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Prevalence of bacteria resistant to antibiotics in reclaimed domestic wastewater used for irrigation in Maasai Mara University African Medicinal Botanical Garden, Narok

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Abstract

Increase in global population and urbanization has resulted in escalation in water consumption and increased generation of waste water which has posed a sanitation challenge. Point sources like wastewater treatment plants are assumed to participate in discharge and availability of bacteria which are resistant to antibiotics into the environment. This study aimed to isolate and determine antimicrobial profile of bacterial pathogens from wastewater in the Maasai Mara University wastewater treatment plant, located at the Maasai Mara University African Medicinal Botanical Garden, Narok, Kenya. Sample collection using quota sampling method and analysis were carried out using standard microbiological protocols. Bacterial isolation was done on Eosin methylene blue and Mac Conkey agar. Identification of isolates was performed using the IMVIC panel of biochemical tests. Additionally, the microbial isolates were exposed to a panel of antibiotics using the Kirby Bauer disc diffusion method to determine their resistance pattern to commonly used antibiotics. Bacterial types obtained from the wastewater used for irrigation at the Botanical Garden included Enterobacter spp, Proteus vulgaris, Proteus mirabilis and Escherichia coli. These isolates were subjected to ampicillin, tetracycline, cotrimoxazole, streptomycin, kanamycin, gentamycin, sulfamethoxazole and chloramphenicol antibiotics. Their activity ranged from susceptible, intermediate to resistant against all the antibiotics for all the isolates under study. The E. coli, P. vulgaris and P. mirabilis strains isolated exhibited multi-drug resistance. 2-way ANOVA results showed that there was no significant difference in antibiotic susceptibility to the panel of antibiotics (p=0.282) by the isolates. It was noted that the reclaimed wastewater utilized for irrigation of the Botanical Garden was contaminated with pathogenic bacteria that were resistant to commonly used antibiotics and posed an obvious threat to the health of animals, visitors, students and staff. Widespread unregulated use of antibiotics maybe prevalent among the University community, the source of the wastewater. Further treatment of the water should therefore be carried out in order to eliminate the microbes before release to the environment.

Keywords: Wastewater, irrigation, pathogenic bacteria, antibiotic resistance, health threat.

Introduction

In some cases, wastewater contains antibiotics. Extensive and indiscriminate use of antibiotics has resulted in multi drug resistant bacteria which carry multiple resistance genes[1,2]. These bacteria are introduced into sewage then can be disseminated into the environment if proper treatment is not carried out[3]. Methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) have been found to survive in treated wastewater. Additionally, utilization of treated wastewater for irrigation is a confirmed route that provides favourable conditions for coliforms to grow, transfer and obtain antibiotic resistance genes[4]. Studies have also reported the presence of *Clostridium*, *Salmonella*, *Streptococci*, viruses, protozoa and helminths in crops that had been irrigated with treated wastewater[4,5]. Also, studies on the possibility of use of treated wastewater in Albacete, Spain, in horticulture crop farming found out that there was presence of pathogenic microorganisms and indicator organisms in the soil and the crops planted in the irrigated area[6].

The U.S Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) already declared the antibiotic resistant bacteria (ABR) as an imminent hazard to human health[7,8]. A WHO published list concerning development of new antimicrobial agents, the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp) pathogens were designated to be “priority status” since their presence in the food chain is a major threat for human health[8]. Use of antibiotics in farm animals and inability to remove these compounds by wastewater treatment processes has seen massive dissemination of antibiotic residues into receiving bodies hence resulting in ultimate accumulation of antibiotic resistant genes in the animal tissues, animal products and the environment[1,2 9].

There is a global concern on emergence of antibiotic resistance in aquatic systems in Africa encompassing surface water, ground water, harvested rainwater systems and wastewater[10]. Presence of antibiotic residues in these water sources will ultimately result in familiarization to pathogens and inherent microbes hence development of antibiotic resistance. In a study conducted to measure the concentration of residues of antibiotics in untreated wastewater used in irrigation of vegetables in Ruai and Njiru small scale farms along Ngong River, Nairobi, Kenya, and it was established that the wastewater was contaminated with sulfamethoxazole and trimethoprim[11]. These were also present in the vegetables. Consumption of these vegetables could result in accumulation by microbes hence resistance to antibiotic residues present in the environment. Studies carried out in selected wastewater treatment plants of Eastern and Mt. Kenya region in Kenya identified six antibiotic residues that could pose a potential risk for antibiotic resistance[12]. Sulfamethoxazole resistant *E. coli* was isolated from groundwater sources in Kisumu County which raised concerns on the spread of resistance to antibioticsto other bacteria in the environment[13].

There is, however, paucity of published literature regarding occurrence of antibiotics in wastewater used for agriculture in Kenya. Such characterization is necessary as increase in use of wastewater in agriculture is likely to increase with increase in water scarcity. The Maasai Mara University African Medicinal Garden Wastewater Treatment plant is composed of a series of shallow ponds filled with wastewater and water hyacinth; *Eichhornia crassipes* (Figure-1). It utilizes a natural gravity- fed process to move the wastewater from one pond to the next[14]. As the wastewater moves through the series of ponds, the water hyacinth acts as a biofilter and it is expected to remove all contaminants from the wastewater including pathogenic microorganisms that could render the water unfit for use for irrigation. The effluent is used in irrigation of the Botanical Garden, orchards, trees and flower beds within the University.

Materials and Methods

Experimental research design was executed and it involved both quantitative and qualitative analytical studies. The wastewater used in the study was collected from the Maasai Mara University wastewater treatment ponds located within the African Medicinal Botanical Garden (AMBG) located in Narok County, Kenya with approximate coordinates of 1.0960°S, 35.8553°E within the Maasai Mara University[14].

Quota sampling[15] was used to determine the wastewater ponds to be studied. In the study location, the total number of posnds were 15 (Figure-1). These were segregated into three categories; raw water- initial wastewater treatment ponds (first five (5) ponds), mid treatment (next five (5) ponds) and treated wastewater - final wastewater treatment ponds (final 5 ponds). From these, 3 ponds were purposively selected from each of the segments. From each of the selected ponds, composite wastewater samples were collected into sterile sample collection bottles in triplicates then transported in a cooler box to the Biological Sciences Laboratory at Maasai Mara University for analysis within 6 hours of collection.

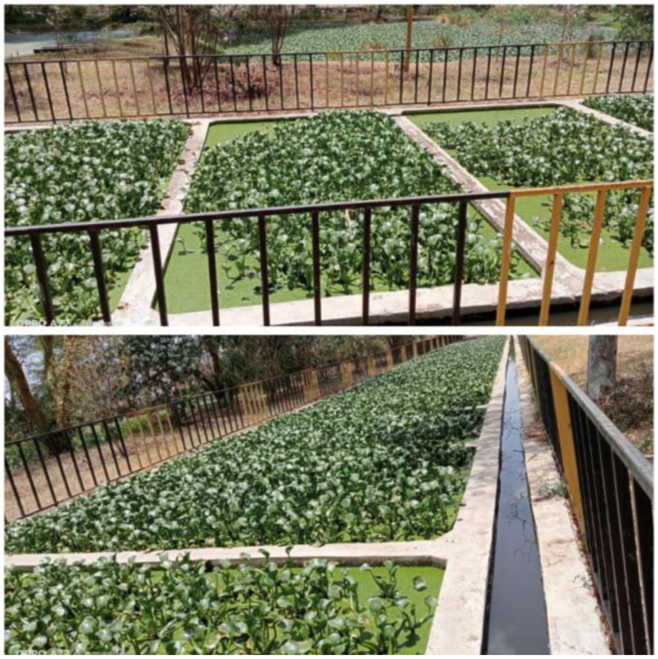

Figure-1: Wastewater treatment ponds at the Maasai Mara University Wastewater Treatment Plant.

Microbial profile and antimicrobial sensitivity of isolates was done at the Maasai Mara University Biological Sciences laboratory. Heterotrophic plate count was carried out to determine presence and absence of bacterial growth[16]. The wastewater samples were inoculated onto Eosin methylene blue agar and Mac Conkey agar to differentiate lactose fermenters from Non lactose fermenters[17]. After a 24hour incubation period, the growth was observed and colony characteristics were recorded. Biochemical tests were carried out by culture onto biotyping media; tryptone water, methyl red- vogesproskauer agar and Simmons citrate agar (IMViC). This enabled identification of the bacteria. Antibiotic sensitivity of the identified bacteria was carried out using the KGL $^{2}/_{4}$ sensitivity discs[18,19].

Analysis of the data obtained was performed using descriptive statistics; percentages and frequency tables in the Statistical Package for the Social Sciences (SPSS) Version 25. Two-way ANOVA was performed to test if there was significant difference in the resistance to antibiotics profile of the bacteria isolated[20].

Results and Discussion

Results on isolation of the bacterial pathogens from the wastewater, identification and antibiotic susceptibility profile are presented below.

Isolation of the bacterial pathogens from wastewater and identification: Indole test, methyl red test, Voges Proskauer test and citrate utilization test (IMVIC) series of biochemical tests was used to identify and differentiate coliforms/ Enterobacteriaceae[21]. After performing these tests, the enterobacteria isolated from the wastewater treatment ponds were identified as indicated in Table-1.

From the initial treatment ponds, *Enterobacter* spp was isolated. This is the section of the wastewater treatment plant where the filtration process had not yet been initiated. These findings are a reflection of earlier studies in Thailand which indicated isolation of *E. aerogenes* from a domestic wastewater collection facility where the bacterium was being explored for its capacity to degrade acrylamide[22]. Further the presence of *Enterobacter* spp bacteria in animals and humans and is isolated from water, sewage, soil and plants has been demonstrated[23]. Poor hand hygiene has been found to disseminate infection. It is responsible for nosocomial infections which result in pneumonitis, bacteremia, post-neurosurgical meningitis, neonatal meningitis, skin and soft tissue infections and urinary tract infections[24]. Therefore, its isolation from the initial wastewater treatment ponds indicated the presence and need for its removal[23]. From the middle treatment ponds, *Proteus vulgaris* and *Escherichia coli* were isolated. These were the ponds where the wastewater had been filtered through a series of ponds with the *Eichhornia crassipes.* From the final wastewater treatment ponds, *E. coli*, *P. mirabilis*, *P. vulgaris* and *Enterobacter* spp were isolated. The final wastewater treatment ponds were the last series of filtration ponds using *E. crassipes* before release into the holding pond for discharge to the irrigation system. Isolation of these pathogenic bacteria is an indication of the hydrophyte not being 100% effective in removal of pathogenic bacteria from the wastewater. Studies have established that for the water hyacinth to be efficient in purification of wastewater, they should be removed from the wastewater after they attain their optimum growth period of 18 days[25]. *Proteus* spp is widely distributed in soil, water and sewage and is an opportunistic pathogen causing urinary tract infection especially experienced in catheterized patients[26]. A microbiological assessment of wastewater treatment plants indicated that *E. coli* counts present in the final effluents poised these wastewater treatment plants as significant point sources of pollution. Pathogenic *E. coli* contamination of the environment has been found to occur from wastewater treatment plants. Presence of pathogenic bacteria in final effluents of a wastewater treatment plant is an indication of lack of effectiveness of the system and calls for employment of a more effective method that will ensure safety of the effluent for discharge into the environment[27].

Table-1: Summary of IMVIC results and identification of probable pathogenic bacteria.

Summary of the IMVIC test results and identification of probable pathogen					
Isolate	Indole	MR	VP	Citrate	Probable pathogenic bacteria
A1	Neg	Neg	Pos	Pos	*Enterobacter* spp
A2	Neg	Neg	Pos	Pos	*Enterobacter* spp
A3	Neg	Neg	Pos	Pos	*Enterobacter* spp
A4	Neg	Neg	Pos	Pos	*Enterobacter* spp
A5	Neg	Neg	Pos	Pos	*Enterobacter* spp
A6	Neg	Neg	Pos	Pos	*Enterobacter* spp
B1	Pos	Pos	Neg	Pos	*Proteus vulgaris*
B2	Pos	Pos	Neg	Neg	*Escherichia coli*
B3	Pos	Pos	Neg	Pos	*Proteus vulgaris*
B4	Pos	Pos	Neg	Neg	*Escherichia coli*
B5	Pos	Pos	Neg	Neg	*Escherichia coli*
B6	Pos	Pos	Neg	Pos	*Proteus vulgaris*
C1	Pos	Pos	Neg	Neg	*Escherichia coli*
C2	Pos	Pos	Neg	Neg	*Escherichia coli*
C3	Neg	Neg	Neg	Pos	*Proteus mirabilis*
C4	Pos	Pos	Neg	Pos	*Proteus vulgaris*
C5	Neg	Neg	Pos	Pos	*Enterobacter* spp
C6	Neg	Neg	Pos	Pos	*Enterobacter* spp

Antibiotic susceptibility profile of isolates: The pathogenic bacteria isolated from the wastewater were exposed to a panel of commonly used antibiotics as indicated in Figure-2. 75% of the isolates showed resistance to ampicillin, an indication that the it was not effective against the isolates. 25% were intermediate hence a higher dose is required for the antibiotic to prevent growth. 25% of the isolates were susceptible to tetracycline, hence the antibiotic was effective against the bacteria while 75% were resistant.

When tested against cotrimoxazole/ sulfamethoprim, 25% of the isolates were intermediate while 75% were resistant. 100% of the isolates were resistant to streptomycin. This shows that streptomycin was ineffective against all of the isolates obtained. 25% of the bacterial isolates were susceptible to kanamycin, 50% were intermediate while 25% were resistant. Gentamicin exhibited the highest susceptibility rate where 75% of the isolates were susceptible while 25% were intermediate. 25% of the isolates were susceptible to sulfamethoxazole while 75% were resistant. Finally, 50% of the isolates were intermediate against chloramphenicol while 50% were resistant. Two- way ANOVA test results revealed that the difference in the antibiotic resistance of the isolates obtained in the study was not significant (p= 0.282). Table-2 shows the zones of inhibition of the bacterial isolates (mm) to the panel of antibiotics.

The isolated pathogenic bacteria were resistant to most of the commonly used antibiotics. *E. coli*, *P. vulgaris* and *P. mirabilis* isolates were multidrug resistant because they were resistant to more than three class of antibiotics[1,2] as indicated in the summary (Table-3).

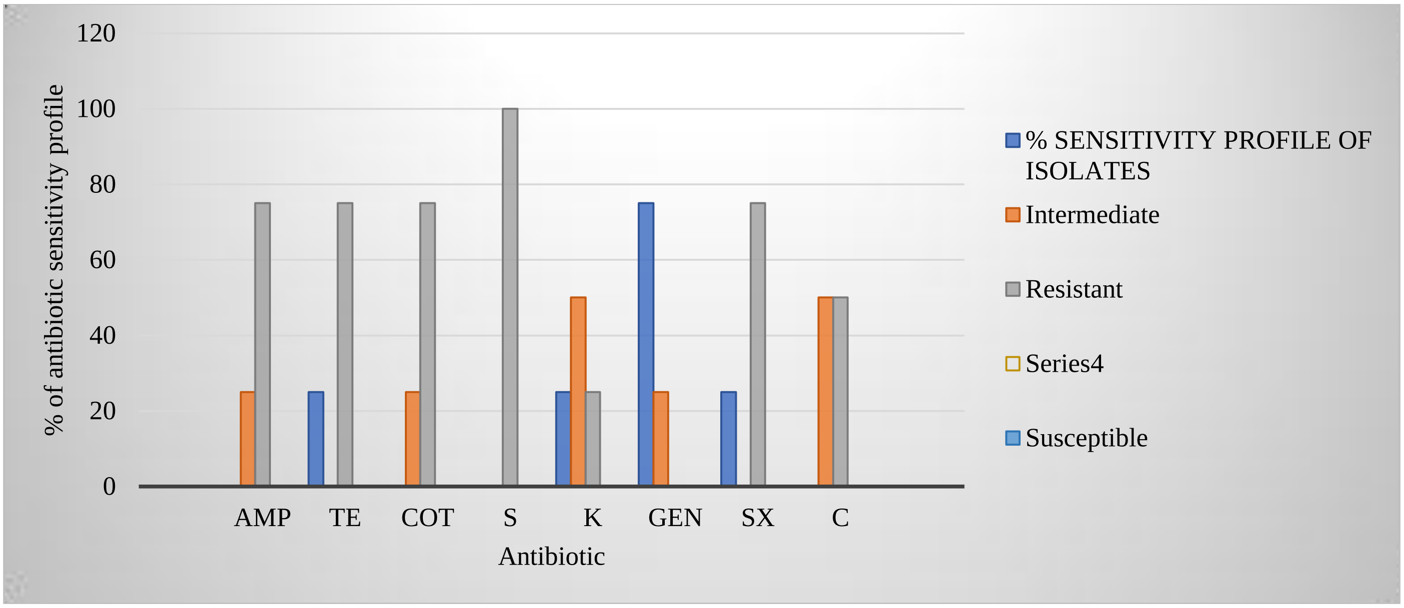

Figure-2: Antibiotic sensitivity profile of the bacterial isolates.

Table-2: Interpretation of zones of inhibition of the isolates to commonly used antibiotics.

	Antibiotic zone of inhibition (mm) and interpretation							
Bacterial isolate	AMP	TE	COT	S	K	GEN	SX	C
Enterobacter spp	8	18	0	11	14	18	0	14
	I	S	R	R	I	S	R	I
E. coli	0	10	15	9	14	18	12	12
	R	R	I	R	I	S	I	R
P. vulgaris	0	0	0	0	12	13	0	12
	R	R	R	R	R	I	R	R
P. mirabilis	0	7	0	8	18	16	5	15
	R	R	R	R	S	S	R	I

S- The bacteria cannot grow in the presence of the drug. It means the antibiotic is effective. R- The bacteria can grow even if the drug is present. It is a sign of an ineffective antibiotic. I- A higher dose of the antibiotic is needed to prevent growth.

Table-3: Bacteria isolates summary of antibiotic resistance to the classes of antibiotics.

Bacteria isolates	Aminopenicillin	Trimethoprim sulfonamides	Aminoglycosides	Chloramphemicol	Tetracyclines	Summary
Enterobacter spp		Resistance	Resistance			
Escherichia coli	Resistance		Resistance	Resistance	Resistance	Multidrug resistance
Proteus vulgaris	Resistance	Resistance	Resistance		Resistance	Multidrug resistance
Proteus mirabilis	Resistance	Resistance	Resistance	Resistance	Resistance	Multidrug resistance

Point sources like wastewater treatment plants have been assumed to have a role in discharge and spread of antibiotic resistant bacteria into the environment[28]. Multidrug resistant bacteria have been isolated from municipal sewage and soil around farms, an indication that they may have a role in the introduction of antibiotic resistance in the environment and may become potential pathogens[3]. Use of reclaimed water as an alternative source for agricultural activities has been a concern of a probable occurrence of antibiotic residues which could result in conferment of antibiotic resistance genes (ARG) to existing microbial community hence posing an environmental and public health risk since soil irrigated with wastewater was found to harbor more antibiotics and ARGs in comparison with soil that was not irrigated at all[29]. An analysis of municipal wastewater treatment plants in Sri Lanka and India found that the percentage of *E. coli* resistant to most of the antibiotics increased from influent to effluent hence a potential risk when released into the environment[30]. Antibiotic resistant *E. coli* has been documented as one of the significant coliform bacteria that reach the soil through irrigated water polluted with wastewater[31]. These findings correspond with the present study and that on study of the microbiological quality of groundwater sources in Kisumu municipality, Kenya where *E. coli* isolated from the wastewater treatment plant was found to be resistant to ampicillin, tetracycline, streptomycin and chloramphenicol[13]. Cotrimoxazole, kanamycin and sulfamethoxazole had intermediate activity hence a higher dosage of these antibiotics would be required in the event of infection with the *E. coli* isolate.

Antibiotic resistant *Enterobacter* spp has been isolated from wastewater and soil, a link that could relate with community-acquired infections and it has been found to be transferred from wastewater in the processes of sewage treatment[32]. Multi drug resistant Enterobacter has been isolated from wastewater in Brazil that had been dumped into a collection system[33]. In the present study, *Enterobacter* spp isolated from the wastewater treatment ponds and was found to be resistant to cotrimoxazole, streptomycin and sulfamethoxazole. It had intermediate action to ampicillin, kanamycin and chloramphenicol while the isolate was susceptible to tetracycline and gentamicin. This indicates that the bacterial isolate is as well headed to resistance to commonly used antibiotics. Findings from a research study on antimicrobial resistance of *P. mirabilis* showed high resistance to ampicillin, sulfamethoxazole- trimethoprim and chloramphenicol[34]. Intermediate resistance was noted against gentamicin and on clinical specimens in Erbil City obtained isolates of *P. mirabilis* and *P. vulgaris* entirely resistant to tetracycline. These findings correspond to those of the present study where 88.1% *P. vulgaris* isolates were resistant to ampicillin, tetracycline, cotrimoxazole, sulfamethoxazole, kanamycin, streptomycin and chloramphenicol whereas 11.1% of the *P. vulgaris* isolates had intermediate resistance to gentamycin. In addition, 55.5% of the *P. mirabilis* isolates were resistant to ampicillin, tetracycline, cotrimoxazole, streptomycin and sulfamethoxazole while 11.1% were resistant to chloramphenicol against 22.2% of the isolates which were susceptible to kanamycin and gentamycin. Isolation of antibiotic resistant bacteria from reclaimed wastewater is an alarming hazard for human, animal and environmental health.

Findings from the present study capture prevalence of possible widespread unregulated use of antibiotics among the University Community; which is the source of the wastewater. It also points out that the existing biofiltration system is not effective hence the release, spread and persistence of bacteria resistant to antibiotics into the environment through irrigation since the filtered effluent is used for irrigation of the Botanical Garden, orchards, flower beds and trees within the University. These could magnify the already existing problem of antibiotic resistance[7,8].

In addition to health effects on humans and animals mentioned in literature above, antibiotic resistant bacteria can lead to transfer of virulence and drug resistance genes between microbes[35]. The findings obtained in the study shed light on the presence of pathogenic bacteria in the treated wastewater[36], and therefore provide information to authorities in charge on the need to utilize more efficient procedures for treatment of the wastewater to in order to generate effluent that is safe for irrigation purposes. It is recommended that public awareness forums to the University community on antimicrobial stewardship be considered and sensitization to the Botanical Garden management on avoiding use of the wastewater for purposes that may be detrimental to human health such as irrigation of vegetables and use as wildlife and domestic drinking water.

Conclusion

While the wastewater treatment plant provides an environmentally sustainable low-tech solution to wastewater treatment in resource scarce countries, prevalence of multidrug resistant bacteria calls for caution in widespread use of the wastewater for purposes that may be detrimental to human health such as irrigation of vegetables and use as wildlife and domestic drinking water. The source of the wastewater used at the University Botanical Garden is from wastewater discharged from the University premises. Results of this study suggest that there may be widespread unregulated use of antibiotics by the University community which should be a cause of concern.

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