 0.17$, $SD = 0.57$). The coliform count for the 4th week was

different from counts at 8th and 10th week. However coliform counts at 6th week (M = 0.60, SD =1.2) was not significantly different from any other week. A similar observation was observed with the urea treated slurry.

4.3.1 Effects of pH, temperature and urea on viability of *Escherichia coli* in peepoo bags

The coefficient of temperature was 0.4314025, which was significantly different from zero ($p < 0.05$). Holding all other factors constant an increase in one unit of temperature resulted to a 0.43 increase in the log odds of *Escherichia coli*, and an odds survival with 35%. The coefficient of pH and urea were not significant ($P > 0.05$) (Table 4.6).

Table 4.6: Effects of independent variables on viability of *Escherichia coli*

Independent variable	b	se	z ratio	Prob	Odds
Temperature	0.4314025	0.1533361	2.81	0.005	1.539415
PH	-1.068296	0.5775036	-1.85	0.064	0.3435934
Urea	-0.649166	1.675721	-0.39	0.698	0.5224813
Constant	-3.692245	6.416952	-0.58	0.565	0.024916
Model X2 =		27.07	p.<0.001		
Pseudo R2 =		0.2943			
n =		126			

A two-way analysis of variance yielded a main effect for the treatment ($F(1, 16) = 5.11$, $p < 0.05$), such that the average viability of the urea treated slurry was significantly lower ($M = .77$, $SD = 2.7$) than the untreated slurry ($M = 2.33$, $SD = 3.52$). The main effect of time was as well significant ($F(6, 16) = 9.91$, $p < 0.05$).

A regression analysis showed that temperature had significant prediction of *E. coli* viability with (b = -0.44, t (24) = -4.43, p<0.05). However, pH had no significant prediction of *E. coli* viability with (b = -0.58, t (22) = -2.07, p<0.05).

4.3.2 Effects of pH, temperature and urea on viability of *Campylobacter spp* in peepoo bags and Slurry

The viability of *Campylobacter* in the peepoo bags was evaluated by the score of 1 = present, 0 = not present, the predictors were temperature, pH and urea. Predicted logit for *Campylobacter spp* was 86.87+ (-4.22) temperature and (-0.02) pH and (2.81) urea. The overall model was statistically highly significant ($X_2=41.14$, p<0.001) (Table 4.7).

Table 4.7: Effects of independent variables on viability of *Campylobacter spp* in peepoo bags

Independent variable	b	se	z ratio	Prob	Odds	Mean	SD
Temperature	-4.22178	5.632535	-0.75	0.454	0.014673	7.17	4.85
PH	-0.02053	0.496925	-0.04	0.967	0.979679	21.11	1.62
Urea	2.806217	1.347833	2.08	0.037	16.54719	-92.6	27.05
Constant	86.86606	117.4641	0.74	0.46			
Model X2 =	41.14 p<0.001						
Pseudo R2 =	0.3911						
n =	121						

The viability of *Campylobacter spp* in the slurry was evaluated by the score of 1= present, 0 = not present, the predictors were temperature, pH and urea. Predicted logit for *Campylobacter spp* was 63.91+ (0.31) temperature and (-15.41) pH+ (-0.19) urea. From this equation, *Campylobacter spp* viability was found to be

positively related to temperature but negatively to pH ($p > 0.05$). The overall model was not statistically significant ($X_2 = 6.77$, $p > 0.05$) (Table 4.8).

Table 4.8: Effects of independent variables on viability of *Campylobacter* in slurry

Independent variable	b	se	Z ratio	Prob	Odds	Mean	SD
Temperature	0.307556	0.304897	1.01	0.313	1.36	7.17	4.85
PH	-15.4066	15.53382	-0.99	0.321	$2.04^{^-7}$	21.11	1.62
Urea	-0.1923	0.307803	-0.62	0.532	$5.7^{^-27}$	-92.6	27.05
Model $X_2 = 6.77$ $p > 0.05$							
Pseudo $R^2 = 0.47$							
n = 14							

4.4 Internalization of microorganisms via plant roots from pathogen spiked soil

At the setup of the green house experiment (day 0), *A. lumbricoides* eggs recovered from the treated and untreated slurry as well as in the soil slurry mixture prepared for planting were 100% viable. *Escherichia coli* in the treated slurry were at average of 3.48 ± 0.07 cfu/g total solids while the untreated slurry had average of 8.01 ± 0.94 cfu/g total solids. The counts reduced to an average of 2.91 ± 0.71 and 8.0 ± 0.52 respectively by mixing with soil. *Campylobacter spp* was isolated from all the preparations except with the negative control. However, *Salmonella spp* was only recovered from the urea treated slurry and consequently from soil mixed with the same slurry (Table 4.9).

Table 4.9: Baseline data at day zero on soil contamination with pathogenic bacteria and STH for plants internalization experiment, 1= present, 0= absent

Time (weeks)	Pathogens	Slurry		Slurry + soil+DAP	
		With Urea	No urea	Urea pots	No urea pots
Day 0	Ascaris (% viable)	100	100	100	100
	<i>E.coli</i> (cfu _{log10})	3.48±0.07	8.1±0.94	2.91±0.71	8.0±0.52
	<i>Campylobacter</i> <i>spp</i> (1/0)	1	1	1	1
	<i>Salmonella spp</i> (1/0)	1	0	1	0

4.4.1 Pathogens

Escherichia coli were isolated from soil and on the surface of coriander roots that were planted in potted soil mixed untreated slurry and slurry treated for two weeks. *Campylobacter spp* was demonstrated on the surface and in plant tissues with both the treated and untreated slurry soil mixtures on the second week of treatment. The bacteria were isolated at harvest from all the plant parts including roots, stem and leaves, both on the surfaces and in tissues through the 10 weeks of untreated slurry storage. *Salmonella spp* was on the other hand isolated on coriander roots surface and in soil mixed with urea treated slurry after two weeks of storage (Table 4.10).

In the control test, using bacteria isolates suspensions, internalized *Escherichia coli* isolates were recovered at harvest from all the tested plant parts (roots, stem and leaves) and residue isolates in soil except from the surface of the stem.

Likewise, *Campylobacter spp* was internalized and recovered at harvest from roots, stem and leaves. There was however no bacterial pathogen isolates recovered from the negative control (soil plus sterile distilled water) Table 4.10.

Table 4.10: Contaminations of coriander by pathogens in soil modified with treated and untreated peepoo at harvest (seven weeks, after planting), 1= present, 0= absent

Time (weeks)	Pathogens	Slurry (storage)		Slurry+ soil+DAP (potted)		Viability tests done at harvest (seven weeks)							
		Urea	No urea	Urea	No urea	Soil		Roots		Stem		Leaves	
						Surface	Tissue	Surface	Tissue	Surface	Tissue	Surface	Tissue
2	<i>Ascaris</i> (% viable)	100	100	100	100	0	0	0	0	0	0	0	0
	<i>E. coli</i> (cfu)log10	3.48	8.01	2.9	8.0	1.3	1.3	0	0	0	0	0	0
	<i>Campylobacter</i> <i>spp</i> (+/-)	1	1	1	1	1	1	1	1	1	1	1	1
	<i>Salmonella</i> <i>spp</i> (+/-)	1	0	1	0	1	1	0	0	0	0	0	0
10	<i>Ascaris</i> (% viable)	0	20	0	19	0	0	0	0	0	0	0	0
	<i>E. coli</i> (cfu)log10	0	0	0	0	0	0	0	0	0	0	0	0
	<i>Campylobacter</i> <i>spp</i> (+/-)	0	1	0	1	1*	1*	1*	1*	1*	1*	1*	1*
	<i>Salmonella</i> <i>spp</i> , (+/-)	0	0	0	0	0	0	0	0	0	0	0	0
Spiked		Recovered at harvest (7 weeks)											
	Controls	10log ₁₀ cfu/ml		Soil		Roots		Stem		Leaves			
				surface	tissue	surface	tissue	surface	tissue	surface	tissue		
Positive	<i>E. coli</i> (isolates no 17,18,19)			3.01	1.78	0.66	0	0.66	0.33	0.33			
	<i>Campylobacter</i> <i>spp</i> (isolates no22,23)			1	1	1	1	1	1	1	1		
Negative	<i>Sterile saline</i>			0	0	0	0	0	0	0	0		

* *Campylobacter* internalized from untreated slurry

4.5 Molecular characterization of *Campylobacter* spp.

All the 294 biochemically characterized *Campylobacter* spp isolates from the 580 baseline stool samples were subjected to PCR using genus and species specific primers. Using genus specific PCR, 106 (18.27%) isolates were confirmed *Campylobacter* spp. Out of the 106 isolates, 28 (4.83%) were *C. coli*, 44 (7.58%) *C. jejuni* while 11 (1.89%) were *C. lari*. Twenty three (3.9%) *Campylobacter* isolates were not species identified as belonging to *C. coli*, *C. jejuni* or *C. lari* (Table 4.11; Figure 4.3a, 4.3b).

Table 4.11: Molecular characterization of *Campylobacter* spp isolates from school going children stool samples

Schools	<i>Campylobacter</i> spp by PCR	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. lari</i>	Other <i>C. spp</i>
A	5	0	0	0	5
B	8	3	0	2	3
C	5	0	0	2	3
D	15	6	7	0	2
E	20	8	9	1	2
F	4	0	3	0	1
G	18	5	10	1	2
H	5	0	3	1	1
I	5	0	4	1	0
J	15	5	6	2	2
K	6	1	2	1	2
Totals	106	28	44	11	23
% Prevalence	18.27% (106/580)	4.83% (28/580)	7.58% (44/580)	1.89% (11/580)	3.90% (23/580)

Fifty-six *campylobacter* spp isolated from stored peepoo bags, slurry soil mixtures and plants were identified by PCR and characterized into 24 (43%) *C. coli*, 15

(27%) *C. jejuni* and zero *C. lari*. Seventeen (30%) of the isolates were not identified as any of the three species. The highest numbers of isolates recovered from the internalization experiment were from the soil mixtures, followed by roots, leaves and lastly stem. The isolates were recovered from both the plants' surface as well as from the plants' tissue in all the tested plant parts. The recovery was through the 10 weeks storage/ treatment period (Table 4.12).

Table 4.12: Molecular characterization of *Campylobacter* isolates from peepoo bag, soil and coriander planted with peepoo fertizer.

Location	<i>C. genus</i>	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. lari</i>	<i>Unidentified</i>
Peepoo-bag	5	1	2	0	2
Soil mix	14	5	7	0	2
Plants roots	14	10	2	0	2
Plants Stem	9	7	2	0	0
Plants Leaves	14	1	2	0	11
Total isolates	56	24 (43%)	15 (27%)	0	17 (30%)

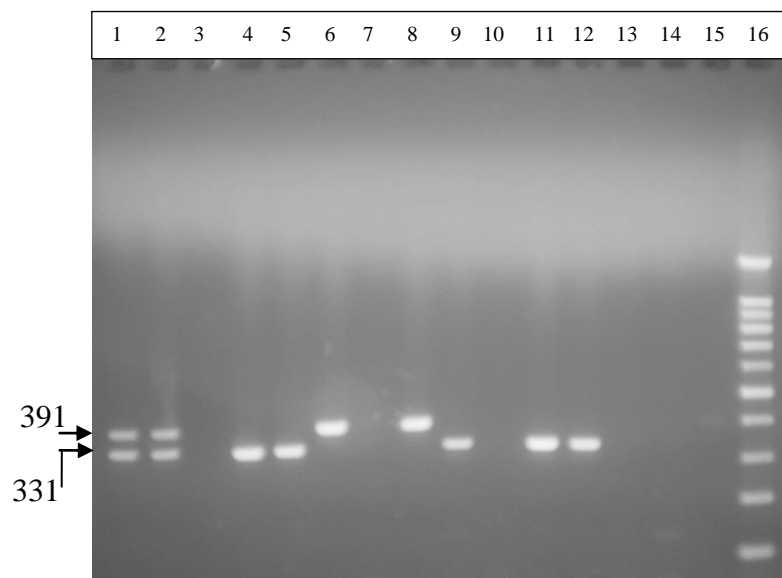


Figure 4.3a: Ethidium bromide stained 1.5% agarose gel electrophoresis of *Campylobacter coli* (391bp) and *C. jejuni* (331bp) in a multiplex PCR with a 100bp ladder. From left to right, lane 1 and 2 positive samples for *Campylobacter jejuni* and *Campylobacter coli* obtained from sequenced laboratory isolates (PHPT 1 &2). Lane 3 negative control: purified water. Lanes 4, 5, 9, 11 and 12: *C. jejuni*. Lanes 6, 8 and 15: *Campylobacter coli*. Lanes 7, 10, 13 and 14: negative samples. Lane 16: 100pb ladder.

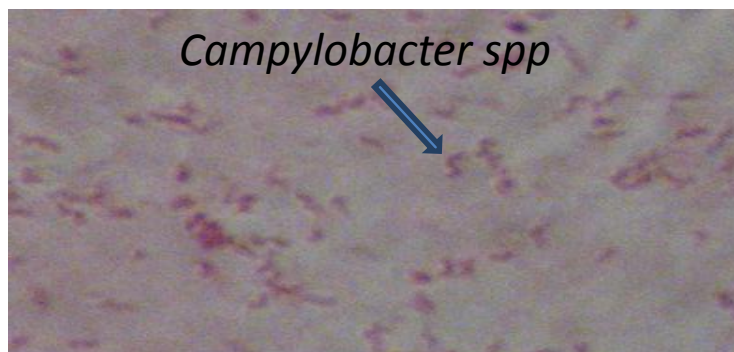


Figure 4.3b: Gram's stained *Campylobacter* spp under 1000 times magnification, showing the characteristic curved rods (→)

4.6 Molecular characterization of *Escherichia coli*

Out of 580 stool samples, 555 pooled isolates (comprising 4 characteristic *E. coli* isolates per fecal sample) were subjected to 12 *Escherichia coli* pathotyping genes for molecular characterization to six *Escherichia coli* pathotypes using multiplex PCR. Out of these, 83 (15%) were shown to have one or a combination of the genes and were thus classified into seven groups. Seventeen (3.1%) of the pools had the *eae* gene and were classified as atypical enteropathogenic. Thirty two (5.8%) pooled isolates were enterohemorrhagic forming the highest number followed by enterotoxigenic at 2.3% (13/555), diffusely adherent at 2.16% (12/555), enteroaggregative at 0.72% (4/555), typical enteropathogenic at 0.54% (3/555) and lastly enteroinvasive at 0.36% (2/555) (Table 4.13; Figure 4.4).

Table 4.13: Molecular characterization of pooled *Escherichia coli* from school stool samples with positive *E. coli* isolation (N=555)

PCR Genotype	Prevalence of pathogenic <i>E.coli</i>	Genes demonstrated
Enterohemorrhagic (EHEC)	5.76%, 32/555	<i>stx1, stx2, eae</i>
Atypical EPEC	3.06%, 17/555	<i>Eae</i>
Enterotoxigenic (ETEC)	2.34%, 13/555	<i>It, stII</i>
Diffuse adherent (DAEC)	2.16%, 12/555	<i>daaE</i>
Enteroaggregative (EAggEC)	0.72%, 4/555	<i>aafII</i>
Typical Enteropathogenic (EPEC)	0.54%, 3/555	<i>eae, bfp</i>
Enteroinvasive (EIEC)	0.36%, 2/555	<i>lpaH, VirF</i>

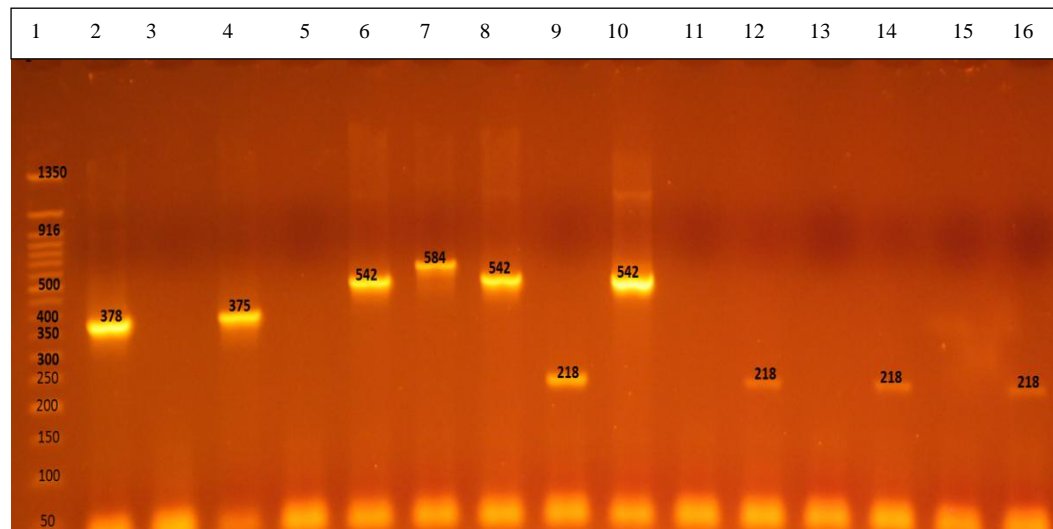


Figure 4.4: Ethidium bromide stained 1.5% agarose gel electrophoresis of pathogenic *Escherichia coli* isolates in a multiplex PCR, Lane1: 50bp ladder. Lanes 2 and 4: *aafII* gene (enteroaggregative *E. coli*). Lanes 6, 8 and 10: *daaE* gene (diffuse adherent *E. coli*), Lane 7: *stx2* gene for the enterohammorrhagic *E. coli* and Lanes: 9, 12, 14 and 16 with *It* gene for the enterotoxigenic *E. coli*. Lanes 3, 5, 11, 13, and 15 were negative samples.

A hundred and twenty three (20%) single *Escherichia coli* isolates from 113 faecal samples were subjected to PCR and characterized into 6 pathotypes, with EHEC

having 33 (27%) isolates, EPEC (typical and atypical) with 30 (24%) isolates, DAEC at 28 (23%) isolates, ETEC at 26 (21%) isolates, EA_gEC with 5 isolates and lastly EIEC had only one isolate. Multiple infections with more than one pathogenic *E. coli* were observed in 10 (8.8%) individuals. Three (2.0%) pupils had EHEC and EA_gEC combined infection, another 4 (3.5%) pupils had EPEC and EIEC. A combined infection of EHEC, EA_gEC and DAEC was observed in one (0.9%) pupil. Also observed in single individual pupils were EHEC together with EIEC and EPEC with EA_g EC.

4.7 Serological characterization of *Salmonella* spp

Using the API 20E biochemical system and confirmed with serological antisera, four salmonella isolates were identified as *Salmonella enteric* subspecies *arizonae* *IIIa*. Two of the isolates were isolated from school samples, translating to a prevalence of 0.34% (2/580), while one isolate was from treated slurry at two weeks of incubation and later on coriander roots at harvest.

4.8 Phenotypic and genotypic antimicrobial sensitivity, of bacteria pathogens isolated along the peepoo handling chain

Disk diffusion method as described by EUCAST (2016) was used to determine the resistance patterns on characterized *Campylobacter* spp. Seventy two isolates (*Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari*) were subjected to 30 mg tetracycline disks and 68 of them were resistant while 4 isolates were sensitive. Fifty-seven of the isolates were resistant to 5mg

ciprofloxacin, while 15 isolates were sensitive. Using 30mg nalidixic acid, sixty one isolates were resistant while 11 isolates were sensitive. Erythromycin (15mg) gave similar results to nalidixic acid but with different isolates (Table 4.14).

Of the four *tet* genes tested, *tet(A)* was most frequently identified have been seen in 20 of the isolates followed by *tet(O)* (8 isolates), *tet(B)* (6 isolates) and *tet(C)* with only 2 isolates. None of the isolates had more than one *tet* genes demonstrated. Quinolones' gene *gyrA* was demonstrated in all 72 isolates, seven of which were isolated from peepoo, 8 from soil mixtures and 3 from plant tissues. The remaining were demonstrated in isolates from baseline isolates. Gene *gyrB* was in 69 isolates with the peepoo big bag isolates and soil having 6 each and three from plant tissues. Sulfonamides' *sul1* gene was found in 36 isolates with 6 from the peepoo big bag, 5 from soil and none from plant tissue while *sul2* gene was found in 27 isolates, one from plant tissues and the rest 26 from school baseline samples (Table 4.14).

Table 4.14: Drug resistant patterns of pathogenic *Campylobacter spp* isolates from school stool samples (n=65)

Antimicrobial agent	CLSI subclass	Reaction patterns (EUCAST, 2016)		Resistance encoding genes by PCR
		Resistant (%)	Sensitive	
Tetracycline, (30mg)	Tetracyclines	68 (94.4)	4 (5.5)	tetA (20), tetB (6), tetC (2), tetO (8)
Ciprofloxacin (5mg)	Quinolones	57 (79.1)	15 (20.8)	gyrB (69); bb (6), s (6), p (3)
Naladixic acid (30mg)		61 (84.7)	11 (15.2)	gyrA (72) bb (7), s (8), p (3)
Erythromycin (15mg)	Macrolides	61 (84.7)	11 (15.2)	Genotyping not done
Trimethoprim-sulfamethoxazole (30mg)	Sulfonamide	na	Na	sul1 (36), bb (6), s (5), p (0) sul2 (27), bb (0), s (0), p (1)

Key: peepoo big-bag (n= 5) soil (n=2) and plants (n=3), N=72, using EUCAST disk diffusion method (2016), Key: tetA, tetB, tetC & tetO = tetracyclines encoding genes; gyrA, gyrB= Quinolones encoding genes; sul1 & sul2 = Sulfonamide encoding genes; bb = peepoo big bag; s = soil, p = plants, na= not applicable

4.8.1 Multidrug resistant profiles in *Campylobacter spp* isolates

Campylobacter jejuni had the highest number of isolates that were MDR with 26 out of the 28 tested being resistant to all the four antibiotics tested, namely; Ciprofloxacin (5mg), Nalidixic acid (30mg), Tetracycline (30mg) and Erythromycin (15mg). *Campylobacter coli* had 16 of the 17 isolates tested resistant to all the four antibiotics while *C. lari* had the least isolated but all the 13 isolates were resistant to the four antibiotics. Twelve of the unidentified isolates were resistant to Nalidixic acid (30mg), Tetracycline (30mg) and Erythromycin (15mg) (Table 4.15)

Table 4.15: *Campylobacter spp* multidrug resistance (MDR) profile with the four tested antibiotics

<i>Campylobacter jejuni</i> n = 28		
Drug profile	No of MDR Isolates (%)	No. of sensitive isolates (%)
Ciprofloxacin (5mg)	26 (93)	2 (7.1)
Nalidixic acid (30mg)	28 (100)	0
Tetracycline (30mg)	28 (100)	0
Erythromycin (15mg)	28 (100)	0
<i>Campylobacter coli</i> n=17		
Drug profile	MDR Isolates	Sensitive isolates
Ciprofloxacin (5mg)	17 (100)	0
Nalidixic acid (30mg)	16 (94)	1 (5.9)
Tetracycline (30mg)	16 (94)	1 (5.9)
Erythromycin (15mg)	17 (100)	0
<i>Campylobacter lari</i> n=13		
Drug profile	MDR Isolates	Sensitive isolates
Ciprofloxacin (5mg)	11 (85)	2 (15)
Nalidixic acid (30mg)	12 (92)	1 (7.6)
Tetracycline (30mg)	13 (100)	0
Erythromycin (15mg)	13 (100)	0
<i>Campylobacter</i> genus (unidentified species) n=12		
Drug profile	MDR Isolates	Sensitive isolates
Ciprofloxacin (5mg)	3 (25)	9 (75)
Nalidixic acid (30mg)	12 (100)	0
Tetracycline (30mg)	12 (100)	0
Erythromycin (15mg)	12 (100)	0

4.8.2 *Salmonella spp*

All the four isolates were only phenotypically resistant to tetracycline (30mg) according to CLSI breakpoints, they were sensitive to all the other 11 antimicrobial agents. However, genotypically, genes encoding for the other antimicrobial subclasses were found in the isolates. Isolate 7c that was isolated from school baseline samples had *gyrA*, *sul1* and *tetC* genes, Isolate 120a, also from school baseline samples had *gyrA*, *gyrB*, *tetA* and *sul2* genes. Isolate 108sa

recovered from treated slurry had only the *gyrA* gene, while isolate 109sb recovered from plant roots had *tetA* and *gyrA* genes (Table 4.16).

Table 4.16: Drug resistant patterns of *Salmonella* spp isolates from school stool samples (n=2), treated slurry (n=1), plants (n=1)

Isolates	Storage period in urea (weeks)	Antibiotic susceptibility pattern	Expressed resistant gene
7c	0	Te ^r , CIP ^s , NA ^s , SXT ^s , CRO ^s , ANC ^s , CAZ ^s , AMP ^s , C ^s , CN ^s , S ^s , CXM ^s	<i>gyrA</i> , <i>sul1</i> , <i>tetC</i>
120a	0	Te ^r , CIP ^s , NA ^s , SXT ^s , CRO ^s , ANC ^s , CAZ ^s , AMP ^s , C ^s , CN ^s , S ^s , CXM ^s	<i>gyrB</i> , <i>gyrA</i> , <i>sul2</i> , <i>tetA</i>
108sa	2	Te ^r , CIP ^s , NA ^s , SXT ^s , CRO ^s , ANC ^s , CAZ ^s , AMP ^s , C ^s , CN ^s , S ^s , CXM ^s	<i>gyrA</i>
109sb	2	Te ^r , CIP ⁱ , NA ^s , SXT ^s , CRO ^s , ANC ^s , CAZ ^s , AMP ^s , C ^s , CN ^s , S ^s , CXM ^s	<i>gyrA</i> , <i>tetA</i>

Key: Te^r; Tetracycline (30mg), CIP^s; Ciprofloxacin (5mg), NA^s; Naladixic acid (30mg), SXT^s; Trimethoprim-Sulfamethaxone (25mg), CRO^s; Ceftriaxone, AMC^s; Amoxicillin-clucianic acid (10mg), CAZ^s; Ceflazime (30mg), AMP^s; Ampicillin (10mg), C^s; Chlonamphenical (30mg), CN^s; Gentamycin (10mg), S^s; Streptomycin (10mg), CXM^s; Cefuroxicin (30mg) ^r =Resistant; ⁱ = Intermediate; ^s = Sensitive

4.8.3 *Escherichia coli*

Phenotypically, the antimicrobial subclass with the highest number of resistant isolates was sulfonamides with 115 (70%) isolates, this was followed by aminopenicillin with 97 (59%) isolates, tetracyclines had 91 (55%), aminoglycoside with 74 (45%) isolates, fluoroquinolones at 37 (22%) and phenicols had 24 (15%) resistant isolates. The other three subclasses had less than 10 (6%) resistant isolates. Genes encoding resistance to three antimicrobial agents subclasses were tested in 74 out of 165 phenotypically resistant isolates. One

(1.4%) isolate had *tetA* gene, 6 (8.1%) had *tetB* gene, 14 (19%) had *tetC* gene and none had *tetO* gene. Quinolones encoding gene (*gyrA*) was found in 56 (76%) resistant isolates while 4 (5.4%) isolates had *gyrB* gene. Sulfonamides gene (*sulI*) was only found in 10 (14%) isolates while none of the isolates had the *sul 2* gene (Table 4.17).

Table 4.17: Drug resistant patterns of pathogenic *Escherichia coli* isolates from stool samples, N=165

Antimicrobial agents used for disc diffusion	CLSI subclass	Reaction patterns (CLSI, 2007) N=165 (%)			Resistance encoding genes tested, n=74
		R	I	S	
Tetracycline, (30mg)	Tetracyclines	91(55)	0	74	tetA (1), tetB (6), tetC (14) tetO (0)
Ciprofloxacin (5mg)	Fluoro-quinolones	10(6)	7	148	gyrA (56)
Naladixic acid (30mg)		27(16)	8	130	gyrB (4)
Trimethoprim-sulfamethoxazole (30mg)	Sulfonamides	115(70)	0	50	sul1 (10), sul2 (0)
Ceftriaxone (30mg) 3rd gen'	Cephalosporin	7(4)	0	159	Genotype not done
Cefuroxime (30mg) 2nd gen'		1(0.7)	4	136	Genotype not done
Amoxillin-clavulanic acid (30mg)	Aminopenicillins + betalactamase	7(4)	14	144	Genotype not done
Ceftazidime (30mg)	Cephalosporin	3(2)	0	162	Genotype not done
Ampicillin (10mg)	Aminopenicillin	97(59)	6	62	Genotype not done
Chloramphenicol (30mg)	Phenicol	24(15)	5	136	Genotype not done
Gentamycin (10mg)	Aminoglycosides	6(3.6)	5	154	Genotype not done
Streptomycin (10mg)		68(41)	43	54	Genotype not done

Key: tetA, tetB, tetC and tetO = tetracyclines encoding genes; gyrA, gyrB= Quinolones encoding genes; sul1 and sul2 = Sulfonamide encoding genes. R= resistant; I= intermediate; S= sensitive; gen'=generation

4.8.4 Multidrug resistant profiles of *E. coli* isolates

One (0.6%) *E.coli* isolate was resistant to the eight antibiotics tested, 3 (1.8%) were resistant to seven drugs, 6 (3.6%) were resistant to six antibiotics, 18 (10.9%) were resistant to 5 drugs while 33 (20%) were resistant to 4 drugs. Thirty five (21.2%) were shown to be resistant to 3 drugs while those resistant to 2 antibiotics were 16 (9.6%) (Table 4.18).

Table 4.8: Multidrug resistant *E.coli* profiles with 45 different drug profiles

Antibiotic resistance profiles	
No. of resistant drugs	No. of resistant isolates, N= 165 (%)
0	31 (18.7)
1	21 (12.7)
2	16 (9.6)
3	35 (21.2)
4	33 (20)
5	18 (10.9)
6	6 (3.6)
7	3 (1.8)
8	1 (0.6)

4.9 Molecular sequencing of selected pathogens isolated along the Peepoo handling chain.

4.9.1 *eae* gene

The *eae* gene encoding the outer membrane protein, was demonstrated in 5 *Escherichia coli* isolates submitted for gene sequencing. The *eae* gene in the 5 isolates had BLASTN alignment identity of 99%, 98% and 94% with standard *E. coli* O157:H7, EP 057 isolate and *E.coli* O157:H7 respectively. These similarities

confirmed the identity of the PCR products obtained from *Escherichia coli* isolated from asymptomatic school going pupils (Table 4.19 and Appendix viii).

4.9.2 *Lt* gene (heat labile enterotoxin)

The genes were demonstrated as *eltB* and *eltA* flanked by IS600 and IS1294. Two isolates with the heat labile enterotoxin B encoding gene were submitted for sequencing. The sequences alignment with *blastn* gave a 99% and 84% identity for isolates 103a and 582a respectively. Isolate 103a had no nucleotide gaps upon alignment when 582a had 1% nucleotide alignment gaps (Table 4.19 and Figure 4.5).

4.9.3 *St II* gene (heat stable enterotoxin)

Two isolates, 15b and 2d recovered from a soil samples from school compound and from internalization test, soil peepoo mixture were submitted for sequencing. The PCR products for the *stII* gene was sequenced and the sequences aligned using *blastn* giving 74% and 100% alignment for 15b and 2d respectively (Table 4.19 and Appendix viii).

4.9.4 *bfpA* gene

One isolate with the *bfpA* gene encoding for major structure subunit of bundle forming pilus, had gene alignment identity at 99% and an expert value of $3e-123$ (Table 4.19 and Appendix viii).

Table 4.19: *Escherichia coli* pathogenic genes in selected isolates and their sequenced homologue and identity obtained from NCBI genebank using BLASTn, SGC=school going children

Isolates information			Alignment information (NCBI genebank)	
Pathogenic genes by PCR (accession No)	<i>E. coli</i> isolates ID	Source of the isolates	Homologue obtained by BLASTn	% identity of isolates with homologue
eae (EF540941.1)	143c	Peepoo SGC	<i>E. coli</i> O157:H7 strain ATCC 43890	429/432(99%)
eae (KT591233.1)	250c	Peepoo SGC	<i>E. coli</i> eae gene, serovar: O153:H7	432/435(99%)
eae	158d	Peepoo SGC	<i>E. coli</i> strain EP057_beta1 intimin (eae) gene	432/435(99%)
eae (AB647617.1)	188a	Peepoo SGC	<i>E. coli</i> eae gene for intimin	86/88(98%)
eae	216d	Peepoo SGC	<i>E. coli</i> strain EP057_beta1 intimin (eae) gene,	412/436(94%)
Lt (LN870273.1)	103a	Peepoo SGC	<i>E. coli</i> eltB and double eltA flanked by IS600 and IS1294	173/174(99%)
Lt (AB011677.1)	582a	Peepoo SGC	<i>E. coli</i> (from human) heat-labile enterotoxin subunit B gene (eltB),	130/154(84%)
stII (M35586.1)	15b	Soil-school	<i>E. coli</i> heat-stable toxin II (STII) gene	117/158(74%)
stII (M357229.1)	2d	Green house soil	<i>E. coli</i> heat-stable enterotoxin II (STII) gene	19/19(100%)
bfp (AB247934.1)	158b	peepoo	<i>E. coli</i> bfp[A,B,C,D,E,F,G,H,I,J,K,L,P,U] genes	248/250(99%)

4.9.5 Drug susceptibility genes for tetracycline (*tetA* and *tetB*) genes

Three isolates 68t, 103a and 310a submitted for sequencing were analyzed by blastn for nucleotides alignment. Tetracycline resistance protein (*tetA*) gene aligned with *E. coli* strain EPEC for isolates 103a and 310a at 99% identity. Isolate 68t was *Campylobacter jejuni* recovered from coriander roots, which aligned at

84% identity with transposon Tn10 *tetB* gene, a tetracycline efflux MFS transporter *Tet (B)* (Table 14.20 and Appendix viii).

Table 4.20: Drugs susceptibility encoding genes demonstrated in selected isolates and their sequenced homologue and identity obtained from NCBI genebank using BLASTn, SGC= school going children

Isolates information			Genebank alignment information	
Pathogenic genes demonstrated by PCR	isolates ID	Source of the isolates	Homologue obtained by BLASTn	% identity of isolates with homologue
<i>Tet A</i> (CP0176546.1)	68t	roots	Transposon Tn10 (<i>tetB</i>) gene for tetracycline efflux MFS transporter	53/63 (84%)
<i>Tet A</i> (KU892720.1)	103a	Peepoo SGC	<i>E. coli</i> strain EPEC tetracyclin resistance protein (<i>tetA</i>) gene	525/530 (99%)
<i>Tet A</i> (KU892720.1)	310a	Peepoo SGC	<i>E. coli</i> strain EPEC tetracyclin resistance protein (<i>tetA</i>) gene	525/530 (99%)

The phylogenetic analyses showed four distinct groups of the pathogenic *E. coli*. Cluster 1 had one EHEC isolate number 158d clustering with reference EPEC strains 0157H7, accession no. EF540941.1 and two EPEC reference isolates U95502.2 and AB647523. Cluster 2 had two EHEC isolates 250c and 216d. Cluster 3 had two EPEC, isolates 103a and 158d with one ETEC isolate 582a. cluster 4 was made up two EHEC isolates 250e and 188e and one EPEC isolate 143e. (Figure 4.5).

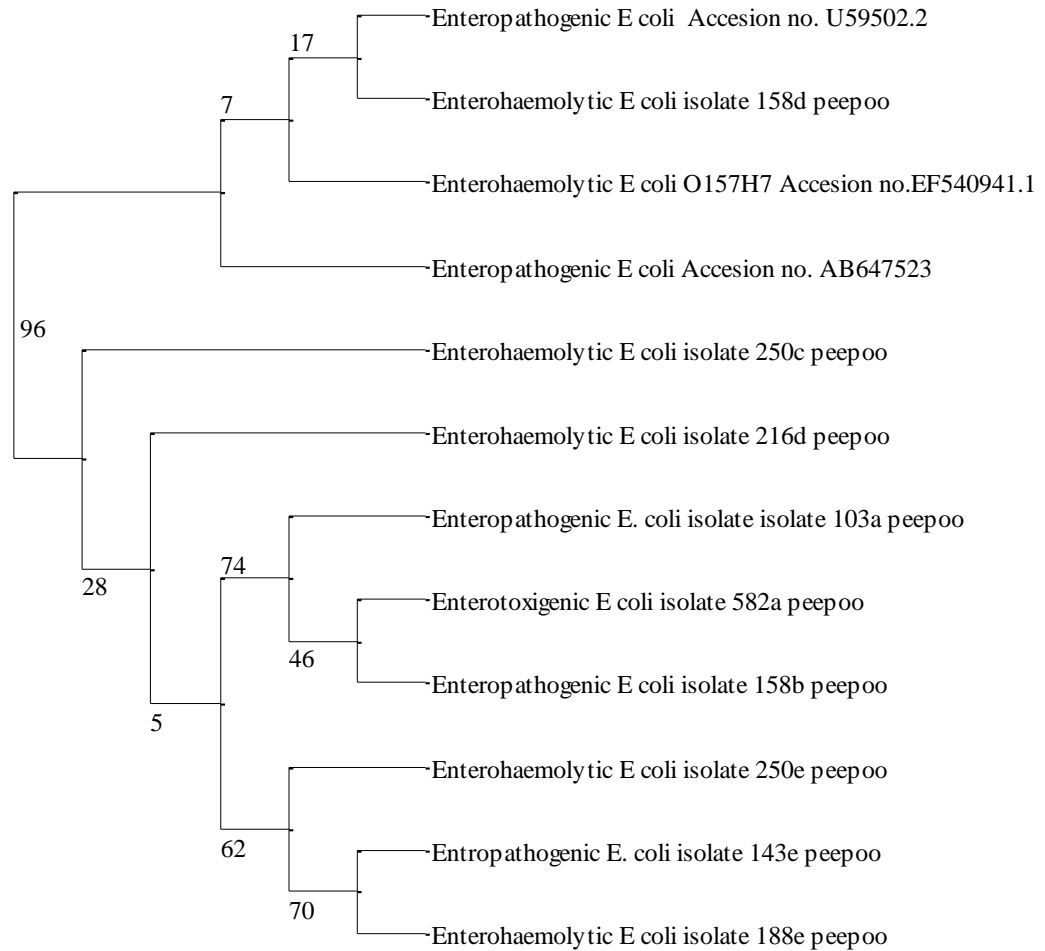


Figure 4.5: Phylogenetic tree of 9 pathogenic *E. coli* isolated from different school going children (peepoo) that were distributed across the study schools. Three pathogenic *E. coli* from GenBank with accession numbers U59502.2, EF540941.1 and AB647523 were used as reference. Bootstrap confidence levels are indicated as percentage of replicates occurring at the tree nodes.

CHAPTER FIVE: DISCUSSIONS

5.1 Prevalence of geo-helminths and protozoa in the study population

In this study, *A. lumblicodes* and *T. trichura* were detected at 20% and 12.7% respectively. Hookworms were, on the other hand not detected at all in the 580 tested samples. These findings are consistent with Davis *et al.* (2014), who reported an infection heterogeneity prevalence of 40.7% for school-aged children at Kibera slums. The authors also reported *A. lumblicodes* infection at 22.7%, *T. trichura* at 28.8% and hookworm was less than 0.1% out of 692 tested samples. Infection of school going children with *E. histolytica* was at 34.1% in this study, which was in agreement with those of Kavili *et al.* (2016), who reported a prevalence of 41.6% *E. histolytica* in school going children in a rural set up. Davis *et al.* (2014) also reported no significant difference in infections at Kibera slums.

There was a marked difference in parasitism distribution among study schools. School E (GPS, -1.319225821, 36.7954909), that is located at lower grounds near Nairobi dam with poor drainage, had the highest prevalence of *Ascaris lumblicoides* in stool at 54% and in both the water and soil samples. School B, located at higher grounds with good, drainage (GPS, -1.311242166, 36.79197589), had a prevalence of *Ascaris lumblicoides* in stool at 10.5%, and none in water (Figure 4.1a). The observed differences in parasitic prevalence infections could then be partly a factor of topography and personal hygiene. High infections were

observed in schools at the lower end of the drainage. These schools have high flooding episodes during rainy seasons. Those located at higher altitudes had relatively low infections. The argument is strengthened by demonstration of STH in soil and domestic water samples obtained from the study schools.

5.2 Prevalence of bacteria pathogens, Rotavirus and Adenovirus in stool samples

This study had *Salmonella enterica* isolated from two asymptomatic school going children out of the 580 samples analyzed, giving a prevalence of 0.34%. Similar findings have been reported by Kariuki *et al.* (2006), who reported a prevalence of 6.9% of non-typhoid *Salmonella* (NTS), 0.42% of which were *Salmonella enterica* from asymptomatic children and adults.

A prevalence of 18.3% PCR confirmed *Campylobacter spp* isolates was demonstrated in this study. The bacteria were isolated from asymptomatic school going children. *Campylobacter spp* isolation from healthy children has been reported in developing countries as well by Coker *et al.* (2008) at 14.9%, which was close to this study's findings. The authors attributed the infections with *Campylobacter* to close contact with reservoir animals like chicken as well as poor sanitation. Both of these factors are prominent in this study area, where chickens share housing with their owners. Pathogenic *Escherichia coli* were characterized using multiplex PCR to demonstrate the presence of characteristic virulent genes. A prevalence of 17% obtained in this study was much lower compared to what

was reported by Rono *et al.* (2014), a prevalence of 34.2% in diarrhea samples. The difference in the observations could be that while this study sampled asymptomatic pupils, Rono *et al.* (2014) worked with diarrhea patients.

In the current study, all the 90 asymptomatic children tested were negative for Rotavirus by both the Prospect-Rotavirus enzyme immunoassay (Oxoid, Hants, UK) and one step rapid immunochromatographic assay (SD standard diagnostics inc, Kyonggi-do, Korea). Seasonal variation of rotavirus has been reported (Parashar, 2008; Breiman *et al.*, 2014). This could explain the reason for the undetected infections during this study and the discrepancies to a study by Breiman *et al.* (2014), who reported a peak incidence during the cool, dry months of July and August in the four years of study (2009-2010). The authors detected Rotavirus in 11.9% of diarrhea samples and at 2.1% of non-diarrhea samples from children below 5 years at the current study area. The current study collected samples in 2 weeks of early July 2015 and worked with non- diarrhea asymptomatic children, which could also be a reason for the none detection. It is therefore important as echoed by Breiman *et al.* (2014), to carry out multiple years surveillance while applying different study approaches to get true picture of rotavirus infection. Periods of vaccination and sample testing should also be considered as a factor of viral infection detection and surveillance.

It is assumed that children below 5 years are the main culprit of rotavirus infection. It is postulated that effective infants immunization could go a long way in protecting children as well as adults. This will also reduce transmission of rotavirus (Desai *et al.*, 2011). The findings of Breiman *et al.* (2014) agrees with earlier reports that rotavirus crude incidence rates has an annual substantial variation, demonstrating up to 53% difference in prevalence between two years (Parashar, 2003). Diagnosis of rotavirus infection by ELISA is an established method, with a sensitivity of 100% and specificity of 89%. The method has been adopted by WHO as the standard diagnostic technique for epidemiological studies.

Adenovirus was tested using Prospect-Adenovirus enzyme immunoassay (Oxoid, Hants, UK) and one step rapid, immunochromatographic assay (SD standard diagnostics inc, Kyonggi-do, Korea) in 90 under five years asymptomatic children. Only one (1.11%) was positive for Adenovirus. Magwalivha *et al.* (2010) reported 16.4% Adenovirus in non-diarrheal stool specimens while Moyo *et al.* (2014) reported much lower non-diarrhea Adenovirus at 2.4%, both in Tanzania primary school children. Studies on Adenovirus have shown seasonality, with Moyo *at el.* (2014) having detected nothing in three months during one-year surveillance study. This could as well be an explanation of the low detection during this study period.

5.2.1 Prevalence of bacteria pathogens, rotavirus and adenovirus in water and soil samples

The high level of soil contamination with both thermo-tolerant *E. coli* (100%) and *Campylobacter spp* (100%) in this study is in agreement with findings by Christabel *et al.* (2012). The authors reported 88.9% soil contamination with enteric bacteria pathogens. Water was also contaminated though to a lesser extent, since 50% of the schools had water with thermo-tolerant coliforms out of which 2 (20%) were thermos-tolerant *E.coli* while 4 (40%) of the schools had *Campylobacter spp*. Higher water contamination at 76.1% and 95% coliforms have been reported at Kibera slums (Chemuliti *et al.*, 2002; Christabel *et al.*, 2012). This could be attributed to the difference in sampling seasons between the two studies and the fact that this study specifically detected TTC as opposed to the previous studies that reported total coliforms.

Drinking water should be void of *E. coli* and TTC in any 100ml sample according to WHO guidelines as well as Kenya standard (KS-05-459) on bacteriological quality of drinking water. The presence of TTC in water is a clear indication that Kibera residents are exposed to a great health risk. Infection with pathogenic *E.coli* is already an established bacterial risk that is of great public health importance. Other enteric pathogens are bound to infect the population as well. This is evidenced by the 40% *Campylobacter spp* identified in drinking water in this study.

Waterborne outbreaks due to *Campylobacter spp* and to some extent, pathogenic *E. coli* has been reported world over. Other common identified agents, to a lesser extent, include *E. histolytica*, *Cryptosporidium spp* and *Salmonella spp* (WHO, 2001). Most waterborne outbreaks in Kenya go unreported or the causative agents are not identified most of the time.

All the soil samples obtained from the study school compound tested positive for both *Campylobacter spp* and *E. coli*. This clearly indicates a high level of human and of animal excreta contaminated environment. The exposure to disease causing agents found in human excreta is thus transmitted through the environment with ease. This poses a public health problem and conditions in the study area qualify as a breeding ground for future's pathogens (Humphrey, 2009; IRIN, 2010).

5.4 Treated peepoo bags and slurry

The process of human faecal treatment aimed at pathogen reduction to acceptable levels of unrestricted reuse as suggested by WHO (2006) guidelines on human excreta reuse and EC (2006). The guidelines allow reduction to less than 1000 cfu per a gram of total solids for *E. coli* and less than one viable helminth egg (*Ascaris spp*) per one gram of total solids and zero *Salmonella spp* in 25g w/w as well as 3 log₁₀ in heat resistant viruses (EU, 2006; WHO, 2006). In this study, treated excreta in both peepoo bags and in the slurry preparation achieved and surpassed the WHO (2006) guidelines at the 6th week of treatment. There were zero viable

Ascaris spp compared with the initial 100% viability, which considering the number of eggs counted, corresponds to more than a $3\log_{10}$ reduction of egg viability.

Temperature was shown to have effect in the peepoo bags and slurry treatments, giving a significant prediction of *E. coli* and coliforms viability. A similar observation had been made by Nordin *et al.* (2009) and Vinneras *et al.* (2008), who attributed micro-organisms inactivation to a combination of ammonia and storage temperature. Apart from indicator organisms, pathogens like *Campylobacter spp* and *Salmonella spp* behaved in a similar way as *E.coli*, showing a positive prediction with temperature but not with pH. This observation is in agreement with that of Nordin *et al.* (2009), who reported sufficient reduction of *Salmonella spp* and consequently, other enteric pathogens within 8 months and 1 month when 2% urea was added at 24 °C and 34 °C respectively.

Ascaris spp inactivation in peepoo bags was observed to be affected by ammonia concentration and storage time with pH and temperature having no significant predictive value. However, with the urea treated slurry, temperature had a significant prediction on the storage time needed for inactivation of viable *Ascaris spp* eggs.

The Peepoo bag can be used as a safe toilet bag bearing in mind that the bag is restricted to a single use and by only one person. The prove that urea added in the bag is able to inactivate pathogens even at high concentrations as shown in the current study is a good indication of safety and ability to contain pathogens thus protecting both the environment and people from faecal contamination an observation also made by Ahmad (2012).

It is an observation of the current study that urea treatment of human excreta in Peepoo bags makes it safe for reuse as a soil conditioner and as a cheap readily available fertilizer. Treated human excreta particularly by use of urea has been shown to contain plants nutrients at comparatively higher levels than animal manure. This is due to the added urea and partially utilized plant nutrient released in human excreta. The peepoo bag used in Kenya has been shown to contain high crop nutrients required for crop production. On average, a peepoo bag will have 5.57g nitrogen (N), 0.50g phosphorous (P) and 0.57 g potassium (K) (Jonsson *et al.*, 2004; Nordin, 2010; Zhang, 2010; Ahmad, 2012).

Human excreta an average of 250-350g per person in one-day in low income countries. This translates to an average of 2500g nitrogen and 400g phosphorous per year, which is a huge amount of crop nutrients. The ratios may differ depending on the type of food in different communities and cultures (Jonsson *et al.*, 2004). However, human excreta is made safe by treatment and shown to

relatively improve soil for crop production. Its applicability in the wet state as present in Peepoo bags will be a challenge (Ahmad, 2012). Therefore, there is need to modify the appearance and to some extent balance the nutritional value with readily available organic materials hence increasing its dry matter content. This will not only mask its appearance but will also improve its application as an alternative fertilizer (Nordin, 2010; Ahmad, 2012).

5.5 Internalization of micro-organisms via plant roots

In this study, it was evident with the control experiment using $10 \log_{10}$ cfu/ml of bacteria suspension that *Campylobacter spp.*, *E.coli* and *Salmonella enterica* gained entry through the roots. All the three were detected in roots, stem and leave tissue of coriander plant at harvest in 7 weeks. Coriander was also grown on both treated and untreated slurry in potted soil with pathogen bacteria at different concentrations, the lowest being $3 \log_{10}$ cfu/g in treated slurry. The coriander roots surface had *E. coli* and *Salmonella enterica* at $1.3 \log_{10}$ by 7 weeks of planting in slurry-soil mixture. However, *Campylobacter spp.* was recovered in coriander roots, leaves and stem at 7 weeks post inoculation. Kroupiski *et al.* (2009) described the process of internalization of *Salmonella enterica* in lettuce leaves. The authors demonstrated the penetration by chemotaxis through open stomata during the process of photosynthesis and production of plant nutrients that attracted the bacteria cells. They also observed that increased bacteria internalization occurred at elevated temperatures between 25-34 °C.

Similar observations have been made by different authors; Bernstein *et al.* (2007) demonstrated *Salmonella* internalization through roots at 500cfu/g and identified it in leaves at 130 cfu/g of 33 day old lettuce, 2 day post-inoculation. Franz *et al.* (2007) also reported internal colonized *Salmonella typhimurium* and *E. coli* O157:H7 in leaves of lettuces at 35 days post-inoculation in potted soil. Ge *et al.* (2012) demonstrated internalization of *Salmonella spp* in lettuces and green onions leaves at 2 days post-inoculation. They also reported increased internalization in water stressed lettuce as opposed to green onions. Onions were not affected by water stress at a contamination of 4 log₁₀ cfu/g. Wright *et al.* (2013) contaminated growth media with 7 log₁₀cfu/g *E.coli* and planted lettuce and spinach. The authors demonstrated internalization 10 days post-inoculation in 81% spinach and 31% lettuce roots and leaves respectively. The authors observed formation of internal bacteria colonies in the plant apoplast at the 9th day of inoculation. This followed bacteria association with plant epidermal cells of the plant roots at day 6, which progressed to bacteria penetration below root surface and formation of internal colonies.

The coriander plants were planted in soil mixed with human excreta prepared as slurry using either untreated slurry or slurry with treated urea at different storage periods, ranging from 2 to 10 weeks. The concentration of pathogenic and indicator bacteria were reduced by the treatment process or/and mixing with potted

soil to a concentration of 3 log₁₀ cfu/g being too low to gain entry and colonize plant tissues. This was demonstrated in this study, since the control, plants that were inoculated with bacteria suspension at 10 log₁₀ had bacteria internalization but those grown on excreta slurry which had reduced inoculum showed only contaminated root surface.

5.6 Molecular characterization of *Campylobacter*

This study obtained 18.27% prevalence of *Campylobacter spp* from asymptomatic children by PCR method. These were further characterized as 4.8% *Campylobacter coli*, 7.6% *Campylobacter jejuni*, 2% *Campylobacter lari* and other unspecified species at 4%. Similar findings on *Campylobacter spp* has been reported by Coker *et al.* (2002), who reported an isolation rate of 20% in developing countries, 14.9% of which was from asymptomatic children. The high prevalence can be attributed to close contact with *Campylobacter* reservoir animals, including chicken early in life as well as poor sanitation (Nguyen *et al.*, 2016). No seasonal variations have been reported with *Campylobacteriosis* in developing countries, most likely due to lack of extreme temperature variation.

Campylobacter jejuni was isolated more frequently as compared to *C. coli* and *C. lari* in this study. This observation agrees with other reports on both developed and developing countries including Kenya (Oberhelman and Taylor, 2000; Coker *et al.*, 2002; Brooks *et al.*, 2003). Isolation of *Campylobacter spp* from asymptomatic

children would be an issue of virulence factors, the cytolethal distending toxin able to induct host cell apoptosis. Pathogenesis could also be influenced by host immune system and pathogens adaptation strategies (Dasti *et al.*, 2009). Other factors like motility and chemotaxis affect effective *Campylobacter* colonization and pathogenesis, which have been shown to vary in mutants (van Vliet and Ketley, 2001).

5.7 Molecular characterization of *E. coli*

Molecular analysis of *E.coli* isolates revealed that 13 (2.3%) samples had the enterotoxigenic (ETEC) genes, LT or the ST 2. Vidal *et al.* (2005) described ETEC having one or more enterotoxins; heat labile (LT) LT1 and LT2 or the heat stable (ST) as STa and STb. Three children had typical EPEC with both the *eae* and *bfp* genes. Another 17 pupils had atypical EPEC having only the *eae* gene. These findings compare well with those reported by Bugarel *et al.* (2011) and Vidal *et al.* (2005), who observed a ratio of 1:3 and 1:4 typical to atypical EPEC respectively. EPEC has proteins involved in attaching and effacement of the host cells microvilli. It has the EPEC adherence factor (EAF) plasmid and gene cluster encoding the bundle-forming pili (*bfp*) gene. Typical EPEC are those isolates with the EAF plasmid. Those with the *bfp* gene but lack the EAF plasmid are classified as atypical EPEC.

The least number of isolates was with EIEC which had only 2 (0.36%) children positive. This compares with other findings where none out of 509 stool samples yielded EIEC (Vidal *et al.*, 2005). Rono *et al.* (2014) made comparable observations, with EIEC isolation being the second last frequently isolated, at 3 out of 100 samples. EIEC possess a gene located in a virulence plasmid (PInv) 140 MDa encoding type III secretion system.

Enterohemolytic *Escherichia coli* had the highest number of isolates, with 32 out of 555 children being positive. The isolates had a combination of *eae* gene with either *stx1* or *stx2* genes. Several authors have reported different distributions in the prevalence of different pathogenic *E. coli*. Some have reported EAEC as the most frequent, others had EAggEC being the most frequent, while ETEC was shown by a different study as the most frequent. EPEC has also been identified by a different study as the most prevalent in Kenya (Bii *et al.*, 2005; Makobe *et al.*, 2012; Sang *et al.*, 2012a; Sang *et al.*, 2012b).

Unlike Vidal *et al.* (2005), who observed one patient out 509 with mixed enteropathogenic *E. coli*, this study had 10 individuals out of 555 with mixed enteropathogenic infections. Out of the 10, nine had combinations of two different enteropathogenic *E. coli*, one of the children had three pathogenic *E.coli* characterized (EHEC, EAggEC and DAEC).

5.8 Serological characterization of *Salmonella* spp

This study isolated 2 (0.34%) non Typhi *Salmonella* (NTS) from 580 stool cultures from asymptomatic school going children between ages 5-13 years. Tabu *et al.* (2012) reported a comparable finding in urban Kenya, on blood cultures where only 1 out of 77 (1.3%) samples ages 5-9 had NTS. The authors also reported NTS in ages 10-17 and ages 0-5 at 0% and 7.2% respectively. However, the authors did not isolate the serotype *arizonae* from blood and stool samples that was isolated in this study. Yegon *et al.* (2012) reported similar findings in a study on asymptomatic food handlers in Nairobi, with a percentage occurrence of serotypes Typhimurium and Typhisuis at 0.25%.

Association between malaria infection and NTS bacteremia has been described, with higher bacteremia in individuals harboring malaria infection and those with malarial anemia (Graham *et al.*, 2000; Scott *et al.*, 2011). Malaria infection result in dysfunctional macrophages phagocytosis, leading to release of free iron, which promotes growth of *Salmonella* spp (Morpeth *et al.*, 2009; Mackenzie *et al.*, 2010). This is a possible explanation of the low infection with NTS in this study area, which has very low to no malaria infections, an observation also made by Tabu *et al.* (2012).

Salmonella arizonae IIIa has been reported to cause severe enteritis and septicemia in chicks and turkey pouts. The subspecies is commonly reported in reptiles, and

most frequently in snakes. It has however been isolated from malnourished infants and immunocompromised individuals, with close contact with reptiles (Mahajan, 2003). In humans, the subsp. *arizonae* causes gastroenteritis and systematic infection. The only reported *Salmonella arizonae* IIIa so far is by Muthumbi *et al.* (2015), who isolated one subsp. *arizonae* from 521 bacteremia samples in a study at Kilifi, Kenya.

5.9 Antimicrobial Sensitivity Tests (AST)

Selection of number of disks per plate is guided by plate size and the intended use of the results (clinical or epidemiological). Ordinarily, no more than 5 disks should be placed on a 10 cm agar plate and no more than 12 disks should be placed on a 15 cm agar plate. In a clinical setting, only ampicillin, a quinolone and/or fluoroquinolone, and trimethoprim-sulphamethoxazole should be reported for faecal isolates of *Salmonella* and *Shigella*. Chloramphenicol and a third-generation cephalosporin should also be tested and reported for extra-intestinal isolates of *Salmonella*. Other agents and drug classes (e.g. aminoglycosides) may provide valuable epidemiologic data; however, *in-vitro* susceptibility of *Salmonella* to these agents may not correlate with *in-vivo* efficacy (CLSI, 2015).

Sometimes when disks are placed closely together, interaction between antimicrobials may produce distortion of inhibition zones (i.e. antagonism, synergism, inhibition and/or induction). Such valuable additional information

should not be considered in the reading of the inhibition zones but provides important information about the putative mechanism of resistance, bacterial identity among others.

5.9.1 *Campylobacter spp* AST

Human *campylobacter spp* resistant to tetracycline had more tet (A) genes than tet (O) gene which were found in 20 and 8 isolates respectively. Nguyen *et al.* (2016) echoing these findings, reported to have identified more tet (A) genes than tet (O) genes in Kenya *Campylobacter spp* isolates from chicken at 35% and 13% respectively. Multidrug resistant *Campylobacter* isolates were demonstrated in this study, with the highest number showing resistance to tetracyclines with 64 (88.8%) isolates of the 72 tested being resistant to tetracycline 3mg. Quinolones (Ciprofloxacin, 5mg) had relatively few resistant isolates with 57 (79%) of them, while macrolides (erythromycin, 15mg) had 61 (84.7%) resistant isolates and 11 sensitive isolates. The high resistant rates obtained in this study are in agreement with Nguyen *et al.* (2016) and Coker *et al.* (2002) findings on chicken and human *Campylobacter* isolates respectively. These authors reported more than 70% resistance to ciprofloxacin, nalidixic acid and tetracycline. However these results contrasts a report on human *Campylobacter* from diarrhea cases in Western Kenya, which had ciprofloxacin resistance at 6%, nalidixic acid 26%, and 18% for tetracycline. Erythromycin resistance, in this study was also high which contrasts Nguyens *et al.* (2016) finding on chicken *Campylobacter*. Ciprofloxacin and erythromycin are the drugs of choice for *Campylobacter* treatment. These drugs

are often used for self-treatment in Kenya for infections other than gastroenteritis. For this reason, resistance is on the increase in developing countries (Coker *et al.*, 2002).

5.9.2 Antibiotic sensitivity test for *Salmonella enterica* subspecies *arizonae*

The rare human *Salmonella* subsp *arizonae* isolated in this study was only resistant to tetracycline by the Kirby-Bauer disk diffusion method. This was in contrast to a reported subsp *arizonae* isolate that was MDR to chloramphenicol, tetracycline, nalidixic acid and gentamicin (Tabu *et al.*, 2012). The isolate in this study however, had genes encoding for resistant to quinolones, sulfonamides and tetracycline that were demonstrated using PCR method. In a study on characterization of antibiotics resistance in environmental enteric pathogens from Kibera slum in Nairobi, Kenya, Christabel *et al.* (2006) reported MDR *Salmonella* Typhimurium isolated from food commodities. The isolates were resistant to tetracycline, ampicillin and sulphamethoxazole+trimethoprim. However, *Salmonella* subsp *arizonae* being a rare human isolate may not have been extensively exposed to antibiotics and hence the resistance pattern observed in this study.

5.9.3 Antibiotic sensitivity test for *Escherichia coli*

Resistance to sulfonamides was the highest at 70%, this was followed by ampicillin at 59%, tetracycline 55%, streptomycin 41%, nalidixic acid at 16% and

chloramphenicol at 15%. The findings in this study agree with Christabel *et al.* (2012) who reported high resistance of environmental isolates from Kibera slums. In her study, ampicillin had the highest frequency at 57% while sulfonamides come second at 30% and tetracycline at 19%. Though the frequencies were not in agreement with the current study, the general profile of resistance is comparable. In this study cephalosporin (ceftriaxone, cefuroxime and ceftazidime) were the most sensitive with the resistance frequencies at 4%, 0.7% and 2% respectively. These are second and third generation antibiotics and resistance by *E. coli* is mainly by the extended spectrum beta-lactamases (ESBLs) enzymes. The enzymes are able to destroy most of beta-lactam antibiotics. ESBLs can be transferred between bacteria species. In a report on resistance to antibacterial drugs in selected bacteria of international concern, WHO (2014); the report gave a range of resistance *E. coli* to 3rd generation cephalosporin in African region at 2-70%. This was within the levels of this study which had 4% resistance to ceftriaxone.

Only 31 (18.7%) pathogenic isolates were sensitive to all the 12 antibiotics tested, 21 (12.7%) were resistant to only one of the 12 antibiotics while the other 113 (68.5%) were resistant to more than 2 antibiotics thus classified as multidrug resistant (MDR) isolates. Isolates resistant to 3 antibiotics were 35(21.2%). Those resistant to any four of the 12 antibiotics were 33 (20%), resistant to 5 antibiotics were 18 (10.9%), 6 antibiotics were 6(3.6%) and those resistant to 7 antibiotics were 3(1.8%) isolates.

Multidrug resistant *E. coli* has been reported on environmental isolates at 40% from the same study area. These findings have slightly lower frequency compared to those reported in this study, which could be attribute to the differences between human isolates and environmental isolates. The findings however agrees with Kipkorir *et al.* (2016) who reported 42.2 % MDR *E. Coli* isolates from fecal specimens obtained from patients ages 2weeks to 82 years. The patients had gastroenteritis and the study was in Kitale, Kenya. Oundo *et al.* (2008) also reported 65.5% MDR *E. coli* isolates from asymptomatic food handlers in Kenya, which was very close to this study's 68.5%. They reported resistance to cefuroxime at 6.9% which was much higher than 0.7% in the current study.

5.10 Phylogenetic analysis

Using PCR products specific genes were targeted for sequencing to confirm their true identity. Sequencing information is used for in-depth genetical analysis including establishing genetical relationship among study isolates, identification of genetic variations and gene mapping (Bando *et al.*, 2007). Gel electrophoresis incorporating molecular weight markers, gives an indication of the PCR product identity by their molecular size. This technique is limited in revealing underlying genetical information of the amplified gene (Yildirim *et al.*, 2011). It was therefore necessary to have selected PCR products submitted for sequencing. Due to cost, only ten isolates were submitted for sequencing.

Phylogenetic analysis showed two divergent EPEC groups, atypical and typical types, with the atypical EPEC occupying a unique cluster as was also described by Bando *et al.* (2007). The second cluster had both the *Lt* and *eae* genes bearing ETEC and EHEC respectively. This was a heterogeneous cluster with two distinct pathotypes that may have originated from same descendant and thus clustering together. Similar observations of ETEC clustering together with EHEC has been made by other authors, though they used virulence genes and serotypes (Bando *et al.*, 2007; Bugarel *et al.*, 2011).

The *eae* gene characteristic for EPEC was sequenced and blastn alignment gave 99% identity with *E. coli* O157:H7 strain ATTC 43891, accession No. EF540941, this homology confirmed identity of the sequenced PCR product. Similarly, the *Lt* gene and *stII* gene for the EHEC gave sequence alignment identity at 99% and 100% respectively with *E. coli* accession No. LN870273, a clear indication of virulence in the isolates. The *bfp* gene encoding for major structure subunit of bundle forming piles, that was found in EPEC together with the *eae* gene had a 99% blastn alignment with GenBank *E. coli* accession No. AB247934, proving that diarrheagenic *E. coli* were in did isolated from asymptomatic children in the current study. Similar findings have been reported by Quiroga *et al.*, (2000), who demonstrated the presence of EPEC, DAEC, ETEC and EA_gEC in symptomatic infants. The authors also described 7.5 months of life as the earliest time of *E. coli*

colonization. The presence of diarrheagenic *E. coli* in asymptomatic individuals has been reported in several studies and it is attributed to a number of observations including, development of protective antibodies early in life against diarrheagenic *E. coli*. This has been shown in children growing in endemic areas (Quiroga *et al.*, 2000). Host susceptibility is also proposed as a course. Though the actual process leading to diarrhea is not clearly understood (Donnenberg and Finlay, 2013). Non-specific host barriers, as internal microbiota, intact mucus layer and epithelial cell layer prevents diarrheal episodes (Levine and Robin, 2012). Bacterial factors inclining some pathogens to diarrhea has also been shown in EPEC (Hu and Torres, 2015).

Sequenced *tet a* gene from enteropathogenic tetracycline resistant *E. coli* showed 99% homogeneity with related *tet a* sequences in GenBank. The fact that 55% of tested *E. coli* and 89% *Campylobacter spp* were resistant to tetracycline and the demonstration of *tet a* genes in some of the isolates. Would suggest that genes were freely transferable between bacteria *in-vivo* in cases of multi-bacterial infections observed in this study. The homogeneity observed is a frequent finding with tetracycline resistance encoding genes in *E. coli* and really in *Campylobacter*. *Tet a* gene has been reported by AbdiHachesoo *et al.* (2014) to be located on bacterial mobile elements and thus horizontally transferable among bacteria strains.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The following conclusions were made from this study

- i) School going children in Kibera slums have parasitic infections of *Ascaris lumbricoides* (20%), *Trichuris trichiura* (13%), *Entamoeba histolytica* (28%), *Entamoeba coli* (34%), *B. coli* (3.4%), *H. nana* (1.9%) and *Cryptosporidium spp* (2.4%). Pathogenic *Escherichia coli* were at a prevalence of 7.0%. *Salmonella enterica* subspecies *arizonae* IIIa, a rear subsp was the only *Salmonella* isolated at 0.34%, while 50% of the children had *Campylobacter* infection (*Campylobacter jejuni* (44%), *Campylobacter coli* (28%), *Campylobacter lari* (11%) other *Campylobacter spp* (23%). Adenovirus was at 1.11% while none of the asymptomatic school going children had Rotavirus.

- ii) Peepoo bag with urea reduced the viability of thermo-tolerant coliforms, *Escherichia coli*, *Campylobacter spp*, *Salmonella enterica* and *Ascaris lumbricoides* to the WHO (2006) recommendations on reuse of treated human excreta. Eight weeks of urea treatment, in peepoo bags and faecal slurry achieved the recommended levels of reuse of human excreta. Human pathogenic bacteria contaminating soil at a minimum of 3log₁₀ cfu/g only allowed bacteria attachment to coriander roots with no evident internalization. However higher soil contamination with human pathogenic bacteria (10log₁₀ cfu/g) allowed internalization through coriander roots and detection in the

roots, stem and leaves. Treating of faecal matter to WHO guidelines on reuse of treated human excreta will thus stop pathogenic bacteria from plant internalization.

iii) Pathogenic *Escherichia coli* (69%) including EHEC (6.0%), EPEC (3.5%), ETEC (2.0%), DEAC (2.0%), EAEC (0.7%), EIEC (0.4%) and *Campylobacter jejuni* (92%), *Campylobacter coli* (88%), *Campylobacter lari* (76.9%) and *Campylobacter spp* (25%) isolated and genetically identified were antibiotic resistant. Viral infection (Rotavirus and Adenovirus) was not identified as a health issue among the school-going children during the study period. Using DNA sequences analytical programs, Basic Local Alignment Search Tool (BLAST) it was possible to confirm identity of pathogenic *E. coli* and show relatedness between isolates obtained from different individual and environments.

6.2 RECOMMENDATIONS FROM THIS STUDY

i) School going children and by extension, the general population at Kibera slum should undergo mass deworming. This should be conducted by the Ministry of Health with close consultations with stakeholders in the area. The deworming program should be undertaken every three months followed by annual testing to evaluate its impact.

- ii) Personal hygiene programs should be an ongoing process in schools.

- iii) Use of Peepoo bag should be encouraged as a form of human waste management and for public health with particular emphasis in slums, camping sites as well as military training and operation camps.

6.3 RECOMMENDATIONS FOR FURTHER RESEARCH

- i) An in-depth study should be undertaken on isolates obtained from this study to establish the relationship between pathogenic bacteria and asymptomatic children.

- ii) Human excreta corrected and inactivated in peepoo bags should be further processed to remove smell and form to encourage reuse as soil conditioner (fertilizer).

- iii) The ability of enteric pathogens to survive in plant tissue and adopt effectively with possibilities of gene exchange needs more study.

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APPENDIX I**Approval letter by Graduate School**

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke

Website: www.ku.ac.ke

P.O. Box 43844, 00100

NAIROBI, KENYA

Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 28th October, 2014

TO: Mr. Nduhiu Gitahi Johnson
C/o Zoological Sciences Dept.
Kenyatta University

REF: I84/25743/2011

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board at its meeting of 29th September, 2014 approved your Research Proposal for the Ph.D. Degree. Entitled "Molecular Characterization and Serotyping of Enteric Pathogens for Validation of "Peepoo" Sanitization along Handling Chain at Urban Informal Settlement, Kenya".

You may now proceed with data collection, subject to clearance with the Permanent Secretary, Ministry of Higher Education, Science and Technology.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking forms per semester. The form has been developed to replace the progress report forms. The supervision Tracking Forms are available at the University's website under Graduate School webpage downloads.

Thank you.


REUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Zoological Sciences Dept.

Supervisors:

1. Dr. Micheal M. Gicheru
C/o Zoological Sciences Dept.
KENYATTA UNIVERSITY
2. Dr. Peter B. Gathura
Public Health Pharmacology and Toxicology
C/o Zoological Sciences Dept.
KENYATTA UNIVERSITY
3. Dr. Annika Nordin
Department of Energy and Technology
Swedish University
C/o Zoological Sciences Department
KENYATTA UNIVERSITY

APPENDIX II Ethical clearance by Kenyatta University



KENYATTA UNIVERSITY ETHICS REVIEW COMMITTEE

Email: chairman.kuerc@ku.ac.ke
secretary.kuerc@ku.ac.ke
ercku2008@gmail.com
 Website: www.ku.ac.ke

P. O. Box 43844 - 00100 Nairobi
 Tel: 8710901/12
 Fax: 8711242/8711575

Our Ref: KU/R/COMM/51/416

Date: 26th February, 2015

Nduhiu Gitahi Johnson
 Kenyatta University
 P.O. Box 43844 -00100, Nairobi.

Dear Nduhiu,

APPLICATION NUMBER PKU/278/I274 – “MOLECULAR CHARACTERIZATION AND SEROTYPING OF ENTERIC PATHOGENS FOR VALIDATION OF “PEPOO” SANITIZATION ALONG HANDLING CHAIN AT URBAN INFORMAL SETTLEMENT, KENYA.”

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic, “Molecular Characterization and Serotyping of Enteric Pathogens for Validation of “PEEPOO” Sanitization along Handling Chain at Urban Informal Settlement, Kenya.” Received on 6th January, 2015, discussed on 17th February, 2015.

2. APPLICANT

Nduhiu Gitahi Johnson

3. SITE

Kibera Informal Settlement, Nairobi County, Kenya.

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines AND APPROVED that the research may proceed for a period of ONE year from 26th February, 2015.

5. ADVICE/CONDITIONS

- i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
- ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.
- iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
- iv. Submit an electronic copy of the protocol to KUERC.

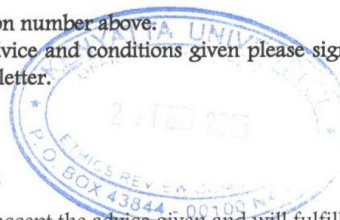
When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.


 PROF. NICHOLAS K. GIKONYO
 CHAIRMAN ETHICS REVIEW COMMITTEE

I Nduhiu Gitahiaccept the advice given and will fulfill the conditions therein.


Signature Nduhiu Gitahi Dated this day of 29/03 2015.
 cc. Vice-Chancellor



APPENDIX III Research permit - NACOSTI

THIS IS TO CERTIFY THAT:
MR. JOHNSON NDUHIU GITAHI
of UNIVERSITY OF NAIROBI, 29053-625
Nairobi, has been permitted to conduct
research in Nairobi County
on the topic: MOLECULAR
CHARACTERIZATION AND SEROTYPING
OF ENTERIC PATHOGENS FOR
VALIDATION OF "PEEPOO"
SANITIZATION ALONG HANDLING CHAIN
AT URBAN INFORMAL SETTLEMENT,
KENYA.
for the period ending:
31st December, 2017

Permit No : NACOSTI/P/14/6545/4121
Date Of Issue : 27th November, 2014
Fee Received :Ksh 2,000




[Signature]
Applicant's
Signature


[Signature]
Secretary
National Commission for Science,
Technology & Innovation

CONDITIONS

- 1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit**
- 2. Government Officers will not be interviewed without prior appointment.**
- 3. No questionnaire will be used unless it has been approved.**
- 4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.**
- 5. You are required to submit at least two(2) hard copies and one(1) soft copy of your final report.**
- 6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice.**



REPUBLIC OF KENYA



National Commission for Science,
Technology and Innovation

RESEARCH CLEARANCE
PERMIT

Serial No. A 3756

CONDITIONS: see back page

APPENDIX IV Research Authorization – NACOSTI



**NATIONAL COMMISSION FOR SCIENCE,
TECHNOLOGY AND INNOVATION**

Telephone: +254-20-2213471,
2241349, 310571, 2219420
Fax: +254-20-318245, 318249
Email: secretary@nacosti.go.ke
Website: www.nacosti.go.ke
When replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No.

Date:

27th November, 2014

NACOSTI/P/14/6545/4121

Johnson Nduhiu Gitahi
University of Nairobi
P.O. Box 30197-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on *“Molecular characterization and serotyping of enteric pathogens for validation of “PEEPOO” sanitization along handling chain at urban informal settlement, Kenya.”* I am pleased to inform you that you have been authorized to undertake research in **Nairobi County** for a period ending **31st December, 2017.**

You are advised to report to **the County Commissioner and the County Director of Education, Nairobi County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.


SAID HUSSEIN
FOR: SECRETARY/CEO

Copy to:

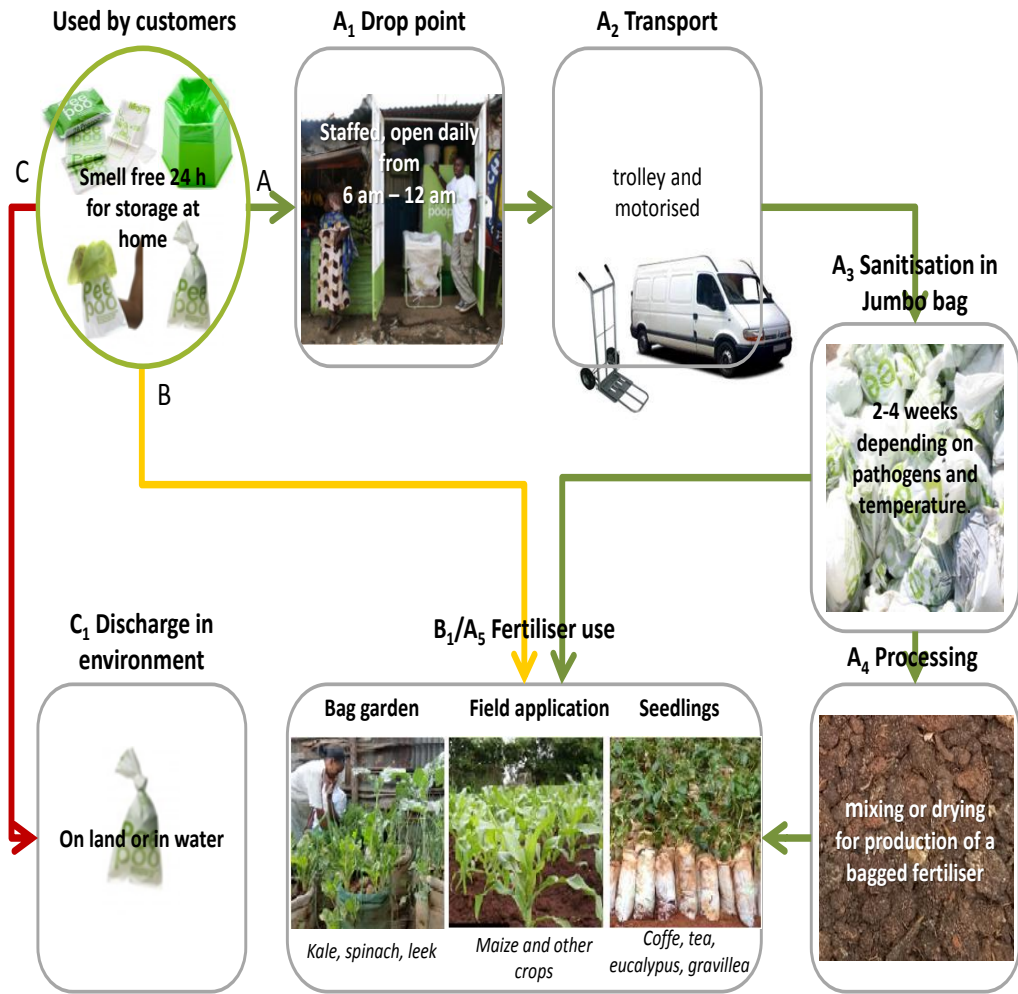
The County Commissioner
Nairobi County.

The County Director of Education
Nairobi County.

COUNTY COMMISSIONER
NAIROBI COUNTY
P. O. Box 30124-00100, NBI
TEL: 341666



APPENDIX V Peepoo sanitization and reuse chain



Overview of the Peepoo sanitization and reuse chain (A) and direct use as of Peepoo as fertilizer (B) and discharge in the environment (C), (Peepople Kenya Ltd.)

APPENDIX VI. Clinical and Laboratory Standards Institute (CLSI) Guideline M100, Table 2A (Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae.

Table 2A
Enterobacteriaceae
M02 and M07

44

Table 2A. Zone Diameter and Minimal Inhibitory Concentration Interpretive Standards for *Enterobacteriaceae*

<p>Testing Conditions</p> <p>Medium: Disk diffusion: Mueller-Hinton agar (MHA) Broth dilution: cation-adjusted Mueller-Hinton broth Agar dilution: MHA</p> <p>Inoculum: Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard</p> <p>Incubation: 35°C ± 2°C; ambient air Disk diffusion: 16 to 18 hours Dilution methods: 16 to 20 hours</p>	<p>Routine QC Recommendations (See Tables 4A and 5A for acceptable QC ranges.)</p> <p><i>Escherichia coli</i> ATCC® 25922 <i>Pseudomonas aeruginosa</i> ATCC® 27853 (for carbapenems) <i>Escherichia coli</i> ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations)</p> <p>When a commercial test system is used for susceptibility testing, refer to the manufacturer's instructions for QC test recommendations and QC ranges.</p>
--	--

* ATCC® is a registered trademark of the American Type Culture Collection.

Refer to Tables 3A, 3B, and 3C for additional testing recommendations, reporting suggestions, and QC.

General Comments

- (1) For disk diffusion, test a maximum of 12 disks on a 150-mm plate and **no more than 6 disks** on a 100-mm plate; disks should be placed no less than 24 mm apart, center to center (see M02, Subchapter 3.6). Each zone diameter should be clearly measurable; overlapping zones prevent accurate measurement. Measure the diameter of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. Hold the Petri plate a few inches above a black background illuminated with reflected light. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., ignore the thin veil of swarming growth in an otherwise obvious zone of growth inhibition. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.
- (2) When fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be reported routinely. In addition, for extraintestinal isolates of *Salmonella* spp., a third-generation cephalosporin should be tested and reported, and chloramphenicol may be tested and reported if requested. Susceptibility testing is indicated for typhoidal *Salmonella* (*S. Typhi* and *Salmonella* Paratyphi A–C) isolated from extraintestinal and intestinal sources. Routine susceptibility testing is not indicated for nontyphoidal *Salmonella* spp. isolated from intestinal sources.
- (3) The dosage regimens shown in the comment column below are those required to achieve plasma drug exposures (in adults with normal renal and hepatic functions) on which breakpoints were based. When implementing new breakpoints, it is strongly recommended that laboratories share this information with infectious diseases practitioners, pharmacists, pharmacy and therapeutics committees, and infection control committees.

NOTE: Information in boldface type is new or modified since the previous edition.

Table 2A. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria (nearest whole mm)				MIC Interpretive Criteria (µg/mL)				Comments
			S	SDD	I	R	S	SDD	I	R	
PENICILLINS											
A	Ampicillin	10 µg	≥17		14–16	≤13	≤8		16	≥32	(4) Results of ampicillin testing can be used to predict results for amoxicillin. See comment (2).
B	Piperacillin	100 µg	≥21		18–20	≤17	≤16		32–64	≥128	
O	Mecillinam	10 µg	≥15		12–14	≤11	≤8		16	≥32	(5) For testing and reporting of <i>E. coli</i> urinary tract isolates only.
O	Ticarcillin	75 µg	≥20		15–19	≤14	≤16		32–64	≥128	
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS											
B	Amoxicillin-clavulanate	20/10 µg	≥18		14–17	≤13	≤8/4		16/8	≥32/16	
B	Ampicillin-sulbactam	10/10 µg	≥15		12–14	≤11	≤8/4		16/8	≥32/16	
B	Piperacillin-tazobactam	100/10 µg	≥21		18–20	≤17	≤16/4		32/4–64/4	≥128/4	
B	Ticarcillin-clavulanate	75/10 µg	≥20		15–19	≤14	≤16/2		32/2–64/2	≥128/2	
CEPHEMS (PARENTERAL) (Including cephalosporins I, II, III, and IV. Please refer to Glossary I.)											
<p>(6) WARNING: For <i>Salmonella</i> spp. and <i>Shigella</i> spp., first- and second-generation cephalosporins and cephamycins may appear active <i>in vitro</i>, but are not effective clinically and should not be reported as susceptible.</p> <p>(7) Following evaluation of PK-PD properties, limited clinical data, and MIC distributions, revised interpretive criteria for cephalosporins (cefazolin, cefotaxime, ceftazidime, ceftizoxime, and ceftriaxone) and aztreonam were first published in January 2010 (M100-S20) and are listed in this table. Cefuroxime (parenteral) was also evaluated; however, no change in interpretive criteria was required for the dosage indicated below. When using the current interpretive criteria, routine ESBL testing is no longer necessary before reporting results (ie, it is no longer necessary to edit results for cephalosporins, aztreonam, or penicillins from susceptible to resistant). However, ESBL testing may still be useful for epidemiological or infection control purposes. For laboratories that have not implemented the current interpretive criteria, ESBL testing should be performed as described in Table 3A.</p> <p>Note that interpretive criteria for drugs with limited availability in many countries (eg, moxalactam, cefonicid, cefamandole, and cefoperazone) were not evaluated. If considering use of these drugs for <i>E. coli</i>, <i>Klebsiella</i>, or <i>Proteus</i> spp., ESBL testing should be performed (see Table 3A). If isolates test ESBL positive, the results for moxalactam, cefonicid, cefamandole, and cefoperazone should be reported as resistant.</p> <p>(8) <i>Enterobacter</i>, <i>Citrobacter</i>, and <i>Serratia</i> may develop resistance during prolonged therapy with third-generation cephalosporins as a result of derepression of AmpC β-lactamase. Therefore, isolates that are initially susceptible may become resistant within three to four days after initiation of therapy. Testing of repeat isolates may be warranted.</p>											
A	Cefazolin	30 µg	≥23		20–22	≤19	≤2		4	≥8	(9) Interpretive criteria are based on a dosage regimen of 2 g every 8 h. See comment (7). For UTI interpretive criteria, see below under CEPHEMS (ORAL).
C	Ceftaroline	30 µg	≥23		20–22	≤19	≤0.5		1	≥2	(10) Interpretive criteria are based on a dosage regimen of 600 mg every 12 h.

APPENDIX VII. EUCAS Breakpoints for *Campylobacter*.

Campylobacter jejuni and *coli*

EUCAST Clinical Breakpoint Tables v. 6.0, valid from 2016-01-01

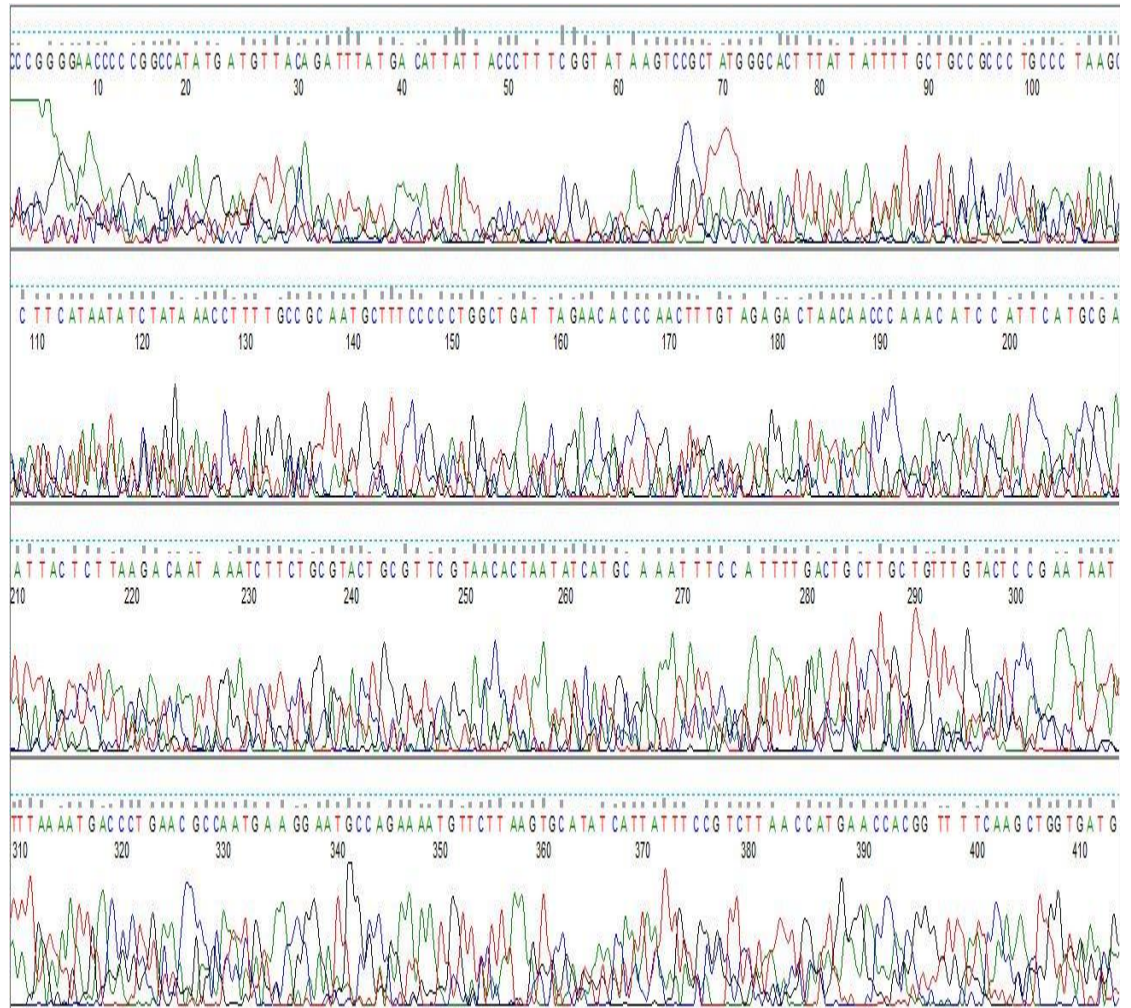
Disk diffusion (EUCAST standardised disk diffusion method)
 Medium: Mueller-Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F). The MH-F plates should be dried prior to inoculation to reduce swarming (at 20-25°C overnight or at 35°C, with the lid removed, for 15 min).
 Inoculum: McFarland 0.5
 Incubation: Microaerobic environment, 41±1°C, 24h. Isolates with insufficient growth after 24h incubation are reincubated immediately and inhibition zones read after a total of 40-48h incubation.
 Reading: Read zone edges as the point showing no growth viewed from the front of the plate with the lid removed and with reflected light.
 Quality control: *Campylobacter jejuni* ATCC 33560

Fluoroquinolones	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method.
	S ≤	R >		S ≥	R <	
Ciprofloxacin	0.5	0.5	5	26	26	

Macrolides	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method.
	S ≤	R >		S ≥	R <	
Azithromycin	Note ¹	Note ¹		Note ^A	Note ^A	1/A. Erythromycin can be used to determine susceptibility to azithromycin and clarithromycin.
Clarithromycin	Note ¹	Note ¹		Note ^A	Note ^A	
Erythromycin, <i>C. jejuni</i>	4 ¹	4 ¹	15	20 ^A	20 ^A	
Erythromycin, <i>C. coli</i>	8 ¹	8 ¹	15	24 ^A	24 ^A	

Tetracyclines	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes Numbered notes relate to general comments and/or MIC breakpoints. Lettered notes relate to the disk diffusion method.
	S ≤	R >		S ≥	R <	
Doxycycline	Note ¹	Note ¹		Note ^A	Note ^A	1/A. Tetracycline can be used to determine susceptibility to doxycycline.
Tetracycline	2 ¹	2 ¹	30	30 ^A	30 ^A	

APPENDIX VIII. Sequences of selected pathogenic *Escherichia coli* isolates



Sequence 1. Isolate 250c enterohaemolytic *Escherichia coli*

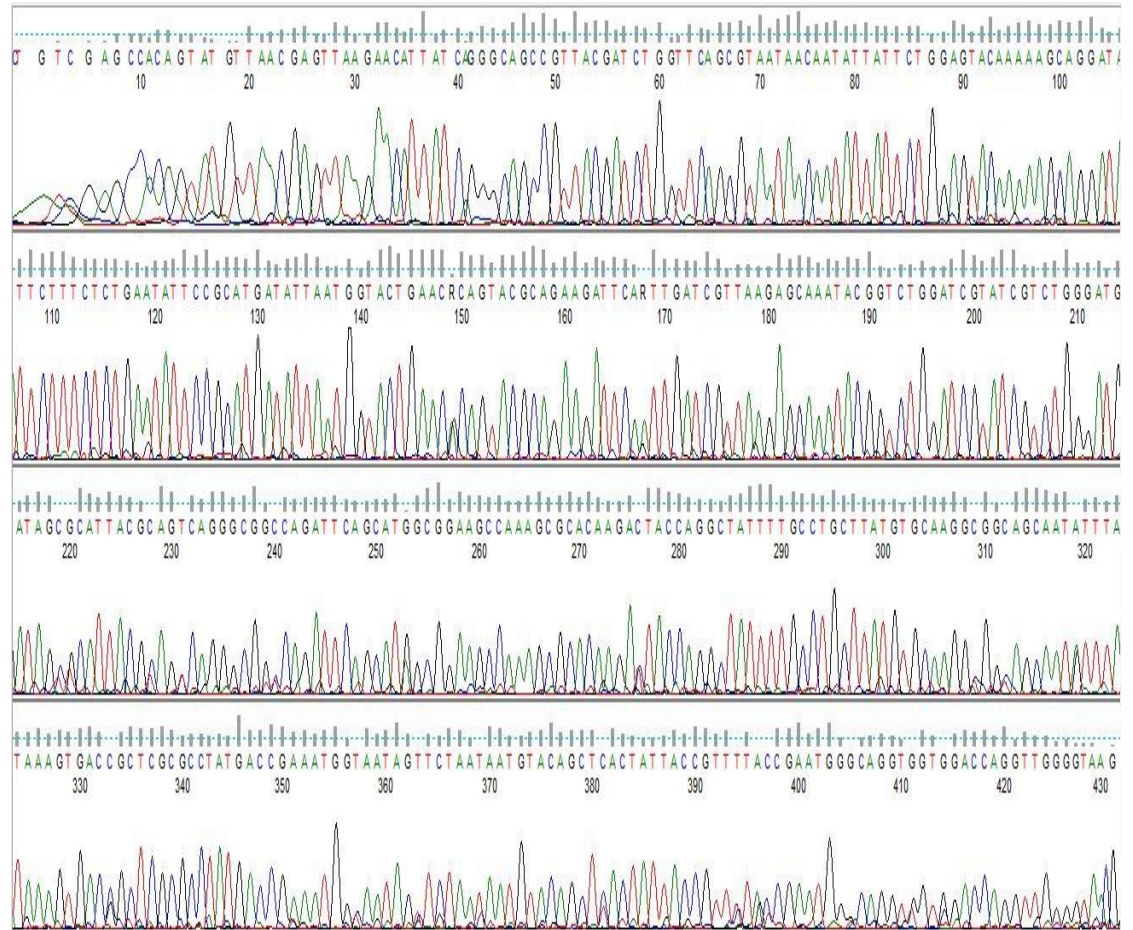
Key: nucleotides colour codes

A adenosine

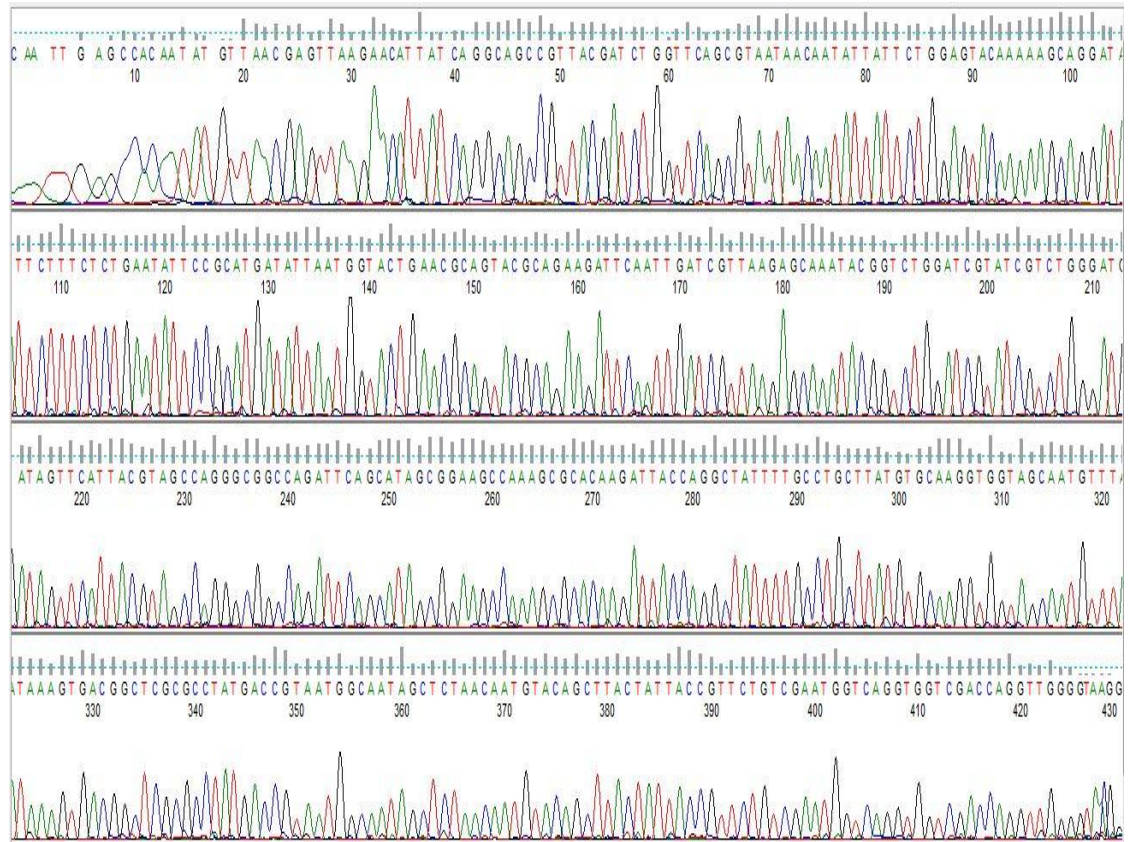
C cytosine

G guanine

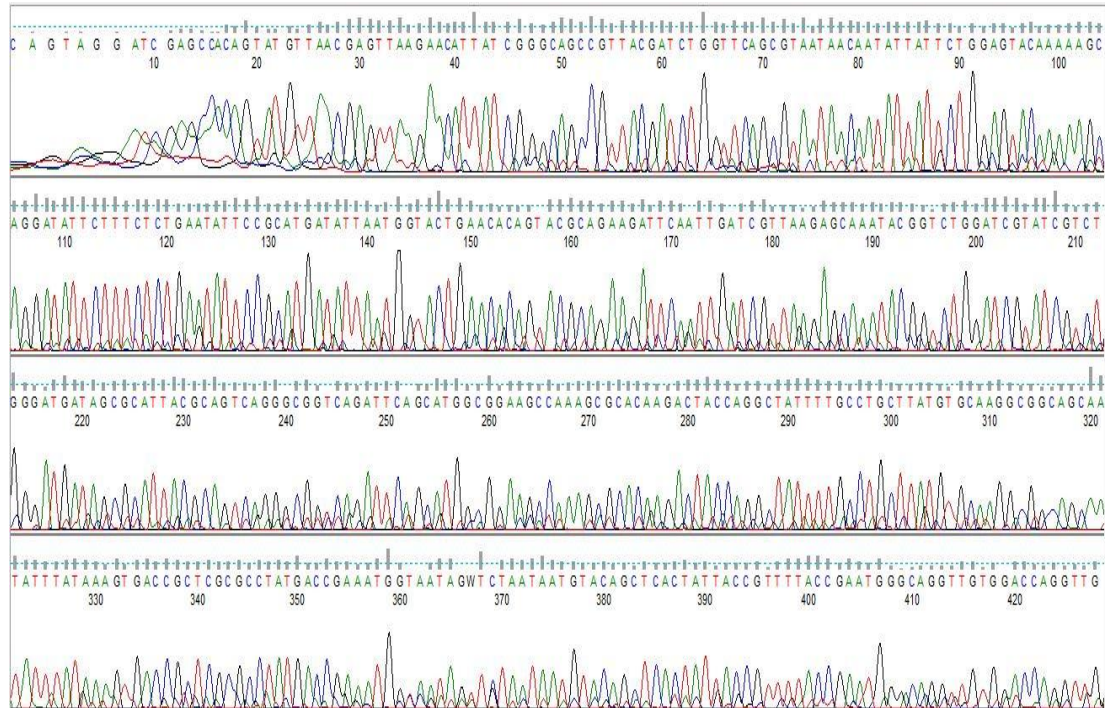
T thymine



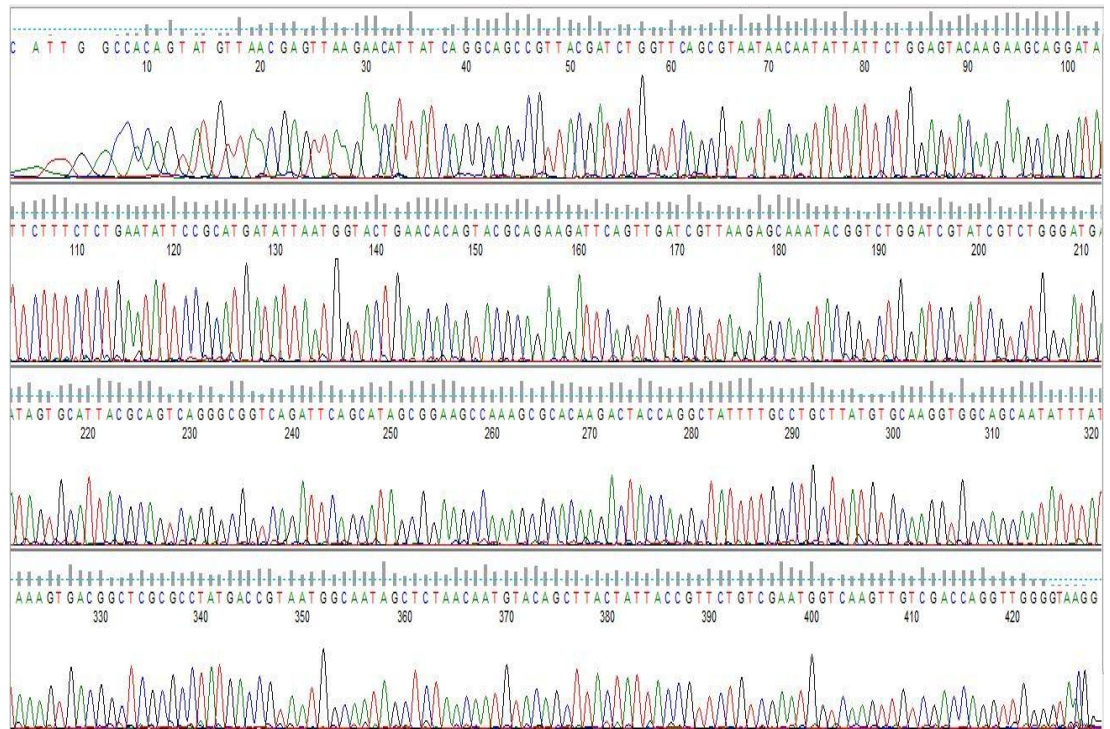
Sequence 2. Isolate 250e enterohaemolytic *Escherichia coli* isolate



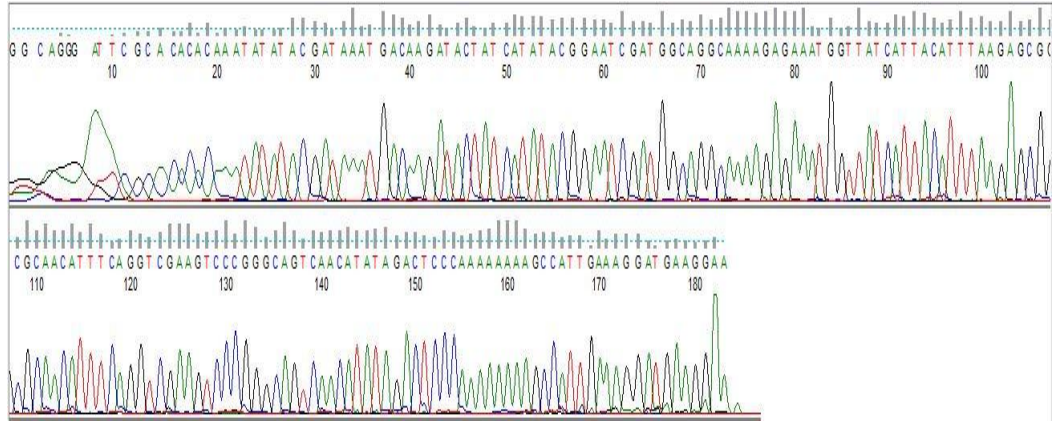
Sequence 3. Isolate 188e enterohaemolytic *Escherichia coli*



Sequence 4. Isolate 159d enterohaemolytic *Escherichia coli*



Sequence 5. Isolate 143e enteropathogenic *Escherichia coli*



Sequence 6. Isolate 103a enteropathogenic *Escherichia coli*