The effect of sanitization methods on human urine collected from people residing in different parts of Addis Ababa, Ethiopia

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List of abbreviations/ Acronyms

AAU	Addis Ababa University
CNCS	College of Natural and Computational Science
AOAC	Association Official analytical chemistry
APHA	American Public Health Association
ARG	Antibiotic resistance genes
CFU	Colony forming unit
EcoSan	Ecological Sanitation
ENPHO	Environment and Public Health Organization
FAO	Food and Agricultural Organization
FC	Fecal coliform
HGT	Horizontal gene transfer
HPC	Heterotrophic plate count
ICE	Integrating conjugative elements
MLS	Membrane Lauryl sulfate
NPK	Nitrogen, Phosphorus, Potassium
PCR	Polymerase Chain Reaction
QRDR	Quinolone resistance determining region
SCF	Synthetic Chemical Fertilizer
SRP	Soluble Reactive Phosphorus
TC	Total coliform
TN	Total Nitrogen
TP	Total Phosphorus
UDDT	Urine diversion dry toilet
UNICEF	United Nation International Children Educational Fund
WHO	World Health Organization

Abstract

Urine is rich in nutrients such as Nitrogen and Phosphorus which are essential for agriculture and therefore worthy of safe recovery. The aim of this study was to evaluate the effectiveness of acidification, storage and pasteurization of source-separated human urine in reducing coliform bacteria. Urine samples were collected using acetic acid-treated portable toilets and jerrycans from four sites of Addis Ababa namely; Addis Ababa University (AAU) Natural Science campus, Merkato portable toilet, Megenagna public toilet and Gelila Elementary school. Treatment of urine was conducted using Hydrochloric acid (HCl) heating to 80 ºC for pasteurization and storage in tight-sealed containers at room temperature. The urine undergoing the three sanitization methods were analyzed for their physico-chemical parameters, coliform bacteria and for the stored samples, PCR-based detection of genes resistant to tetracycine (tetB), sulfonamides (sul2) and quinolones (qnrB) were analyzed. The study showed the pH of fresh urine collected from the four sites to be slightly acidic in the range of pH 5.2 and 5.8, which increased to a maximum of 10.4 during storage for six months. Total coliforms in fresh urine collected from the four sites of Addis Ababa ranged between the highest count of 234,000 cfu/ml to 64500 cfu/ml, which decreased steadily during the storage time reaching to nil at the sixth month of storage. In all the fresh urine samples, fecal coliforms were detected with the maximum count of 1500 cfu/100 ml in samples collected from AAU. Storage for one month was efficient in reducing fecal coliform units to zero in all the samples collected from the four sites. Pasteurization was also efficient in completely removing both total and fecal coliforms. Acidification was didn't show any reduction in total coliform counts of urine collected from all the four sites. Regarding fecal coliforms, a slight decrease was observed resulting in 20% removal of fecal coliforms in the case of urine collected from Merkato .PCR-based detection of tetB resistance gene from the meta-DNA revealed that fresh urine collected from AAU was positive for the tetB which was absent at the sixth month of storage. Similarly, the fresh urine sample collected from Merkato was positive for qnrB which persisted in the stored sample for 4 months and finally absent at the sixth month of storage. It was concluded that storage as a sanitization process is the most effective method in reducing coliform bacteria and some of the horizontally transmitted antibiotic resistance genes, with minimal loss of Nitrogen and Phosphorus.

Keywords/phrases: urine, coliforms, Storage, nutrient recovery, antibiotic resistance genes

1. INTRODUCTION

1.1. General background

Human urine is rich with diverse inorganic and organic compounds. It is a by-product of metabolism in the human body when the body cells produce nitrogenous wastes, many chemicals and water (Heinone-Tansk *et al.,* 2007). All of which are removed through the urinary system.

Urine is rich in nutrients essential for agriculture. It is estimated that the urine of an average person contains 0.9 gram of Phosphorus and 11 Kilogram of nitrogen per day (reviewed in Simha and Ganesapillai, 2016). The average person's yearly urine reaches to 547 liters containing 2.5−4.0 kilo gram of total nitrogen (TN) and 0.22−0.37 kilo gram of total phosphorus (TP), This amount is enough to produce 145 kg of wheat per year (Karak and Bhattacharyya, 2011; Jonsson *et al*., 2004).

Human urine and synthetic chemical fertilizer (SCF) are equivalent in terms of the nitrogen content. In both cases, 90-100% of nitrogen is either in the form of urea or ammonium (Kirchmann and Petterson 1995). Around 75-90% of nitrogen is excreted as urea while the rest is in form of ammonium (Lentner, 1981). Urea in human urine will quickly degrade into ammonium and water and elevates the pH value up to 9 (Pradhan *et al.,* 2007). This rise in pH negatively affects the survival of pathogenic microorganisms (Pradhan *et al.,* 2007). An advantage of urine in comparison with organic fertilizers is that the phosphorus exists in forms that are directly available to plants and less prone to leaching (Richert *et al.,* 2010; Kirchmann and Petterson 1995).

In low income countries, particularly sub-Saharan Africa, most agricultural lands are over- utilized and poor in plant nutrients (Andersson, 2014). The poor soil nutrient content joined with recurrent drought rendered these countries to be less productive. As a result, millions of people in these countries are exposed to food shortage and diseases due to malnutrition. For example, the majority of the world's undernourished people live in developing countries. The proportion of undernourished people remains highest in sub-Saharan Africa (FAO. 2010). Because of fertilization is the most cases, is dependent on synthetic chemical fertilizer (SCF) (Erisman *et al.,* 2008).

Currently, the demand for synthetic chemical fertilizer (SCF) has been increasing with increasing demand for food to support high population size of developing countries. For example, only 10% of the national fertilizer demand was met from 2010-2015 in Ethiopia (Simtowe, 2015). Therefore, this problem should be addressed by using alternative organic fertilizers such as human urine as an affordable natural fertilizer. Various researchers have demonstrated fertilizer value of stored human urine for cultivation of barley (Kirchmann and Petterson 1995); maize (Guzha *et al.,* 2005); wheat (Tidåker *et al.,* 2007); cucumber (Heinonen- Tanski *et al*., 2007). It is used to varying extents for crop fertilization in countries such as Mexico, Germany, Sweden South Africa and Zimbabwe (Pearson *et al*., 2008, Jansen and Koldby 2003, Esrey and Andersson, 2001, Linden, 1997). Human urine can be effectively used to vegetables that can be cultivated in small scale in home gardens. Consequently, local people can easily fulfill their daily nutritional requirements and support their economic value through the supply of vegetables.

In addition to fertilizer application, urine when separated at the toilet decreases the energy exerted to remove nitrogen and phosphorus from the wastewater (Hoglund, 2001). Urine separation is economical since 70-80 % of the nitrogen and 40% of the Phosphorous in the wastewater comes from the urine fraction of the wastewater (Wilsenach and van Loosdrecht, 2006). Human urine collected in separating systems can be used directly as a liquid fertilizer (Kirchmann and Pettersson, 1995).

Unlike fecal matter, urine is usually pathogen-free and low in heavy metal concentrations, thus there is low health risks associated with the use of human urine in plant production. Despite this fact, fecal matter is often detected in source-separated urine, thus leading to the possibility of pathogen contamination. In addition to fecal matter, urinary tract infections can be a source of pathogens in urine. This is particularly of concern due to the high prevalence of antibiotic resistant bacteria associated with urinary tract infections. There has recently been an increased focus on minimizing the release of pathogens and antibiotic resistance genes into the environment.

1.2. History of Source-Separation of Human Urine

During past time when researches conducted human excreta contents were collected in buckets from each household and human urine was collected separately and poured into the drain, because to avoid smells and to prevent the latrine from filling too quickly (Höglund, 2001). At that time in 1867, it was known that “the amount value of the fertilizing ingredients held in solution in urine to that contained in faeces is as six to one (Höglund, 2001). Whereas, Muller, a German scientist who saw it as a necessity to separate the urine from the faeces in order to produce a fertilizer that was of manageable proportions (Vinnerås and Jönsson (2002 a)

According to Hoglund (2001), the old Japanese practice the nightsoil recovery from urban areas to separated urine and faeces, so urine was considered as a valuable fertilizer. In Yemen, the urine is drained away and evaporated on the outer face of multistory buildings to obtain the faeces as a dry fraction without smell for use as fuel . A system that has been in used for hundreds of years (Esrey *et al.,* 1998). In Sweden s since source separation of human urine was beginning of in 1999 (Vinneras *et al*., 2002).So the recent toilet was installed in "holiday homes" and ecovillages, and at that time, numbering at least 135,000, are used in apartment blocks, schools, and detached homes across the country (fig1) (Johansson *et al*., 2000)

Figure 1 Urine-separating materials toilet models: (a) Model from wost man ecology (b) dubbletten™ from bb innovation and (c) Nordic from gustavsberg. (Source: Hoglund 2001)

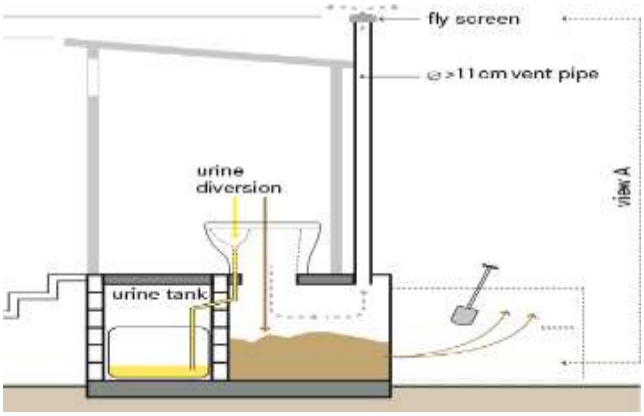

Figure 2 Urine diversion dry toilet (UDDT) internal design (left) a squatting type of UDDT Source: (Rieck et al., 2012).

Separation of urine and faeces collected at the source has additional benefits. A dry fecal fraction is more manageable and thus safer and easier to handle for composting and reuse (Johansson *et al*., 2000). And will minimize leaching when fecal matter is disposed of, for example in pit latrines (Schonning, 2001b). However, disposal of fecal matter by flushing water when source separation practiced is not uncommon. Urine contains the majority of nutrients excreted by humans, is far more hygienic, and contains lower concentrations of heavy metals than faeces (Jonsson *et al.*, 1998). Sludge from the largest wastewater treatment plants in Switzerland contained an average of 55 mg cadmium per kg phosphorus, and chemical fertilizers sold in 1995 contained 26 mg Cd/kg P, while urine contained less than 3.6 mg Cd/kg P (Jonsson, 2002). Worldwide, a number of manufacturers offer versions of the source-separating toilet. These toilets are manufactured both as Western-style porcelain pedestal flush toilets familiar in much of the developed world, and the simpler squat-toilets more common in developing countries. In developing countries, especially India, source-separating squatting pans are available for less than the equivalent of 10 EUR, although costs increase to include materials and labor for the toilet

super-structure and accessories (e.g. urine collection and storage containers). Even at a total cost of 50 EUR, a source-separating toilet is unaffordable for large segments of the population (Rieck *et al*., 2012). Designs using low-cost, locally available materials and subsidies for construction can help increase access to the technology (Fig.2) (Water Aid Nepal, 2008).

Source separation of urine is an act of separation of urine from its origin and treating separately. This is found out-smarts the conventional waste water treatment procedures in different aspects. The fact that urine not mixed with faeces contains a very low amount of heavy metals makes it suitable for application as a fertilizer with less heavy metal contamination risk (Ronteltap *et al*., 2007; Jönsson *et al*., 2000; and Johansson *et al*., 2002). It would be a loss if we mix such a solution with large amount of heavy metal content, the other fractions of the wastewater. Besides urine separated toilets were observed to save flush water by 80% (Stephanie *et al*., 2015 and Vinnerås, 2001a).

Source-separation of urine improves the efficiency of wastewater treatment plants in removal of pollutants. A study in Sweden (Jönsson *et al*., 1999) shows that in a domestic wastewater treatment system in Stockholm, the removal of Nitrogen with no mix of urine was 27 times more efficient than the urine-mixed wastewater. Similarly, Phosphorus removal was 35% higher Potassium removal was 25 times more efficient (Jönsson *et al*., 1999). Together with this, urine separation has a great advantage in the energy saving for the wastewater treatment. It was found to save energy of about 32 MJ/ person per year since it decreases nutrients from waste water system along with urine replaced chemical fertilizers which require 75MJ/ person and year to produce. Thus, urine separation saved a total of 63MJ/ person and year since it only requires 44MJ/ person and year for transporting the urine mixture 33 km with a truck to a farm and spread it as fertilizer (Johansson *et al*., 2002). Therefore, separation of urine from the point of entry reduces the costs of extensive waste water treatment and nutrients become available for recovery and reuse. However, when we see from the monetary perspective both the conventional and urine separation system expenditure is the same (Stephanie and Treavor, 2015).

The potential of urine as nitrogen source was found out 90 % to that of ammonium-nitrate fertilizer according to studies by (Johansson *et al*., 2001). And the phosphorous effect was found out to be equal to the chemical fertilizer (Kirchmann and Petterson, 1995 and Hellström *et al.*, 1999). In Richerts and his colleagues experiment on barely, oat and wheat, no toxic effect was observed from its ammonia which most commonly is toxic to some growing plants. This is due to the hydrolyzed urine contains nitrogen in the form of ammonium/ammonia (Richert *et al*., 2010).

Though source separated human urine has paramount advantage in agriculture there are worries we have to be careful with. These are pharmaceuticals (such as propranolol, ibuprofen, diclofenac and carbamazepine), hormones (such as estrone, estradiol and ethinylestradiol) and heavy metals (such as Co, Cd, Cr, Cu, Ni and Pb). To treat human urine from chemical contaminants the suggested methods are the coast, energy and maintenance intensive these are ozonation, electrodialysis and nanofiltration (Fewless *et al*., 2011). Besides these methods cause nutrient loss during treatment. So according to experiments on the fate of pharmaceuticals scientists have studied (Ronteltap *et al*., 2007) during struvite precipitation and found promising results for future use of struvite for agriculture.

Purposely administered hormones and pharmaceuticals in to collected human urine were found soluble during struvite precipitation. Thus, these soluble can be removed by washing step after filtration from urine solution. This makes struvite a safe fertilizer from hormones and pharmaceuticals. But additional studies must be there to ensure safety of the struvite. However, the heavy metals administered to human urine such as Cd, Cu and Pb were found to precipitate naturally in 7 days stored urine. And others were observed to make carbonates and hydroxides of Co, Cd, Cr, Cu, Ni and Pb in hydrolysed urine. Along with the heavy metals precipitated amounts 20-63%. However, in non-hydrolysed urine Cu and Pb precipitated as phosphate and the others remained in solution.

Struvite precipitated from stored urine was found to contain insignificant amount of heavy metals. Besides it was found for As, Cd, Cr, Co, Cu, Ni, Pb, Zn, Al, and Fe concentration to be lower in urine than commercially available fertilizer and manure (Rogowski *et al*., 1999 cited in Ronteltap *et al*., 2007). The (Ronteltap *et al*., 2007) recommended that optimum phosphorus recovery is obtained from human urine if we reduce primary introduction of magnesium and calcium in the urine since it leads natural precipitation of phosphorous before the struvite is produced. Generally, the (Ronteltap *et al*., 2007) have learned from their experiments that phosphorous recovery from human urine through struvite precipitation yields a struvite that avoids most of hormones, pharmaceuticals and contains a fraction of law amounts of heavy metals which was already in urine.

1.3. Nutrients in human urine

Urine contains the largest proportion of plant nutrients found in the household waste fractions (Fig3). The amount of plant nutrients excreted through urine per person and year has been measured at 2.5-4.3 kg nitrogen (N), 0.4-1.0 kg phosphorus (P) and 0.9-1.0 kg potassium (K) Jönsson et al., 2005; Vinnerås et al., 2006).

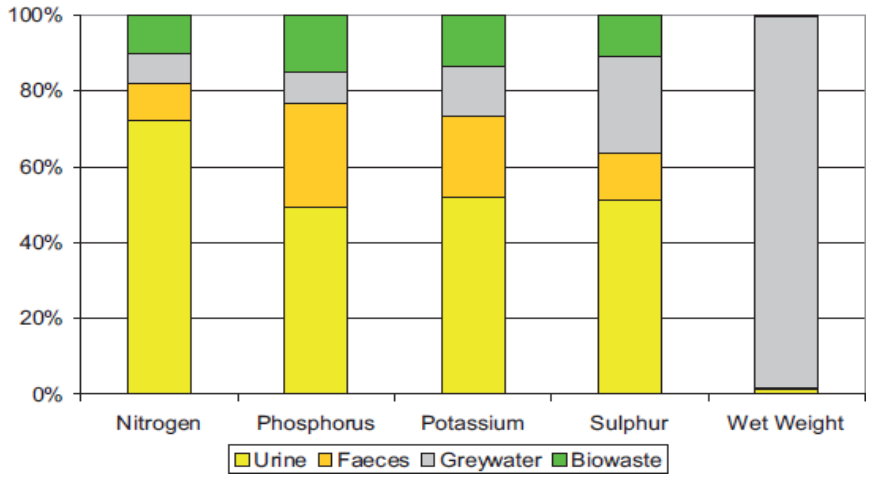

Figure 3 Proportions of nutrients found in household wastewater fractions. Bio waste in Sweden (Source: Jönsson et al., 2005).

Jonsson *et al*. (2005) and Vinnerås *et al*. (2006) analyzed the nutrient content of contents urine. The annual excretion rate per person in Sweden to be about 4000 g N, 330-365 g P and 1000 g K. Together, the nutrients in urine and faeces in Sweden add up to some 4500-4600 g N, 500-550 g P and 1400 g K per person per year (Jönsson *et al*., 2005; Vinnerås *et al*., 2006). Based on FAO data on food supply, Jönsson and Vinnerås (2004) estimated that the quantity of nutrients in Ugandan excreta to be 2500 g N and 400 g per person per year.

The additional constituents in human faces and urine contain trace metals, which is present in excess concentrations could be harmful to humans and to the environment. The amounts of harmful heavy metals in the urine are minute (WHO, 2006). This is a result of the biological uptake being small and their excretion being even smaller (Vinnerås, 2002).

1.4. Use of human urine

Human Urine was used in Europe in the early times for household cleaning, softening

wool, hardening steel, tanning leather and dyeing clothes. The Greeks and Romans used it to color their hair (Esrey and Andersson, 2001). Sweden is probably the country with the most advanced system of collection and reuse of human urine, where it is practiced by farmers on a large, mechanized scale. In a number of settlements (called 'eco-villages') or apartment blocks in the country, the residents have ecological sanitation systems with urine diversion toilets. The human urine from the houses or apartments is collected in large underground tanks and is collected by farmers in road tankers and used for fertilizing their crops.

The usual practice is to spray it onto the lands while they are being prepared for planting and then harrow it into the soil before sowing the seed (Jansen and Koldby, 2003). It has been found to be a valid substitute for mineral fertilizers in growing cereals, with no negative impact on the crop or the environment (Esrey and Anderson, 2001).

According to Drangert (1998) in the Danish countryside in the 19th-century human urine was stored and used as a detergent for washing clothes and dyeing. In a fertilizer experiment on the growth of barley, using human urine in parallel with manure, urine was examined with high quality a fertilizer (Jansen and Koldby, 2003). In a field trial in Sweden in 2002, different application strategies for urine as a fertilizer on leeks were tested (Richert *et al.,* 2010). Fertilizing with urine gave a three-fold yield increase. Neither yield nor nutrient uptake was significantly affected by whether the same total amount of urine was applied in two doses or whether it was divided into smaller doses applied every 14 days (Richert *et al.,* 2010).

The farmer's perception of the use of urine in Sweden is that the more concentrated the urine is the better it (Adewole *et al.,* 2013). They have found out in their study that

establishment of a quality control system, such as certification of urine and other source diverted wastewater fractions is extremely important for the use of fertilizers of organic origin in the Swedish context. The guideline for the use of urine and faces in crops are that urine can be applied neat or diluted. They harassed that the application rate should always be based on the desired nitrogen application rate and the urine or urine mixture should be quickly incorporated into the soil, to minimize ammonia loss (Jonsson *et al*., 2004).

A study by Schönning (2001) for hygienic measurement, recommended that crops consumed raw should not be fertilized with urine closer to harvest than one month. In addition, there might be pathogens that may be present in the urine, thus requiring inactivation the pathogens in urine will be dependent on the pH (naturally increases to 9), concentration and temperature.

Esrey and Andersson (2001) showed that the Chinese pharmaceutical industry used urine to make blood coagulants. They further highlighted anecdotal evidence from several locations that indicated people preferred vegetables grown with urine fertilization, and in China, people were willing to pay more for vegetables grown in urine. Rooftop gardeners used only urine to grow vegetables such as tomatoes, cabbages, beans, and pumpkins. Farmers have commonly used night soil, often untreated, to grow food (Esrey and Andersson, 2001) in Kerkala (India).

In a pilot project with human urine from toilets was diverted into a growing area attached to the back of the toilet (Esrey and Andersson, 2001). In Manipur state (India), harvests of potatoes and chilies, when urine was used as fertilizer, were very good compared to harvests fertilized with chemical fertilizer, such as DAP, urea, and potash (Singh, 2003).

In Sri Lanka human urine was used for plants such as banana, coconut, vegetables, flowers or fuelwood. In Matale town, the Nandawathi family used urine and wash water to grow chilies, but only used the chilies after drying, not fresh (Esrey and Andersson, 2001). Getting people in Thailand to accept the application of human urine as a fertilizer was not easy. The main problem was the sociological difficulty, as the common belief is that human excreta are dirty and a pathway for disease transmission (Pinsem and Vinnerås, 2003).

In Nepal, the Environment and Public Health Organization has conducted experiments on application of urine on various seasonal crops including potatoes, radishes, and rice. The study concluded that there is a possibility of growing potatoes with the application of urine only. The experimentation, on the other hand, showed that higher yields of potatoes were reported with the application of chemical fertilizers than urine (Shrestha and Morgan, 1993).

According to (Chaggu and John, 2002) use of human urine in Africa like the Kagera area in Tanzania, urine has been used as an antidote when somebody has inhaled and ingested poison, by giving that person fresh urine to drink. And the other human urine has also been used as a pesticide to kill banana weevils the tradition of visitors was for visitors to urinate in the host's home garden, which was much appreciated and considered a gesture of respect. This practice has disappeared with the adoption of modern hygiene (Chaggu, 2004).

In Botswana, Pilot trials for the agricultural use of urine were identified as the most important follow-up step towards encouraging responsibility for one's own toilet products, and for demonstrating their fertilizing potential. Trials were conducted whereby three plots

were prepared in each of 16 locations. One was fertilized with urine, the second one with urine and compost, and the third one without any kind of fertilization for comparison purposes.

In all the cases the pilots were planted with spinach. After a certain period, the best results were achieved with the use of compost and urine together. This resulted in participants starting to use urine after the demonstration, and even those without toilets started to collect urine for further use (Richert *et al.*, 2010).

1.5. Antibiotic resistance genes in human urine

Antimicrobial resistance is an emerging global concern to both public and veterinary health. Antibiotics are heavily used to treat disease in both humans and animals, and there is a pattern of antibiotic resistance and transfer emerging among bacterial populations in proportion to the use of antibiotics, especially in agriculture (Levy 1997; Oppegaard *et al.* 2001). Antibiotics have been added to animal feed as growth promoters for some time, and the animal production industry has been identified as a potential reservoir for resistant Enterobacteriaceae (Witte 1997). The use of antibacterial drugs for prophylactic or therapeutic purposes in humans and for veterinary and agricultural purposes has provided selective pressure favoring the survival and spread of resistant organisms.

The wide-spread use of antibiotics to treat microbial infections in humans and to promote animal growth has led to the proliferation of antibiotic resistant bacteria (ARB) in the environment. Antibiotic resistance genes (ARGs) have been detected in surface water (Reinthaler *et al*., 2003), groundwater (Chee-Sanford *et al*., 2001), sediments (Pei *et al*., 2006; Storteboom *et al*., 2010) and wetlands (Cummings *et al*., 2010). High concentrations of multidrug resistant bacteria have also been detected in domestic sewage, hospital

wastewater, and drainage from livestock feeding operations (Pruden *et al*., 2006).

These resistant bacteria may transfer their resistance to earlier non-resistant pathogenic bacteria or directly infect humans with bacterial diseases that cannot be treated by conventional antimicrobial therapies (Bridgett *et al*., 2010). The potential for antibiotic exposure and resistance development in human and animal gastrointestinal tracts, coupled with relatively great abundance in waters contaminated with human and animal waste, makes the fecal coliform bacteria a logical main group for studies of antibiotic resistance and transfer in aquatic environments.

1.5.1. Genetic Basis for Antimicrobial Resistance

Bacteria have a remarkable genetic plasticity that allows them to respond to a wide range of environmental threats, including the presence of antibiotic molecules that may risk their existence. As mentioned, bacteria sharing the same ecological niche with antimicrobial-producing organisms have evolved ancient mechanisms to withstand the effect of the harmful antibiotic molecule and, consequently, their intrinsic resistance permits them to thrive in its presence (Munita and Arias, 2016). From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic which undergo mutations in gene and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

A. Mutational Resistance

Resistant mutant emerges, the antibiotic eliminates the susceptible population and the resistant bacteria predominate. In many examples, mutational changes leading to resistance are expensive to cell homeostasis that are decreased fitness and only maintained if needed in the presence of the antibiotic. In general, mutations resulting in antimicrobial resistance

alter the antibiotic action through the following mechanisms, which are modifications of the antimicrobial target decreasing the affinity for the drug and a decrease in the drug uptake. An activation of efflux mechanisms to extrude the harmful molecule or global changes in important metabolic pathways via modulation of regulatory networks, thus, resistance arising due to acquired mutational changes is diverse and varies in complexity (Munita and Arias, 2016).

B. Horizontal Gene Transfer

Horizontal gene transfer is one of the most important drivers of bacterial evolution and it is frequently responsible for the development of antimicrobial resistance. Most antimicrobial agents used in clinical practice are derived from products naturally found in the environment mostly in soil. Bacteria sharing the environment with these molecules harbor intrinsic genetic determinants of resistance and there is robust evidence suggesting that such "environmental resistome" is a prolific source for the acquisition of antibiotic resistance genes in clinically relevant bacteria Munita and Arias, 2016).

Horizontal gene transfer can take place between different organisms through transformation (uptake of DNA from the environment), transduction (packaging and transport of bacterial DNA by viruses), and conjugation (bacterial mating). Much of the horizontally transferred DNA is part of the flexible gene pool. The role of conjugation in horizontal gene transfer is complicated by the fact that other mobile genetic elements besides plasmids can be transferred by conjugative machinery. Conjugative transposons are transposons which excise themselves from covalently closed circular DNA, such as a plasmid or chromosome, and integrate into another location in the same genome or are transferred by conjugation and then integrated into a different genome. These elements can

also mobilize non-conjugative plasmids or other DNA sequences in a cell and transfer them in to another cell (Slayers *et al*., 1995).

Another level of complexity is added when other mobile genetic elements such as integrons or integrating conjugative elements (ICE) are considered in tandem with plasmids and conjugative transposons (Walsh,2006). Integrons are mobile genetic elements that encode integrase and are capable of site-specific recombination and typically carry antibiotic resistance genes (Hall, 2012). ICE is transferred through cell-to-cell contact followed by transformation and can integrate into the chromosome of the recipient. The main mechanism of horizontal transfer of plasmids is through conjugation (Norma *et al*., 2009) However, natural transformation does allow for the uptake of plasmid DNA as well as chromosomal DNA. Because of this, it is widely accepted that natural transformation is a major mechanism in the evolution of microbes. However, it is still unclear as to how and why the process itself evolved. Natural transformation is the process whereby bacterial cells take up free DNA from the environment and incorporate it into their genomes. There are over 80 known different species of naturally transformable bacteria, which includes members from different lineages including photolithotrophs, chemolithotrophs, heterotrophs, and methylotrophs (Lorenz and Wackernagel ,1994).

1.5.2. Resistance to tetracycline

Tetracycline is bacteriostatic antibiotic that binds reversibly to the 30S subunit of ribosome, there by inhibiting the initiation of protein synthesis (Beceiro *et al*., 2013). The main resistance mechanisms for tetracycline are mono-component efflux systems specific for *tetracyclines* encoded by the *tet* genes. There are several types of *tet* genes. The most common types reported in antimicrobial resistance *gene are tetA, tetB, tetC, tetD* and *tetG*

(Pezzella *et al*., 2004). Other mechanisms of resistance to tetracyclines are ribosomal protection and antibiotic modification (*tetX*). Efflux resistance genes and genes involved in ribosome protection are generally found on plasmids and self-transmissible chromosomal conjugative transposons, respectively which allow for mobilization via horizontal gene transfer (Speer *et al*., 1992). Among the various members of the *tet* gene family, *tetB* is a 1374 bp long gene which translates to 458 aminoacids long protein which is involved in active efflux of tetracycline thus pumping the drug out from the cytoplasm in an energy-dependent manner (http://www.uniprot.org/uniprot/P23054).

1.5.3. Resistance to sulfonamides and trimethoprim

The target of sulfonamides and the basis for their selectivity is the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway. Mammalian cells are not dependent on endogenous synthesis of folic acid and generally lack DHPS. Trimethoprim inhibits dihydrofolate reductase, the enzyme required to convert dihydrofolic acid to tetrahydrofolic acid, the precursor of folic acid. The mechanism of resistance to sulfonamides and trimethoprim is due to the horizontal spread of resistance genes; variants of the target enzymes dihydropteroate synthase and dihydrofolate reductase characterized by reduced affinity to sulfonamides and trimethoprim, respectively. Three sulfonamide resistance genes coding for different types of DHPS (insensitive to sulfonamides) have been identified: *Sul*1, *Sul*2 and the more recently described *Sul*3 gene (Grape *et al.,* 2003). Most multi-resistant gram-negative bacteria harbor Class 1 integrons which carry the *Sul*1 gene. The *Sul*2 gene is frequently associated with the small, multi-copy, non- conjugative IncQ plasmid group. Although less common, resistance can also be due to mutations within the chromosomally located dihydropteroate synthase gene (*folP*) (Perreten and Boerlin, 2003).

1.5.4. Resistance to Fluoroquinolones

Fluoroquinolones are potent, broad-spectrum antibiotics used to treat infections caused by wide range of Gram-positive and Gram-negative pathogenic bacteria. Fluoroquinolones differ from quinolones by the replacement of the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity.

Fluoroquinolones target type II topoisomerase (DNA gyrase) mainly in Gram-negative bacteria and topoisomerase IV in Gram positive bacteria. DNA gyrase contains two subunits *gyrA* and *gyrB* whose function is to relax the supercoiled DNA ahead of DNA replication fork by creating negative supercoiling. The major mechanism of resistance to fluoroquinolones is through mutation in quinolone resistance determining region (QRDR) of DNA gyrase and topisomerase IV subunits mainly *gyr A* and *par C* (Redgrave *et al.*, 2014). Topoisomerase IV contains *par C* and *parE* subunits with the function of decatinating (unlinking) replicated double stranded DNA (Drlica *et al*., 2008). DNA gyrase and topisomerase IV subunits mainly *gyr A* and *par C* (Redgrave *et al.,* 2014).

The other mechanisms of quinolone resistance include efflux pumps (multidrug efflux pump and quinolone specific plasmid mediated efflux pump encoded by *qep* gene), *Qnr* (plasmid mediated quinolone resistance), porins, and quinolone-modifying enzyme (aac(6')-Ib-cr) (Redgrave *et al*., 2014). Plasmid mediated quinolone resistance determinants, especially *qnr* genes, has recently been reported from different parts of the world in different Gram-negative resistance bacteria (Jacoby *et al*., 2014).

Fluoroquinolones These genes encode for pent peptide proteins which protects bacterial

topoisomerase from the effect of quinolones. They do not induce high level resistance but their presence leads to mutation in the QRDR (Robicsek *et al*., 2006).

1.6. Potential risks associated with recovery of nutrients from urine

1.6.1. Salts content of human urine

Human urine contains various salts and the most predominant among them is sodium chloride. The concentration of sodium (Na) and chlorine (Cl) in undiluted fresh human urine evaluated by Kirchmann and Pettersson (1995) were amounting 0.94-0.98 g/L and 2.3-2.5 g/L respectively. While a value of 2.34 g/L of sodium content in urine was recently reported in the literature (Pradhan et al. 2010). In Niger, sodium contents in source-separated urine collection were in the range of 2.9-3.5 g/L (Dagerskog and Bonzi, 2010). Moreover, the evaluation of human urine as source of nutrients for selected vegetables in South Africa (Mekeni et al., 2008) showed a high Na (0.90 %) contents in bulk urine compared to N (0.74%) and P (0.029%) but slightly lower than K (1.62%). Thus, applications of human urine as liquid fertilizer in agriculture may have potential to accumulate sodium (Na) ions in soil and eventually be damaging for plant growth and production, especially in dryland when urine is planned to be reused at several years' scales. Besides, it has been reported earlier that salts including chlorides may be toxic to some plants (Holliman, 1998).

1.6.2. Contamination in human urine by pathogens

In a healthy individual, human urine is sterile in the bladder (Höglund, 2001). However, in general, pure human urine contains very few enteric microorganisms (Heinonen-Tanski, 2007). The pathogens traditionally known to be excreted in human urine are *Salmonella*

Typhi, Salmonella paratyphi, and Schistosoma haematobium. Infections by S. Typhi and S. paratyphi only cause excretion in urine during the phase of typhoid and paratyphoid fevers when bacteria are disseminated in the blood (Feachem *et al*. 1983). These infections happen rarely in developed world (Lewis- Jones and Winkler 1991), while cause about 16 million cases per year in developing countries (Feachem *et al*, 1983).

Urinary Schistosomiasis is one of infections mainly occurring in North Africa and caused by *Schistosoma haematobium;* and their eggs are excreted in urine sometimes during the hold life of the host (Feachem *et al.*, 1983: Höglund, 2001). Nevertheless, it is widely known, that many microorganisms die off during hygienisation of human urine which include storage and pasteurization. The storage of urine in tropical developing countries may not be necessary for a long-term (< 3 months), even though storage for 6 month is recommended in the Nordic countries (Jönsson *et al*., 2004); because the heat (>20^{o} C), U-V radiation and the increase of pH up to 9 caused by urea hydrolysis might be beneficial for the inactivation of pathogenic microorganisms in urine in the tropical world (Höglund, 2001; Heinonen-Tanski, 2007). Besides, the risk of microbial contamination is diminished by the fact that pathogenic microbes do not survive in soil for a long time (Feachem, 1983). Therefore, the reuse of urine in tropical area seems to present low risk of pathogen contamination. However, furthers investigation are required to clarify this negative side of urine (public health issues) in the standpoint of sustainability when is reuse in at large scale at the farmland of tropical developing countries (Richert *et al.*, 2010).

In addition, total and fecal coliform bacteria: total Coliform is a group of bacteria present all everywhere, most of which are not dangerous to human health. However, these bacteria are not naturally present in groundwater and are an indication that more harmful organisms

might be present. Fecal Coliform and *E. coli* are subgroups within the total Coliform group which primarily come from the faeces of warm blooded animals. Presence of *E. coli* indicates that the water has been exposed to faeces and an immediate risk to human health exists. Fecal coliform are gram-negative bacilli, non-sporulated, heat tolerant, strict aerobes or facultative, with the ability to ferment lactose with formation of gas in 48 hours at 44± 5^{o}C. They include the species *E. coli* and certain strains of *Klebsiella pneumoniae* in addition to other enteric bacteria (Höglund, 1998). Therefore, they are considered ideal bacteriological indicators of contamination of an intestinal origin (Jiménez *et al*., 2002) and Jana *et al.,* (2015). The mean count of total coliform of bacteria was maximum (174.66 × 10^4 cfu/ ml) in case of fresh urine and lowest (0. 033 × 10^4 cfu ml) in old urine stored for 638 days.

1.6.3. Pharmaceuticals

Human urine contains pharmaceuticals residues even after prolonged storage of urine as a treatment step (Winker, 2008). Thereby, the reuse of human urine is associated with a risk of transfer of pharmaceutical residues to the agricultures fields. About 70 % of pharmaceuticals taken are excreted in urine and is accounting for 50 % of the Eco toxicological risk (Lienert *et al*., 2007). Little is known about the fate of pharmaceuticals (anti-malarial drug, antibiotics) present in urine regarding their accumulations in soil, transfer in ground water and uptake in plants. It has been reported that, pharmaceuticals, which are polar substances and hardily biodegradables, have potential to be uptake by plants and eventually enter into human food chain (Winker, 2008). While, strictly speaking the environmental fate of pharmaceuticals and their effect on humans, animals and microorganisms is still unknown and remained a big challenge, which needs to be

addressed urgently for a sustainable reuse of human urine in agriculture.

1.7. Sanitization methods of human urine

1.7.1. Treatment by Storage

The factors responsible for the persistence or die-off of microorganisms during storage of human urine are temperature, pH, and ammonia which are also affected by the dilution of the urine (Chandran *et al*., 2009). Treatment of urine by storage has been investigated at 4-5 °C and at 20 °C (Höglund, 2001) and at 4, 14, 24 and 34 °C (Nordin *et al*., 2009). Studies on the die-off of various types of microorganisms in stored urine, combined with a risk assessment, resulted in the recommendation that when urine is stored for 6 months at 20 °C, it should be safe to use as a fertilizer for any crop (WHO,2006).

The other factor depends on the conditions in the nature of the environment. Therefore, the variations in the external environment, for instance, seasonal changes in temperature and humidity, can be an increased number of pathogenic organisms as a result of re-growth, especially the bacteria (Niwagaba, 2009). General Storage is more effective in killing pathogenic microorganism. in dry, hot climates, with dehydration of the material and low moisture contents supporting the pathogen inactivation and the degree of storage is including studied at 4-5 °C and at -20 °C (Höglund, 2001) and at 4°C, 14°C, 24 °C and 34 °C and 6 months at 20 °C, it (Nordin *et al*., 2009 and WHO, 2006).

1.7.2. Treatment by Pasteurization

A study by Hoglund, (2001) the pathogenic microorganism inactivation is faster at higher temperatures (*e.g*. 20, 24 or 30-34 °C) than lower at 4-5 °C or 14-15 °C The temperature often varies between day and night, and temperature amplitude (peak-to-peak) between day

and night can be of the order of magnitude of 10 °C or more. The primary aim of pasteurization is to eliminate the pathogens present in urine such as viruses, bacteria and protozoa. Secondly, as temperature strongly influences enzymatic activity, pasteurization could be a solution to inhibit urease activity, if operating at the right temperature. Pasteurization can be performed either by maintaining the liquid at 80 ^{o}C for 30 minutes (Lahr *et al*., 2016). The pasteurization process must take place in an anaerobic environment in order to be effective. However, control is required to ensure that the pasteurization has been effective and the urine is safe at the end of this stage.

1.7.3. Treatment by Acidification

Acidification is the positive effects with respect to hygiene due to harmful effects on pathogenic organisms at pH values below 4 (Hellstrom e*t al*, 1999). Low pH values can also have an influence on pharmaceuticals present in the urine (Maurer et *al*., 2006). Studied at pH 2, an inactivation level of between 50% and 95% could be found for antibiotics (sulfamethazine, sulfamethoxazole, tetracycline) and the anti-inflammatory drug diclofenac (Maurer et *al*., 2006).

2. Statement of the problem

Farmers in Ethiopia, like in many other low-income countries, cannot afford to continuously buy the imported synthetic fertilizers due to the increasing cost. Data from the Ministry of Agriculture in Ethiopia (IFDC report, 2012) showed that the amount of imported chemical fertilizer increased from 370,000 MT in 2002 to 570,000 MT in 2011, indicating an increasing demand of chemical fertilizers in agriculture. On the other hand, in developing countries like Ethiopia, sanitation systems are managed poorly and recovery of

resources from waste is not commonly practiced. Instead of expending capital and energy to remove agriculturally important nutrients such as Phosphorous and Nitrogen from domestic wastewater, it is better to separate the fraction of the wastewater rich in these nutrients such as urine from the source and recover the nutrients. Recovery of the nutrients from human urine can serve as an alternative and affordable natural fertilizer, which plays a role in sustainable agricultural practice in Ethiopia. While recovering these nutrients (N and P) from urine, different biological contaminants should be given due attention and effective sanitization should be used for safe recovery of N and P from urine. Research thus needs to assess if the different sanitization methods of urine could minimize the risk of contamination of the environment.

3. Objectives of the study

General Objective

The general objective of this study was to evaluate the effect of urine pasteurization, acidification and storage on the quality and safety of source-separated urine.

Specific Objectives

- To characterize fresh human urine collected from the different parts of Addis Ababa in terms of its nutrient and contaminants contents
- To compare the effect of storage, Pasteurization and acidification on the survival biological contaminants
- To assess the persistence of three antimicrobial resistance genes upon storage of urine

4. Hypothesis

Sanitized source-separated urine contains adequate amount of Phosphorus and Nitrogen for use as fertilizer.

5. Materials and methods

5.1. Study design

This study was a laboratory scale survey of nutrients and bacteriological contaminants of urine sample collected from different sites. Longitudinal study was conducted in the laboratory to assess human urine contaminants under three different sanitization methods as described in the Guideline of WHO (2006) for the treatment of safe use of urine in agriculture.

5.2. Sample collection sites

Urine was collected from four different areas in Addis Ababa namely; Addis Ababa University (AAU) College of Natural Science (CNS) campus, Megenagna public toilet, Merkato portable jerrycan-based toilet and Gelila Primary and Secondary School located in main regional bus station ("Atobis Tera") area. The selection criteria for these sites are based on their distance to the laboratory site.

5.3. Urine Collection, Processing, and Characterization

Initially, urine collection was done in CNCS campus, AAU. Urine collection event was held for two days (on January 2-3, 2017) by deploying two portable toilets near the AAU students' toilet (Fig 4 and 5). Following the AAU event, from January – March 2017, urine was also collected from residents in Merkato area, public toilet of Megenagna and Gelila Primary and Secondary School. The collection was done using clean disinfected 10 Liter jerrycans. Before each collection event, the containers used for collection were washed with hypochlorite and rinsed with distilled water and 5% acetic acid or the commercial vinegar. During urine collection from Merkato, the number of users and their demographic and economic profiles were recorded since in other sites it was not suitable to take both

interview and urine sample.

Figure 4 Portable toilets (left: female, right: male) placed in front of CNS campus toilet, AAU (January 2017)

Figure 5 Urine collection from AAU portable toilettes in January 2017

By the end of each collection day, urine in jerry cans was transported in ambient temperature to the bioinstrumentation laboratory, Addis Ababa University. The collection was done separately for male and female which were combined after the physicochemical and biological contaminants analysis was done separately for the male and female samples.

5.4. Physico-chemical and Nutrient analysis of urine

The physicochemical parameters included pH, soluble fraction phosphorus (SRP), total phosphorus (TP) and total Nitrogen (TN). pH was measured using pH meter (Fisher Scientific, Singapore). SRP analysis was done using Ascorbic acid method described by American Public Health Association (APHA, 1998). Total phosphorus (TP**)** was analyzed using the digestion method by Ammonium per-sulfate (APHA, 1998). Briefly, 10 ml of urine samples mixed with 40 ml of distilled water were added into 500 ml sterilize Erlenmeyer flask. Then, 0.05ml (1 drop) of phenolphthalein was added into the urine sample. Concentrated sulfuric acid (1 ml, 2N) was added drop-wise following pink color formation to discharge the color. Then 0.4gm $(NH4)_7$ SO4 was added and was digested until ten ml was left in the sample flask. Then, 20 ml of distilled water was added to each urine sample and 1 drop of phenolphthalein was added. Sodium hydroxide (1N) was added drop-wise until the pink color became faint. Then, 20ml of distilled water and 8ml of combined reagent was added to each urine sample. The absorbance of each urine sample at 880nm was measured spectrophotometrically (HACH DR/2010, Canada) using reagent blank as the reference solution. The absorbance reading was converted to concentration using standard curves followed by (APHA, 1998).

Soluble fractionation phosphorus (SRP) was analyzed following ascorbic acid method of APHA (1998). Briefly, 5 ml of each urine sample mixed with 20ml of distilled water and

then was filtered by using GF/F filtered paper (47 mm, Whatman® ,UK) and into 500 ml sterile Erlenmeyer flask. Then, 4ml of combined reagent was added each urine sample which contained 50ml H_2SO_4, 15ml Ammonium molybdate, 5ml potassium tartrate and 30ml ascorbic acid. Absorbance at 880nm was measured spectrophotometrically (HACH DR/2010, Canada) using reagent blank as the reference solution. The absorbance reading was converted to concentration using standard curves followed by (APHA, 1998).

Total nitrogen (TN) was analyzed in the laboratory of Center for Food Science and Nutrition using the protocol by the Association Official Analytical Chemistry (AOAC, 2005). Briefly, 1gram of urine sample was measured a tector tube. Then, 6ml concentrated sulfuric acid was added to the measured sample and mixing of the sample and the acid was done. Then 3.5 ml of 30% hydrogen peroxide was added step by step. This is followed by observing violet reaction. One the violet reaction caused the tube was shaken few times by hand and tube was put back onto the rack. There after 3g of the catalyst mixture (0.5gm of selenium metal with 100gm potassium sulfate) was added in to each tube and allowed to stand for about 10 minutes before digestion. When the temperature of the digestion attained at 37^{o}C the tubes allowed into the digester, then the digestion was continued until appearance of the clear solution about four hours. The tube on the racks were cooled in a fume hood and then 25ml of de-ionized water was added and shake to a void the precipitation of sulfate in the solution digestion and titration. The digested and diluted urine sample was distilled using boric acid and the distillate was titrated by using 0.1 N hydrochloric acid until reddish color appeared. The total amount of nitrogen in the urine sample was calculated using the formula stated below (AOAC, 2005):

$$\text{Nitrogen (\%)} = \left[\frac{\text{VHCl} \times \text{NHCl} \times 14}{\text{Wo}}\right] \times 100$$

Where: V = volume of HCl in L consumed to the end point of titration,

N = the normality of HCl (used often is 0.1),

Wo = Sample weight on dry matter basis and

14 = the molecular weight of nitrogen

5.5. Biological contaminants analysis of urine

5.5.1. Enumeration of total and fecal coliform bacteria

For the detection of total and fecal coliform bacteria sample was taken from the collected urine using and cultivation of microbes was done immediately after collection. To analyze total coliforms, heterotrophic plate count (HPC) was conducted using spread plate techniques based on Standard Methods 9215C of (APHA, 1998). 1 mL of urine was serially diluted with Phosphate Buffer Saline (0.85% NaCl) in order to reduce the bacterial density. Subsequently;0.1 mL of the appropriate dilutions was spread directly onto the R_2A agar plates. HPC colonies were counted after 48 hours of incubation at 37°C.

Fecal Coliforms were analyzed by Membrane Filtration technique using membrane lauryl sulfate broth (MLS). The urine samples were collected in a sterile glass bottle. After sterile absorbent pads were transferred into sterile Petri dishes, 2 ml of sterile membrane lauryl sulfate broth was added to each Petri dish. The membrane filter forceps were dipped in ethanol and burned off in the flame of the Bunsen burner. Then a sterile membrane filter of 0.45 μm pore size, 44 mm diameter was placed on the membrane filtration unit and a diluted sample was poured and filtered using a manual vacuum pump. The Petri dishes

were placed at incubator maintained at 44 ^{o}C ± 0.5 C. Finally, the number of yellow colonies was counted after 24 HRs of incubation periods (APHA, 1998).

5.5.2. Parasites examination of urine

The urine sample was initially examined for the presence of parasites (protozoa, and helminthes ova) as described in standard methods in APHA (1998). Briefly, the collected urine specimen was shaken and poured into the 1000ml sterilized Erlenmeyer flask and was allowed to sediment for 1-2 hours. Then, the supernatant was decanted and the sediment was transferred into a 15ml centrifuge tube, and centrifuged at 5000rpm for 14 minutes. After centrifugation, the pellet was transferred to a new centrifuge tube and was added equal volume of acetoacetic and ethyl acetate step by step and was centrifuged at 5000rpm for 14 minutes. Finally, the pellet was examined under the microscope using different (X10, X40 and X100 with oil) magnification powers for the presence of ova.

5.6. Evaluation of the persistence of bacteriological contaminants in sanitized urine

5.6.1. Effect of pasteurization

After examination of fresh urine samples for the contaminants described above, 500ml each urine sample was poured into sterilized Erlenmeyer flasks and put in a water bath, then incubated with the temperature set at 80 ^{0}c for 30 minutes. Then the time was completed to allow the sample to cool at room temperature for 30 minutes to 15 seconds, respectively. After pasteurization completed, the different microbial contaminants were examined all the above procedure followed by (Lahr *et al*., 2016).

5.6.2. Effect of acidification

After examination of fresh urine samples for the bacterial coliforms, 500ml each urine sample poured into sterilized Erlenmeyer flasks. The pH of the initial urine sample was measured using pH meter and 1N HCl was dropped into the fresh sample until its pH reached 5.5. After acidification samples were examined for the biological contaminants and the removal efficiency of acidification process was calculated by using the following formula:

$$\text{Removal Efficiency} = \left[\frac{\text{CFU in fresh urine} - \text{CFU in acidified urine}}{\text{CFU in fresh urine}}\right] \times 100$$

Source: (Desta *et al*., 2015).

5.6.3. Effect of storage

The urine samples collected from all the sites were stored in the bioinstrumentation laboratory at room temperature and samples were taken by the end of each month and examined for their faecal and total coliform counts as described above. The sampling was done for six months and removal efficiency was calculated using the formula above.

5.7. Culture-Independent Analysis of Antibiotic Resistance Genes

5.7.1. Extraction of Bacterial DNA

The bacterial DNA extraction protocol by using phenol saturated buffer and chloroform followed by (Lopes da Silva *et al*., 2013). Five ml of human urine collected from four sites mixed, with 600µl of lysis solution which contains (100mM NaCl, 10mM Tris –HCl, and 0.5% SDS, pH 8) and 40µl of proteinase K (20mg/ml was added to falcon tube and incubated 60^{o} C for 1 hour on water bath. After incubation an equal volume of buffer-saturated phenol was added to the DNA solution. hen the samples were centrifuged at

5000rpm for seven minutes and was carefully removed the aqueous layer from each sample and was placed into a new tube. And then an equal volume of a buffer –saturated phenol: chloroform (1:1) mixture was added into the aqueous layer and was centrifuged at 5000rpm for seven minutes. And was carefully remove the aqueous layer from each sample and was placed into a new tube and an equal volume of chloroform was added each aqueous layer and centrifuged at 5000rpm for seven minutes, Repeat the chloroform step twice. There then equal volume of isopropyl alcohol was added each aqueous layer and centrifuge at 5000rpm for seven minutes. After centrifugation discard the supernatant and washed the pellet by adding of 5 ml of 70% ethanol up and down to suspend the pellet completely and was centrifuge at 5000rpm for ten minutes. Then the pellet was dried for 2 days in laminar air flow hood. The DNA samples were diluted at 20µl autoclaved Milli-Q water.

After the extraction of total DNA, the DNA concentrations in the four different sites was quantified using a Nanodrop 1000 (Thermo scientific, USA). From each sample 1 µL was measured and the DNA concentration was measured under 260 nm. In order to look for protein contaminations a purity check was done by measuring the absorbance ratio at A260/A280 wave lengths. The DNA was stored at -20^{o}C until use.

5.7.2. DNA amplification by Thermo cyclic polymerase chain reaction (PCR)

The extracted total DNA was subjected to PCR for the presence of antibiotic resistance genes. The antibiotic resistance genes were *tetB, sul2* and *qnrB,* which are responsible for resistance against tetracycline, sulfonamide and floroquinolone antimicrobials. For tetB, the PCR was carried out (Thermo Cycler, Germany) using premix PCR strips (AccuPower® PCR PreMix, Bioneer, S. Korea) a total 20 µl reaction volume was used containing 1µ1 each primer (reverse and forward), 16 µ1 of Milli-Q water and 2 µ1 of

template DNA. The PCR conditions for *tetB* and *sul2* genes involved an initial denaturation for 3 min at 95 °C followed by 35 cycles of 95 °C for 30s, specific annealing temperature for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 6 min. Specific annealing temperature values for each PCR reaction is shown in Table 1.

The reactions for the amplification of qnrB gene involved an initial denaturation for 3 min at 95 °C followed by 30 cycles of 95 °C for 30 s, specific annealing temperature for 1 min, and extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min. Specific annealing temperature for qnrB reaction is shown in Table 1. The amplification products were visualized after electrophoresis on a 2% agarose gel and 5 µl ethidium bromides for staining and photographed using an Image Master VDS (BioRed Gel Doc XR, USA).

Table 1 List of primers and PCR conditions for amplification of the proposed antibiotic resistance genes

Primer name	Primer direction	Primer sequences 5'-3'	Amplicon size (bp)	Amplicon Temperature (°C)	Ref.
Sul 2	Forward	CCTGTTTCGTCCGACACAGA	420	55	Tadesse Eguale *et al.*, 2017
	Reverse	GAAGCGCAGCCGCAATTCA			
tet A	Forward	GCGCCTTTCCTTTGGGTTCT	826	57	
	Reverse	CCACCCGTTCCACGTTGTTA			
tet B	Forward	CCCAGTGCTGTTGTTGGTCAT	724	57	
	Reverse	CCACCACCAGCCAATAAAAT			
qnr B	Forward	GATCGGCAAAGGTTAGGTCA	469	53	
	Reverse	ACGATGCCTGGTAGTTGTCC			

5.8. Ethical Consideration

To conduct this study, ethical clearance was obtained from Institutional Ethics Review of College of Natural Science, Addis Ababa University with a minute number IRB/024/2017 and letter number CNSDO/238/09/2017.

5.9. Data presentation and analysis

The data for nutrient contents and coliform bacteria counts were entered using Microsoft Excel. Mean values of the nutrients and graphical presentations of the microbial count data were conducted using Microsoft Excel (Microsoft Office V 10).

6. Results

6.1. Physicochemical characteristics of fresh urine collected from different sites

Fresh urine collected from male and female participants in CNCS, AAU campus had pH of 5.5 and 5.8, respectively. The fresh urine collected form Megenagna and Merkato had pH close to 6. The fresh urine collected from Gelila school had the lowest pH of all the sampling sites ranging between 5.2 to 5.6 (Table 2). The total Phosphorus values of fresh urine collected from AAU and Megenagna were close to 4 mg/l while in the case of Merkato and Gelila, the total phosphorus ranged from 1.2 to 2.7 mg/l. The total Nitrogen level of fresh urine in all the sampling sites ranged from 5 – 6 g/l, with the exception of male urine collected from Megenagna. The soluble fraction of phosphorus (SRP) in fresh urine collected from AAU campus was 2.5 and 4 mg/l in female and male samples respectively. The SRP levels of samples from Merkato and Gelila School ranged between 0 and 1 mg/l while the sample from Megenagna had 4.5 mg/l (Table 2)

Table 2 Physicochemical characteristics of fresh urine collected from four different sites in Addis Ababa, January to March 2017

Site		Parameter			
		pH	**SRP (mg/L)**	**TP (mg/L)**	**TN (g/L)**
AAU	Female	5.84	2.47	3.99	5.5
	Male	5.50	4.08	4.13	6.3
	Mixed urine	5.67	3.27	4.06	7.2
Megenagna	Male	5.80	4.48	4.09	1.7
Merkato	Female	5.90	1.07	1.22	5.9
	Male	5.60	0.70	2.64	6.0
	Mixed urine	5.75	0.99	1.93	5.9
Gelila	Female	5.66	2.06	2.60	5.7
	Male	5.28	0.43	2.69	5.4
	Mixed urine	5.46	1.25	2.64	5.6

SRP = soluble reactive phosphorus, TP = total phosphorus and TN= total nitrogen

6.2. Bacteriological characteristics of fresh urine samples collected from different sites

Total coliforms in fresh urine samples collected from the four sites of Addis Ababa ranged between the highest counts of 234,000 cfu/ml in Gelila female urine collection unit to 64500 cfu/ml AAU male urine collection unit (Table 3). Out of the four sites of collection, fecal coliform counts were not detected in both male and female samples from Gelila School and male samples from Merkato. Fresh urine collected from AAU had faecal coliform counts of 1500 cfu/100 ml in female samples and 680 cfu/100 ml in male samples. Samples from

Megenagna had 800 cfu/100ml and Merkato (female sample) had 100 cfu/100ml (Table 3).

In all urine samples collected from the four sites, no parasite ova were detected.

Table 3 Bacteriological characteristics of fresh urine collected from four different sites in Addis Ababa, January to March 2017

Site		Parameter		
		Parasite	Total coliform (TC) (cfu/ml)	Fecal coliform (FC) (cfu/100ml)
AAU	Female	ND	1.4E+04	1500
	Male	ND	6.450E+03	680
	Mixed urine	ND	1.0225E+04	1600
Megenagna	Male	ND	1.30E+04	800
Merkato	Female	ND	2.00E+04	100
	Male	ND	ND	ND
	Mixed urine	ND	2.00E+04	100
Gelila	Female	ND	2.34E+04	ND
	Male	ND	1.70E+04	ND
	Mixed urine	ND	2.02.00E+04	ND

ND = Not detected, FC= Fecal coliform, TC= Total coliform and CFU = Colony Forming Unit

6.3. Efficiency of different sanitization methods in reducing bacterial counts

6.3.1. Effect of acidification and pasteurization in coliform removal

Acidification of fresh urine by addition of 1N HCl until the pH reaches to 5.5 didn't show any reduction in total coliform counts of urine collected from all the four sites. Regarding fecal coliforms, a slight decrease was observed resulting in 20% removal of fecal coliforms in the case of urine collected from Merkato (table 4). Urine collected from the other three sites did not show any decrease in fecal coliforms. On the other hand, pasteurizing the fresh urine resulted in the total and fecal coliform units to null in all the samples from the four sites, indicating 100% removal (Table 4).

Table 4 Removal efficiency of acidification in reducing bacterial count from fresh urine at four different sites of Addis Ababa, January to March 2017

Site	Initial		Final (Acidified)		RE (%)	
	TC (cfu/ml)	FC (cfu/100ml)	TC	FC (cfu/100ml)	TC	FC
AAU	1.0225E+04	1600	1.0225E+04	1600	0	0
Megenagna	1.30E+04	800	1.3E+04	800	0	0
Merkato	2.00E+04	100	2.00E+04	80	0	20
Gelila	2.02.00E+04	ND	2.02 E+04	ND	0	ND

Table 5 Removal efficiency of pasteurization in reducing bacterial count from fresh urine at four different sites of Addis Ababa, January to March 2017

Site	Initial		Final (Pasteurized)		Removal Efficiency (%)	
	TC (cfu/ml)	FC (cfu/100ml)	TC (cfu/ml)	FC (cfu/100ml)	TC	FC
AAU	1.0225E+04	1600	0	0	100	100
Megenagna	1.30E+04	800	0	0	100	100
Merkato	2.00E+04	100	0	0	100	100
Gelila	2.02.00E+04	ND	0	0	100	∄

ND = Not detected, FC= Fecal coliform, TC= Total coliform and CFU = Colony Forming Unit, **∄ = undefined due to division by zero**

6.3.2. Effect of Storage in reducing total and fecal coliforms

Total coliform count from the four samples showed variable decreasing patterns during storage for six months. Highest total coliform counts in fresh urine was found in samples collected from Gelila school followed by Merkato with counts of 20200 and 20000 cfu/ml, respectively (Fig. 6a). From the first to the fourth month of storage, urine samples from Gelila School showed steady pattern of decrease in total coliforms. On the fifth and sixth months, total coliforms were completely removed in urine samples from Gelila School. In sample from Merkato, drastic decrease of total coliform was observed on the first month and the decreased continued up to the fourth month. Similar to Gelila school sample, total coliform count was null on the fifth and sixth months.

In urine collected from Megenagna, total coliform count in the fresh urine was the third highest of all the samples with the count of 13000 cfu/ml. During storage, drastic decrease (4300 cfu/ml) was observed on the first month followed by a steady decrease until the

fourth month and with complete removal from the fifth month onwards. The fresh urine sample from AAU showed the least total coliform count (10255 cfu/ml) of all the urine samples freshly collected from the sites. During storage, urine from AAU showed a steady decreasing pattern up to the 5th month and completely removed on the sixth month of storage (Fig. 6a).

Storage for one month was efficient in reducing fecal coliform units to zero in all the samples collected from the four sites. For the sample collected from AAU, 100% removal of total coliform bacteria was achieved in the sixth month of storage (Fig. 6b). For the urine samples collected from Megenagna, Merkato and Gelila School, five months of storage resulted in complete removal of the total coliform bacteria. Fecal coliforms did not survive after one month of storage in all samples (Fig. 6b).

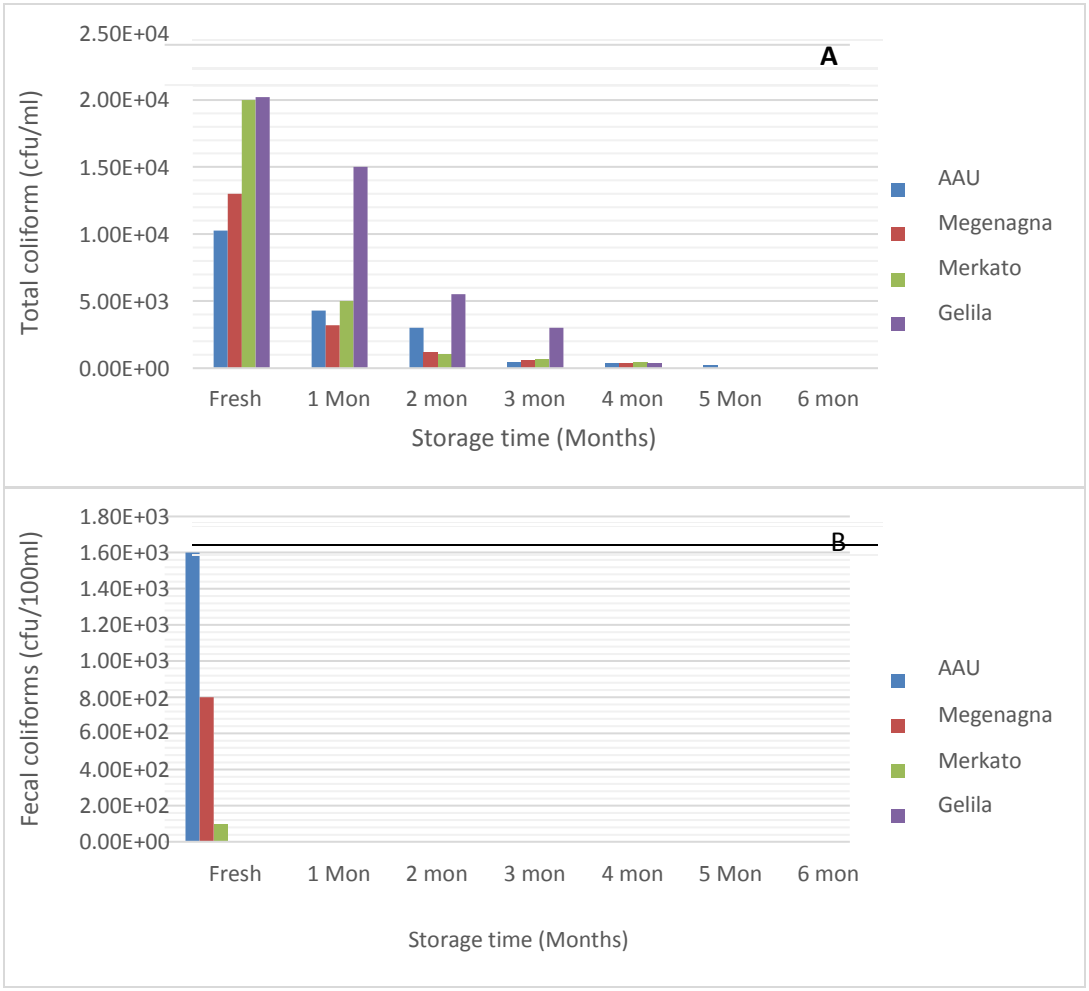

Figure 6 Monthly count of total coliforms (A) and fecal coliforms (B) in urine samples collected from four different sites during six months of storage, January to August 2017.

Table 6 Removal efficiency of storage from 1 to 6 months of time in reducing bacterial count from fresh urine at four different sites of Addis Ababa, January to August 2017

Site	Parameter	Removal efficiency at different storage time (%)					
		Mon1	Mon2	Mon3	Mon4	Mon5	Mon6
AAU	Tc	55.9	65.8	95.5	96	98	100
	Fc	100	100	100	100	100	100
Megenagna	Tc	75	90.7	95.7	97	100	100
	Fc	100	100	100	100	100	100
Merkato	Tc	75	95	96.7	97.7	100	100
	Fc	100	100	100	100	100	100
Gelila	Tc	25.5	72.5	85	98	100	100
	Fc	ND	ND	ND	ND	ND	ND

ND = Not detected, FC= Fecal coliform, TC= Total coliform and CFU = Colony Forming Unit

The removal efficiency of storage on total coliforms of urine sample collected from, Megenagna and Merkato was more than 90% for from month 2 to month 4 storage times, which became 100% on the fifth and sixth months of storage. The removal efficiency of urine samples from AAU and Gelila School was lower than that of Merkato and Megenagna, with 72% removal for AAU and 65% removal for Gelila School. 100% removal of total coliforms was observed on the sixth month for AAU and fifth month for Gelila School (Table 6). For all urine samples except Gelila School, 100% efficiency for fecal coliform count was observed from the first month onwards (Table 6).

6.4. Effect of the different sanitization methods on the nutrient concentration of urine

6.4.1. Effect of pasteurization in Nitrogen and Phosphorus contents of urine

Total phosphorus (TP), soluble fraction of phosphorus (SRP) and total Nitrogen (TN) did not show any variation upon pasteurization in urine collected from AAU. A slight decrease in SRP and TN was observed upon pasteurization on urine collected from Merkato, while TP from the same site remained stable (Table 7).

Table 7 Nutrient contents of urine undergoing pasteurization from fresh urine from AAU and Merkato in January to March 2017.

Sites	Parameter	Mean Nutrient concentration	
		Before Pasteurization	After pasteurization
AAU	SRP (mg/l)	57.2	57.2
	TP (mg/l)	56.5	56.7
	TN (g/l)	5.18	5.18
Merkato	SRP (mg/l)	56.67	56.0
	TP (mg/l)	56.8	56.8
	TN (g/l)	5.46	5.00

SRP = Soluble Reactive Phosphorus, TP = Total phosphorus and TN =Total Nitrogen

6.4.2. Effect of storage in Nitrogen and Phosphorus contents of urine

6.4.2.1. Total Phosphorus (TP) and soluble reactive Phosphorus (SRP) in stored urine

Total Phosphorus (TP) in fresh urine collected in AAU campus was 4mg/l and by the end of its first and second month's storage, the amount of TP increased to 26 mg/l and 51 mg/l, respectively. The increase in TP did not continue to increase in the rest of the storage times, with the TP value of 57mg/l on the sixth month of storage. Equal amount of TP was measured in urine sample collected from Megenagna (4mg/l) but its increase was not as high as that of AAU on the first two months, amounting to 15mg/l on the first month of storage and 40 mg/l on the second month of storage. By the end of the sixth month, the TP was 56 mg/l (Fig 7). The fresh urine collected from Merkato had the lowest TP of all the samples (2 mg/l) but during storage, the TP increased steadily and by the end of the sixth month, TP value was 57 mg/l. Similarly, the fresh urine collected from Gelila School had TP close to 3 mg/l, which increased steadily and by the end of the sixth month of storage, TP reached 46 mg/l (Figure.7)

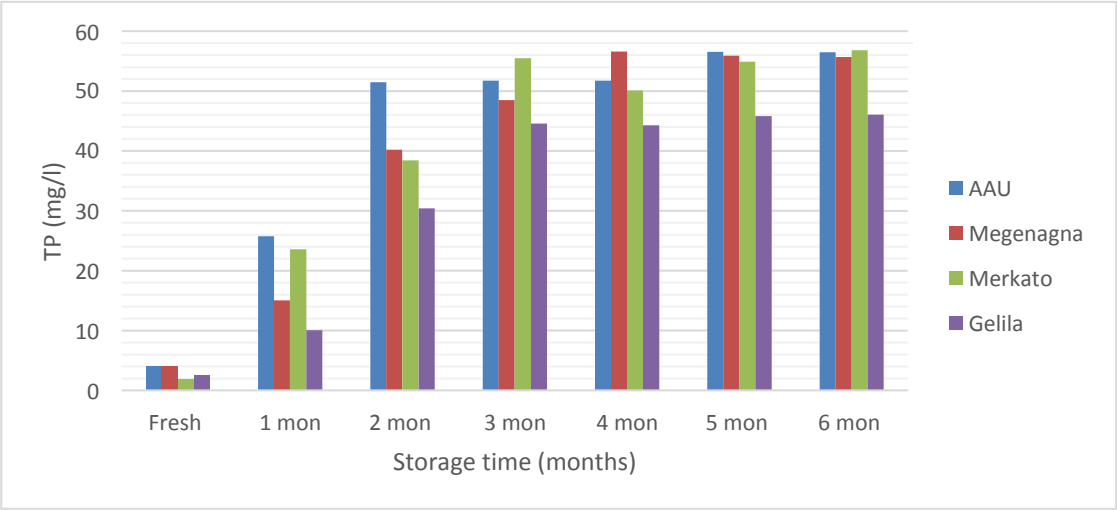

Figure 7 Monthly analysis of total Phosphorus in urine samples collected from the four sites in Addis Ababa undergoing storage at room temperature January to August 2017

The soluble reactive phosphorus (SRP) also followed increasing trend upon storage. Fresh urine collected from Megenagna had the highest amount of SRP (4 mg/l) and gradually increased to 14mg/l on the second month of storage. Drastic increase of SRP was observed from the third month onwards which was 49 mg/l on the third month and increased to 57 mg/l on the sixth month of storage (Fig. 8). Fresh urine collected from AAU had the second highest amount of SRP (3 mg/l) which gradually increased to 13 mg/l by the end of the second month of storage. SRP reached 51 mg/l by the third and 57 mg/l by the sixth month. For the sample from Merkato, the SRP increased drastically from 1 mg/l in the fresh urine to 19 mg/l by the end of the first month, reaching 57 mg/l by the end of the sixth month of storage. Urine collected from Gelila school showed a gradual increase of SRP during the first two months of storage, from 1 mg/l in the fresh urine amounting to 17 mg/l by the second month of storage. The SRP was 45 mg/l by the end of the sixth month, which was the lowest of all the stored samples (Figure. 8).

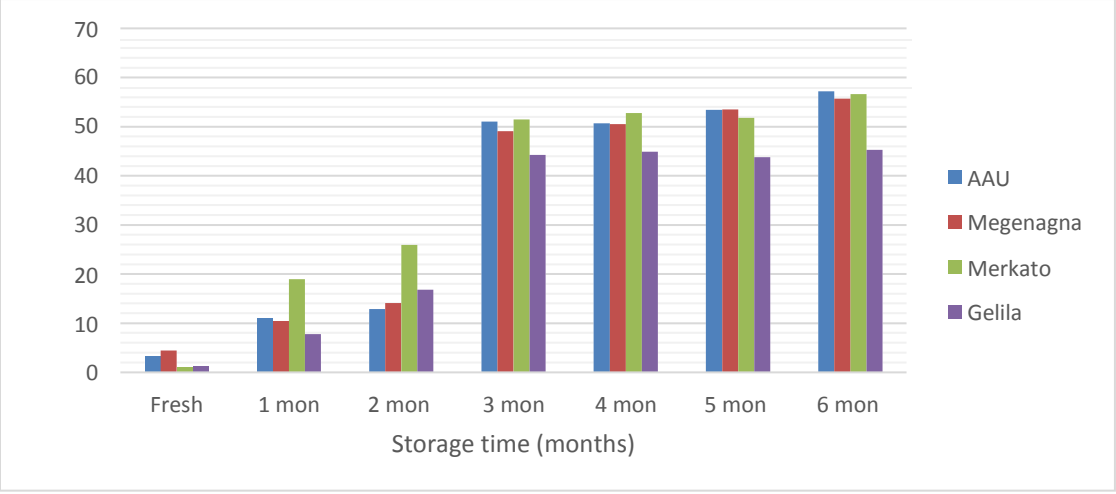

Figure 8 Monthly analysis of soluble reactive phosphorus in urine samples collected from the four sites in Addis Ababa undergoing storage at room temperature, January to August 2017

6.4.2.2. Total Nitrogen in fresh and stored urine samples

The amount of total Nitrogen (TN) in the urine samples collected from the four sites showed variable pattern within the six months of storage time. The fresh sample collected from AAU had the highest TN value (7 g/l) which then decreased during the first three months of storage time reaching 5 g/l by the third month. By the fourth month, the TN increased back to 7 g/l and then decreased to 5 g/l by the end of the sixth month of storage time (Fig. 8). TN in urine sample collected from Merkato decreased from 5.9 g/l (fresh) to 4 g/l by the second month. Between the fourth to sixth months of storage TN was 4 g/l (4^{th} month), 7 g/l (5^{th} month) and 5 g/l (6^{th} month). In the sample collected from Gelila school, TN was initially 5.7 g/l in fresh urine which remained the same by the first month and decreased to 3.8 g/l by the second and fourth months. TN increased back to 6 g/l by the end of the sixth month (Fig. 9). TN measured in urine collected from Megenegna was nearly 2 g/l until the end of the second month of storage which then gradually increased to 3 g/l by the end of the fourth month and reached to 4 g/l by the end of the sixth month (Fig. 9).

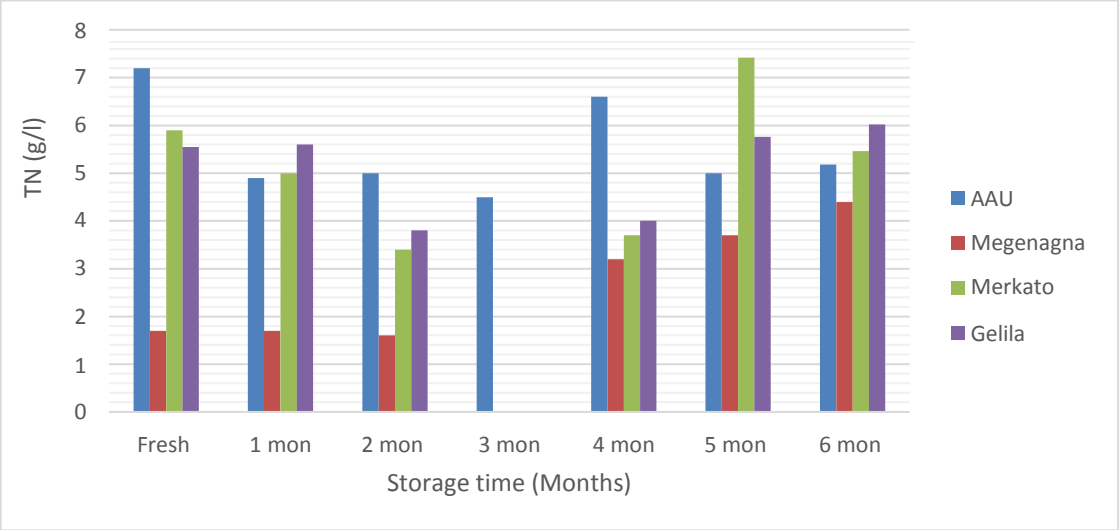

Figure 9 Monthly analysis of total Nitrogen in urine samples collected from the four sites in Addis Ababa undergoing storage at room temperature. (3rd month sample was analyzed for AAU sample only) from January to August 2017

6.5. Culture-independent detection of antibiotic resistance genes in urine samples

6.4.3. Detection of tetB gene in fresh and stored urine samples

Fresh urine collected from AAU was found positive for the resistance gene *tetB* and in the sample from the sixth month of storage; the gene was not detected by PCR (Plate 1). In the fresh samples from all the other sites, *tetB* was not detected. The urine sample collected from Gelila School and stored for 4 months showed positive amplification for tetB gene (Plate 1). All the stored samples from the other sites did not show positive result for this gene.

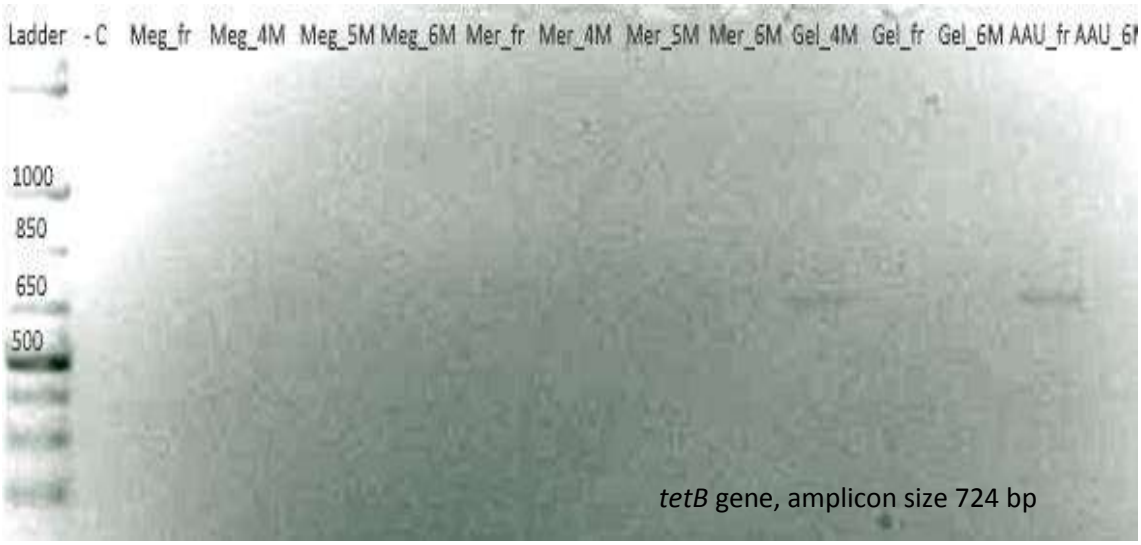

Plate 1 Amplification of tetB gene in fresh and stored urine samples of the four sites in Addis Ababa, January to August 2017

6.4.4. Detection of Sul2 gene in fresh and stored urine samples

Fresh and stored urine samples collected from Megenagna were positive for Sul2 resistance gene. Urine sample collected from Merkato and stored for four months was also positive for this gene. Fresh urine collected from Merkato was negative for this gene. The urine collected from Merkato and stored for six months was positive with a very faint amplification difficult to visualize. All the fresh and stored samples from AAU and Gelila School did not show amplification for this gene (Plate 2).

Plate 2 Amplification of sul2 gene in fresh and stored urine samples of the four sites in Addis Ababa, January to August 2017

6.4.5. Detection of QnrB gene in fresh and stored urine samples

Among the fresh urine samples collected from the four different sites, quinolone resistance genes named as qnrB were detected in the sample from Merkato. The other three sites did not give positive amplification for qnrB gene. The gene was present in the sample collected from Merkato and stored for four months while in the sample stored for six months, the gene was not amplified (plate 3). Stored samples from the other sites did not show any amplification for qnrB gene.

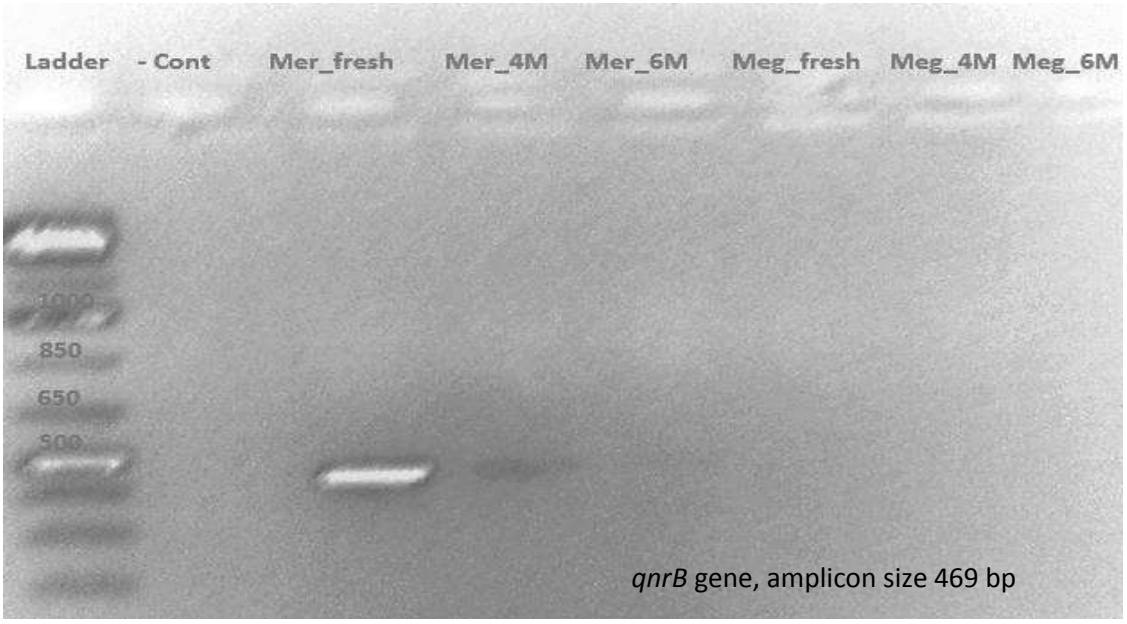

Plate 3 Amplification of qnrB gene in fresh and stored urine samples of the four sites in Addis Ababa, January to August 2017

7. Discussion

In the current study, pH of fresh urine ranged between 5.5 to 5.9 indicating acidic nature. In the study by Srinivasamurthy and colleagues (2012), the pH of fresh urine was reported to be in the range of 4.97 to 6, indicating acidic nature of the collected urine. In contrast, a recent study by Zhou and colleagues (2017) reported that pH of fresh urine was 7.15 which is in the neutral range and higher than our finding. In clinical medicine, the normal pH range of human urine is from 4.8 up to 8.0, which varies at different hours of the day (Cook *et al*., 2000).

The observed increase in pH to a maximum of 10.4, becoming alkaline, during storage, is due to production of urease by cross-contaminating bacteria which hydrolyzes the urea into ammonium, hydrogen carbonate and hydroxide (Vinneras and Jonsson, 2002; Scnecal *et al*.,2017).

The total Phosphorus (TP) of fresh urine collected from the four sites of Addis Ababa, which ranged from 1.9 to 4.1 mg/L was much lower than the amount of total Phosphorus reported by Etter and colleagues (2011), in which the average Phosphorus concentration of fresh urine collected in Nepal was 388 mg/L. Similarly, TP of urine collected from Vietnam was 816 mg/L (Wohlsager et al., 2010), which was much higher than our TP value. Similarly, a study on urine collected from four communities in Nigeria reported an average TP of 260 mg/L (Akpan-Idiok et al., 2012), much higher than our TP value. The variation in the level of Phosphorus and Nitrogen in human urine is dependent on the food intake, body size, physical activity, the amount of drinking water and environmental factors. It varies from country to country, regions to regions and person to person as well as on the time of excretion (Jonsson and Vinneras, 2004). Although our nutrition and fluid

intake can attribute for the low phosphorus amount in the present study, methodological flaws in the adopted protocol for the TP measurement should be validated for urine samples.

Also, the maximum soluble reactive Phosphorus (SRP) in the present study was 4.5 mg/L. However, a much higher concentration of SRP was observed in many other studies. For example, in urine collected in Nepal, a mean SRP of 388 mg/L was reported (Etter et al., 2011). Similarly, in a study by Golder and colleagues (2007) SRP of collected fresh urine in India was 703 mg/L. As a whole, the SRP in the current study was much lower than those reported from all other countries. Although the low phosphorus nutrition and fluid intake can attribute for the low phosphorus amount in the present study, the method adopted might have some flaws which requires validation for urine samples.

In the present study, the total Nitrogen (TN) in fresh urine in all sampling sites ranged from 5 – 6 g/L. Other studies also showed TN values comparable to our study. For example, a study in Nigeria by Akpan-Idiok and colleague (2012) reported TN as an average of 4.58 g/L, which is within the TN range in our study. Likewise, a study in Sweden on urine collection for crop production reported TN in the range of 11 to 13.9 g/L (Jonsson et al., 2004), much higher than our TN value. A TN value lower than the present study was also reported by Sangare and colleagues (2014) which was 2.70 g/L. The contribution of protein-rich food intake in the TN content of urine is well established in country-wide study conducted to estimate N excretion in urine per capita (Richert et al., 2010). Considering Sweden, with an estimated excretion of Nitrogen of 4.0 kg/capita/year, has nearly twice as much Nitrogen as Uganda (N = 2.2 kg/capita/year) (Richert et al., 2010). The present low content of TN in urine may be a reflection of food intake and the general

economic status of the participants. Another study by Sirivasamurthy and colleagues (2012) revealed that urine collected from age range of 20 to 40 years and different diet options (vegetarian and non-vegetarian) resulted in much higher nutrient levels from the non-vegetarian group, indicating the contribution of nutrition to urine Nitrogen level.

The finding that the coliform counts in urine collected from female participants was higher than that of the male participants can be explained by the anatomy of the female urinary tract which may expose the urine to cross-contamination with fecal bacteria. Furthermore, urine from a healthy female may get contaminated in the vagina where different types of microflora reside, depending on age, pH and hormonal level of the female individual (Larsen and Monif 2001).

The study finding that acidification was inefficient in the removal of total and fecal coliforms was in contrary with previous report that coliforms can survive in urine environments in the pH range of 6 to 7 (Schonning, 2001). In sea water, a matrix different from urine, acidic pH was shown to decrease the survival rate and did not totally eliminate coliform bacteria (Wahyuni, 2015), suggesting the need for a neutral pH for optimal growth and survival in the environment. The existence of acid resistant coliforms from various market foods in Pakistan was demonstrated by identifying members of the genera Escherichia, Klebsiella, Shigella, Salmonella and Enterobacter. These bacterial groups survived acidic environments of pH 2, 3 and 4 upon exposure for ten minutes at 37 ^{o}C (Saleem et al., 2014). In addition to the difference in the environment (ie. food vs urine), the length of exposure time to the acidic environment also determined the survival of bacteria. In the present study, the short time of exposure (<10 min) and low acid concentration (1N HCl; pH=1) may have contributed for the survival of the coliforms.

Furthermore, acidification in urine is more related to minimizing loss of ammonia than reduction of microbes as indicated in the study by Hellstrom and colleagues (1999).

The pasteurization study finding was in agreement with the well-established efficiency of pasteurization in the removal of coliform bacteria in food processing industries. Disruption of the metabolic activity of microbes in the urine is the most likely effect of pasteurization on microbial physiology (Lahr *et al*., 2016).

Storage of urine has long been a well-established and suitable method of hygienization of source- separated urine. Studies have indicated that increase in pH is one of the key storage parameters contributing to microbial contamination reduction (Hoglund et al., 2002). Storage facilitates the breakdown of urea into ammonia and hydroxide which contributes greatly to the increase in pH of the urine environment, thus inactivating the resident microorganisms (Hoglund and Stenstrom 1999). Another study by Makaya and colleagues (2014) reported that urine storage for one month under the exposure to sunlight was 100% efficient in removing members of the family Enterobacteriaceae and Gram-positive spore-forming bacteria. The possible mechanism of the efficient removal of bacteria could be the synergistic effect of the storage causing high pH and the high temperature making the urine environment unfavorable for most members of Enterobacteriaceae.

Because of the short survival time of fecal coliform bacteria in stored urine, the fecal coliforms did not survive until the end of the first month of storage. Members of the fecal coliform bacteria belong to the species - E. coli, Klebsiella pneumoniae, Enterobacter cloacae and E. aerogenes (Cabral, 2010). Most members of the group were inactivated rapidly in urine, indicating a low risk for transmission of gastrointestinal infections caused

by bacteria when handling diverted urine (Hoglund *et al*., 1998).

In the pasteurized urine from AAU and Merkato, none and minimal Nitrogen loss was recorded, respectively. This is because of metabolic arrest in urine microbial communities as explained by Lahr and colleagues (2016). Pasteurization is reported to have some limited effect on the concentration of Nitrogen since bacteria can be killed through hydrolysis of urea (Fearn *et al*., 2015).

The concentration of total Phosphorus (TP) and soluble reactive Phosphorus (SRP) increased steadily during the first two months and then leveled off from the third to the sixth months of urine storage. The increase is a result of the transformation of phosphorus into mineral precipitate which is composed of amorphous and crystalline solids. According to the study by Wohlsager and colleagues (2010) on urine collected in Vietnam, precipitation of elements in urine under tropical conditions (temperature 23 ^{o}C and above) occurs within the first day of storage. On the second month of storage, 44% of the Phosphorus is precipitated. The precipitated Phosphorus was present as hydroxylapatite (Ca10 (PO4)6(OH) 2) or hydrated struvite (NH4MgPO4.xH2O) (Wohlsager et al., 2010). In the present study, the storage jerry cans had a sampling pipe at the bottom, which facilitates the collection of these hydrated precipitates despite the vigorous shaking before each sampling time, contributing to the increasing trend of the level of phosphorus throughout the storage time.

Similarly, loss of total Nitrogen (TN) in the urine by the end of the sixth month did not exceed 25%. Wohlsager and colleagues (2010) evaluated the loss of total Nitrogen in urine stored in open containers and compared them with urine stored in closed containers and

found out 85% TN loss in open container storage and 2.9% TN loss in the closed container, suggesting the importance of closing storage containers to conserve Nitrogen in urine.

Surveillance of biological contaminants in source-separated urine undergone sanitization is important for minimizing the risk of disease transmission. The Information on the effect of storage in reducing the risks associated with antibiotic resistance genes (ARG) in microbial pathogens is lacking. The current study on culture-independent ARG detection from urine was a first attempt to get an overview of the fate of some ARGs in urine during storage. Studies on antibiotic resistance genes in cultured microorganisms isolated from various environmental matrices, such as drinking water (Fernando *et al*., 2016), soil fertilized with waste-derived fertilizer (Ross and Topp, 2015), and wastewater effluents from treatment plants (Rizzo *et al*., 2013) indicate that wastewater is an important reservoir of ARGs, which might potentially pose a risk to the spread of antimicrobial resistance in microbial communities of agricultural soils.

tetB genes encode for energy-dependent efflux proteins and these genes are reported in gram positive, facultative or obligate anaerobic bacteria such as Streptococcus spp. (https://www.ncbi.nlm.nih.gov/nuccore/?term=tetB). The possible reason for the appearance of this gene in stored sample from Gelila School, without appearing in the fresh counterpart samples, might be that these genes flourished as the Gram-positive bacteria became abundant in stored urine.

Sul2 gene is a plasmid encoded and integron-mediated gene responsible for the synthesis of modified dihydrofolate synthase, the enzyme required for folic acid synthesis and therefore

evicts the action of the drug sulfamethoxazole (Carattoli and Villa , 2005). Sulfamethoxazole is commonly prescribed for urinary tract infections and several members of the family Enterobacteriaceae are reported to confer resistance to this drug (Teichmann *et al*., 2014). The observed persistence of sul2 genes in 6-month stored urine from Merkato cannot be explained based on the bacterial abundance but might be indicative of their presence in the plasmids without the bacterial host.

qnrB genes are involved in protecting DNA gyrase by producing methylase. NCBI-ntBLAST- based similarity search showed that these genes are identified in plasmids from Escherichia, Salmonella and Enterobacter(https://www.ncbi.nlm.nih.gov/nuccore/?term=qnrB). These genera are members of the total coliforms which were indicated to decrease in abundance during storage. The observed persistence of these genes until the fourth month and its disappearance on the sixth month stored urine is similar to the survival pattern of the total coliforms during storage for six months.

8. Limitation of the study

The study was limited by methodological flaws. Additionally there was limitation of time to conduct this research even if I was used additionally one extra year but it was insufficient.

9. Conclusion and Recommendation

Both pasteurization and storage have provided the advantage of sanitizing urine without much loss of nutrients.

Storage is the best method of sanitization of source-separated urine collected from the four sample points. It was found to decrease the survival of potentially pathogenic microorganisms in urine after five months of storage. It was also observed that fecal contaminants could not survive in the urine after one month of storage.

If urine is intended to be used as a fertilizer for crop production, the safest way of application is after storage for at least five months.

For ease of collection and minimal fecal cross-contamination, it is recommended that urine collection can be done involving male participants only.

Pasteurization was found to eliminate total and fecal coliforms in urine and therefore has a potential advantage for quick application of urine. However, the energy required for raising the temperature to 80 ^{O}C in large scale requires the use of alternative energy sources for urine pasteurization to have practical value. Therefore, technological developments on the application of renewable energy for pasteurizing urine are required.

The formation of precipitation of phosphorus in stored urine is an indication of the potential production of urine-derived fertilizer in the solid form. Therefore, it is recommended that further study on urine-derived fertilizers in solid form should be

conducted.

Although most coliforms were removed during storage of urine, some antibiotic resistance genes (ARGs) remained persistent in the stored urine. Although the information at this point is not enough to conclude on the risk of ARGs horizontal transmission during urine application on soils, it is recommended that further study should be conducted to understand the fate of antibiotic resistance genes and the specific bacterial hosts harboring the genes using culture- based study.

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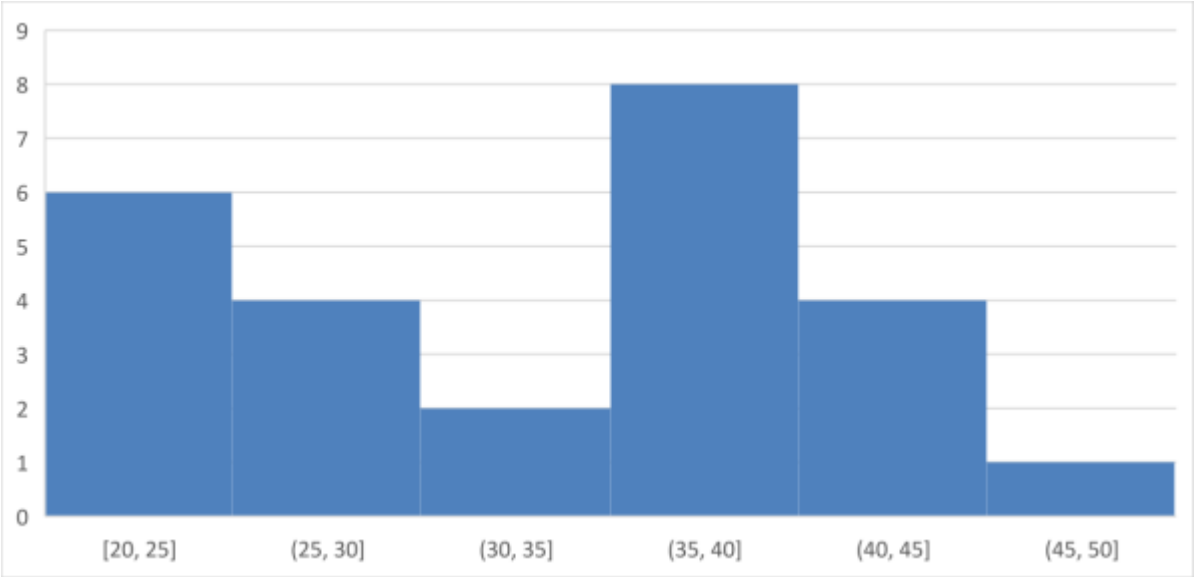
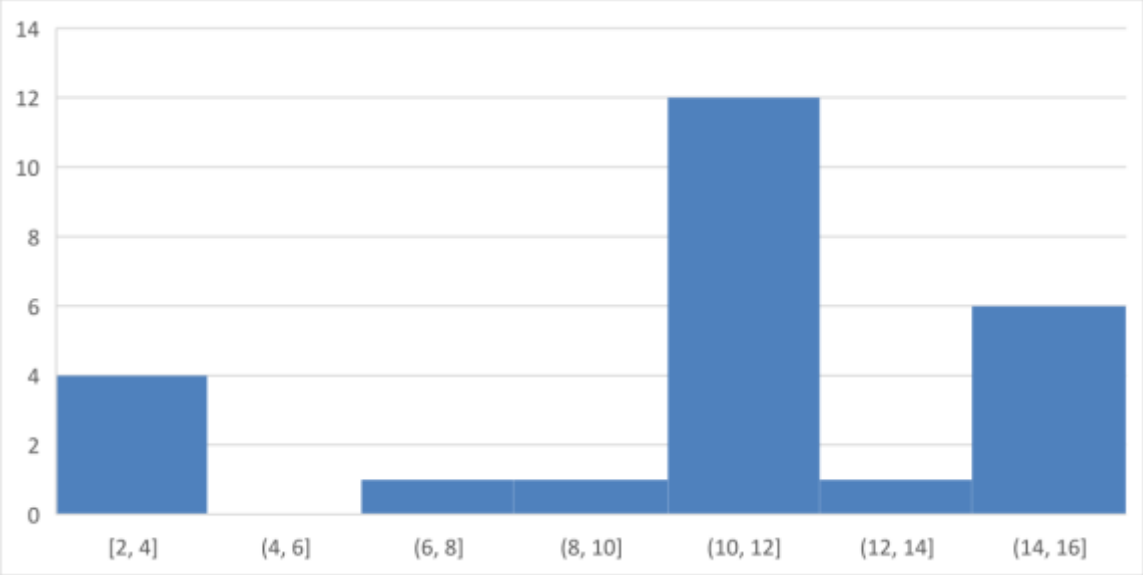
Appendices

Appendix 1 Age group of urine donors (N=25) from Merkato area. All donors were females for ease of collection



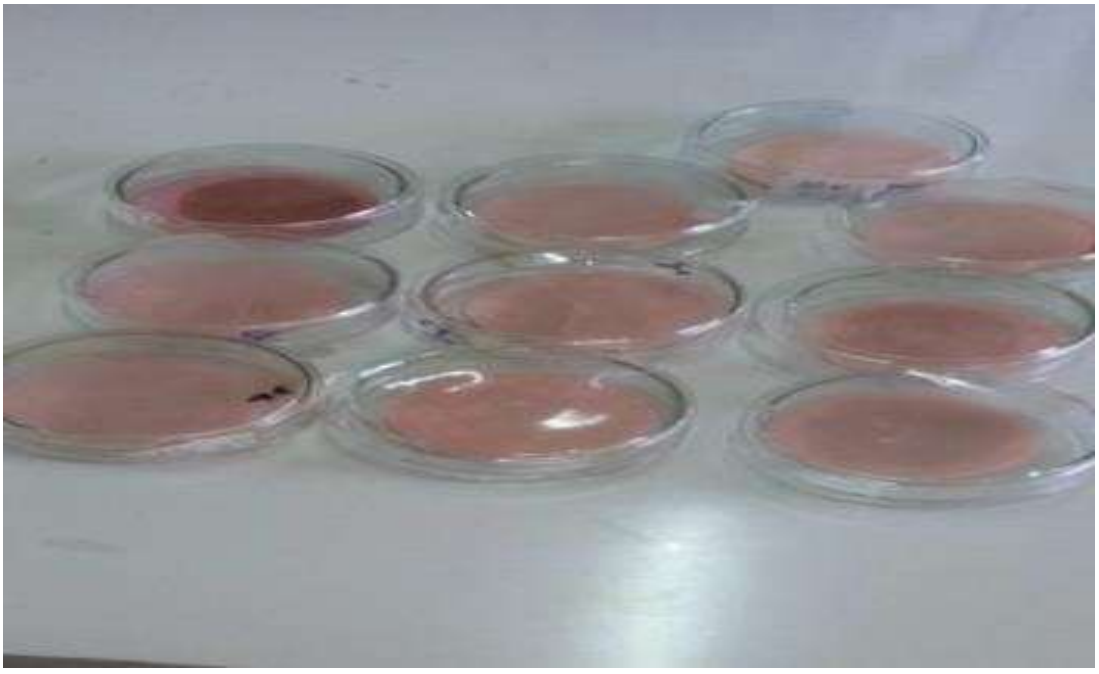
Appendix 2 Education level of the 25 female urine donors from Merkato area: (12,14] means those with 12+2 Diploma; (14,16] means those with degree (former 12+4) education.



Appendix 3 Pasteurization methods at 80 ^{O}C for 30 minutes by using water bath

Appendix 4 Result of total coliform in fresh urine (eft) and after storage for one month (right)

Appendix 5 Total coliform bacteria grown from fresh urine (Top) and absence of colonies after storage for one month (Bottom)



Declaration

I, the undersigned, declare that this thesis is my own original work and has not been submitted or presented for the fulfillment of degree in any institution. All the source of materials used for this thesis has been duly acknowledged.

Name Birtukan Getnet

Signature _____

This thesis has been submitted to the department with our approval:

Name Date Sign

1. _____

2._____