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Occurrence of Multiple Antibiotics Resistant *Enterobacteriaceae* in Sewage Treatment Plants of Oluyoro Catholic Hospital, Ibadan Oyo State

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Abstract

Globally, the widespread development of resistance to antibiotics in bacteria is a major challenge in drug therapy. Unregulated wastewater discharge aid in transiting antibiotics resistant bacteria in the environment, and exposure to these organisms constitute a significant threat to public health. This project studied the resistance profile of *Enterobacteriaceae* from sewers of Oluyoro Catholic Hospital, Ibadan. Wastewater samples from sewer drains were obtained from four different sampling points which included: the Maternity ward, the Theater, Laundry and Obstetrics and Paediatrics (OPD). The Biochemical Oxygen Demand (BOD) values of all sampling points were well above the permissible limit set by World Health Organization (WHO). The results of the microbiological analysis of the wastewaters showed variations in the wastewater quality. The total heterotrophic bacteria count ranged from 1.56 × 10^7 to 1.65 × 10^9 cfu/ml. The coliform count ranged from 0.51 × 10^7 to 0.79 × 10^9 cfu/ml. A total of 67 isolates from 7 genera were recovered from these samples, in the following proportion: 32.8% *Escherichia coli*, 17.9% *Proteus* sp, 16.4% *Klebsiella* sp., 14.9% *Enterobacter* sp, 7.5% *Salmonella* sp, 6% *Serratia* sp and 4.5% *Providentia* sp. These isolates were tested against 8 different classes of antibiotics which included; Chloramphenicol, Tetracycline, Imipenem, Gentamycin, Sulfamethoxazole/Trimethoprim, Streptomycin, Ceftazidime, Ciprofloxacin. The isolates displayed the following resistance pattern: Chloramphenicol-37%, Imipenem-10%, Ciprofloxacin-3%, Sulfamethoxazole- 80%, Tetracycline- 67%, Gentamycin- 12% and Ceftazidime- 22%, Streptomycin- 70%. More than 95% of the test isolates showed Multiple Antibiotics Resistance (MAR) distributed among two to six classes of antibiotics, with MAR index ranging from 0.25 - 0.75. The high prevalence of Multiple Antibiotics Resistant (MAR) *Enterobacteriaceae* obtained from these sewers presents the environment as a high risk source. Wastewaters can serve as a direct or indirect source of disease to both humans and animals by conveying virulent multiple antibiotics resistant pathogens. Measures should therefore be in place to avert the likelihood of an epidemic.

Keywords: *Enterobacteriaceae; Sewage; Antibiotics Resistance; Multiple Antibiotics Resistance*

Introduction

The high prevalence of diseases caused by organisms resistant to antibiotics in different regions of the world indicates an alarming global problem that requires rapid adequate action [1,2]. Resistance to antimicrobials has been reported with a notable frequency in species belonging to the family *Enterobacteriaceae,* among other bacterial families, in sources including animals and food [1,3,4]. *Enterobacteriaceae* family contains a large number of genera that are biochemically and genetically related to one another. This group of

organisms include several that cause primary infections of the human gastrointestinal tract. Members of this family are major causes of nosocomial and opportunistic infections [1]. The spread of *Enterobacteriaceae* that produce extended-spectrum β-lactamases (ESBLs) and other multidrug-resistant (MDR) organisms in both community and hospital based populations potentially limits the availability of suitable antimicrobials to treat such infections [5]. In the USA alone, ESBL resistance among *Enterobacteriaceae*, was reported to have caused approximately 1,700 deaths with medical costs per infection in excess of $40,000 US dollars [6].

The role of the environment as an important source and dissemination route of resistance has been increasingly recognized [7]. One of the main reservoirs of antibiotics resistant bacteria (ARB) and antibiotics resistant genes (ARG) and the source of their spread in the natural environment are wastewater treatment plants (WWTPs) [8-10] and treated wastewater discharged to surface water bodies [11-13]. WWTPs receiving high concentrations of microbial contaminants of wastewater from hospitals, agriculture, and industry stimulate the transfer of genetic information between pathogenic and environmental microorganisms. In addition, the conditions prevailing in wastewater treatment plants, such as a high content of microorganism populations, the relative abundance of nutrients, and the presence of sub-threshold levels of antibiotic substances in wastewater [11], provide an environment favourable for the survival of ARB and the transfer of ARGs. This study therefore sought to determine the occurrence and antibiotics susceptibility profile of common pathogenic bacterial species belonging to the family *Enterobacteriaceae* in wastewater originating from an hospital environment in Ibadan.

Materials and Methods

Description of study sites

Oluyoro Catholic Hospital Ibadan is a Catholic Church owned Hospital in Ibadan North-East local government area of Oyo State, Nigeria. Oluyoro Catholic Hospital Ibadan is located at Oluyoro, Oke-Ofa, Ibadan, Oyo State.

Sample collection

According to the standard methods of [14] 500 ml sewage samples were collected on four sampling days between July and August 2016 at the following sampling sites: the Maternity ward, the Theater, the Laundry; and the Obstetrics and Paediatrics (OPD). The collections were done in the early hours of the morning and transported to the laboratory for bacteriological analysis.

Physico-chemical analysis of the water samples

The physico-chemical analyses of the water samples were performed using standard analytical methods. Parameters with extremely low stability such as temperature and pH were determined on the field using thermometer and pH meter respectively. Chemical oxygen demand (COD), Total suspended solid (TSS), pH, Determination of nitrate, Chloride determination, Determination of Dissolved Oxygen (DO) and Biochemical Oxygen Demand were determined using standard methods of APHA [14]. Turbidity was determined using Secchi disc method and nitrate was determined using salicyclic acid.

Culture media

Nutrient agar, MacConkey and Eosin methylene blue agar were used for enumeration and isolation of enteric organisms. These media were prepared according to the manufacturer's specification.

Isolation and identification procedures

Serial dilution was carried out on the water samples up to a 6-fold dilution factor. Pour plate method was used for both heterotrophic bacteria count and isolation of enteric bacteria. The total heterotrophic count was done on Nutrient agar while isolation of the enteric bacteria was done on Eosin Methylene Blue Agar and MacConkey Agar. The plates were incubated for 24 - 48 hours at 37°C. Distinct colonies were subcultured on freshly prepared MacConkey agar plates and repeated streaking was done to obtain pure cultures. Furthermore, the bacterial isolates were characterized according to morphological characteristics and biochemical tests (Gram staining,

Catalase test, Oxidase test, Citrate utilization, Sulphide test, Indole and Motility test, Starch hydrolysis, Nitrate reduction test, Methyl-Red, Voges-Proskauer test, Urease test, Oxidation/Fermentation of glucose) were performed according to the method of [15] Olutiola., *et al.* 2000, and identified using the guidelines of Bergey's Manual of Determinative Bacteriology [16].

Antibiotic susceptibility tests

Antibiotic susceptibility screening was performed based on Kirby-Bauer disc diffusion method [17]. The Isolates were grown on Mueller Hinton Agar for 24 hours, three to five colonies of each isolate was suspended in 0.85% saline solution to achieve a turbidity equivalent to 0.5 McFarland standard. Using sterile cotton swab sticks, the isolates were swabbed on the entire surface of the freshly prepared Mueller Hinton Agar and then eight antibiotics impregnated discs which included: Ceftazidime, Chloramphenicol, Gentamycin, Ciprofloxacin, Imipenem, streptomycin and Sulfamethoxazole/Trimethoprim and Tetracycline were aseptically placed on the inoculated Mueller Hinton agar plates using sterile forceps. The plates were incubated for 18 hours at 37°C. Zones of inhibition were measured (mm) and classified as resistant, intermediate or sensitive in accordance with the standard set by Clinical Laboratory Standards Institute [18] guidelines.

Results

Physico-chemical analysis of Oluyoro hospital wastewater

The physico-chemical parameters of the wastewaters showed several variation with WHO standard for wastewaters. The results are presented in table 1.

Parameters	Maternity	Theater	Laundry	OPD	WHO guide value
pH	6.56	6.67	6.69	6.73	6.5 - 8.5
Temperature (°C)	26.1	26.7	26.5	26.5	< 35°C
Salinity (mg/L)	180.1	180.7	182.00	190	80-200
Turbidity (NTU)	16.89	25.78	24.75	34.73	5.0
Total Dissolved Solids (mg/L)	147	139	53	151	2000
Dissolved Oxygen(mg/L)	24	20	41.5	23	> 10
Biochemical Oxygen Demand	51.27	9.5	42.12	40.65	30
Chemical Oxygen Demand(mg/L)	65.2	68.00	79.7	85.6	600
Lead (mg/L)	0.03	0.07	0.01	0.01	< 1.0
Zinc (mg/L)	0.02	0.02	0.03	0.05	< 1.0

Table 1: *Physico-chemical analysis of Oluyoro hospital wastewater.*

Total heterotrophic bacterial count and coliform count of Oluyoro hospital wastewater

The total heterotrophic bacteria count ranged from 1.56×10^7 to 1.65×10^9 cfu/ml; while the coliform count ranged from 0.51×10^7 to 0.79×10^9 cfu/ml. The results of the heterotrophic bacterial count and the coliform count are presented in table 2 and 3 respectively.

Frequency of occurrence of Enterobacteriaceae from Oluyoro catholic hospital wastewater

A total of 67 isolates were recovered from these samples, in the following proportion: 32.8% *Escherichia coli*, 17.9% *Proteus* spp, 16.4% *Klebsiella* spp., 14.9% *Enterobacter* spp, 7.5% *Salmonella* spp, 6% *Serratia* spp and 4.5% *Providentia* spp. The results are presented in figure 1.

Sampling points	July (15/7/2016) ($\times 10^7$ cfu/ml)	July (29/7/2016) ($\times 10^7$ cfu/ml)	August (12/8/2016) ($\times 10^7$ cfu/ml)	August (28/8/2016) ($\times 10^7$ cfu/ml)	Mean coliform Count ($\times 10^7$ cfu/ml)
Maternity	1.65	1.75	1.69	1.75	1.71
Laundry	1.87	1.83	1.83	1.80	1.83
Theater	1.56	2.65	1.67	2.51	2.09
OPD	158	165	150	135	152

Table 2: *Total heterotrophic bacterial count of Oluyoro hospital wastewater.*
Legend: cfu/ml: Coliform Forming Units per ml.

Sampling points	July (15/7/2016) ($\times 10^7$ cfu/ml)	July (29/7/2016) ($\times 10^7$ cfu/ml)	August (12/8/2016) ($\times 10^7$ cfu/ml)	August (28/8/2016) ($\times 10^7$ cfu/ml)	Mean coliform Count ($\times 10^7$ cfu/ml)
Maternity	0.65	0.55	1.0	0.67	0.72
Laundry	0.51	0.59	0.73	0.54	0.59
Theater	0.8	0.93	0.71	0.59	0.76
OPD	75	79	69	12	59

Table 3: *Total coliform count of Oluyoro hosiptal wastewater.*
Legend: cfu/ml: Coliform Forming Units per ml.

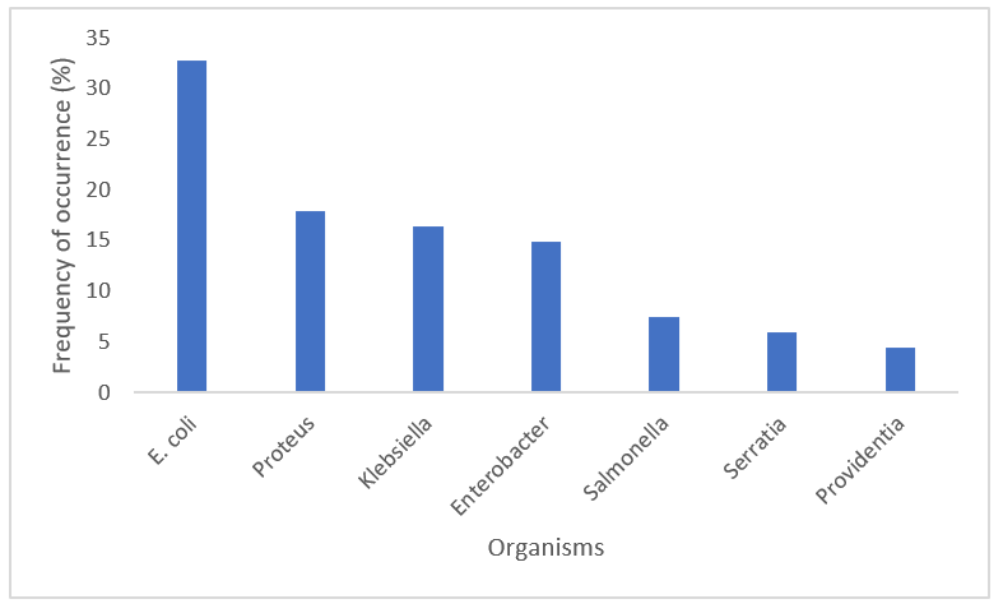

Figure 1: *Percentage occurrence of Enterobacteriaceae from Oluyoro catholic hospital wastewaters.*

Antibiotics susceptibility profile

The isolates displayed the following resistance pattern to various antibiotics employed: Ceftazidime-22%, Imipenem-10%, Ciprofloxacin-3%, Gentamycin-12%, Streptomycin-70%, Sulfamethazine/Trimethoprim-80%, Chloramphenicol-37% and Tetracycline-67%. The result is shown in figure 2.

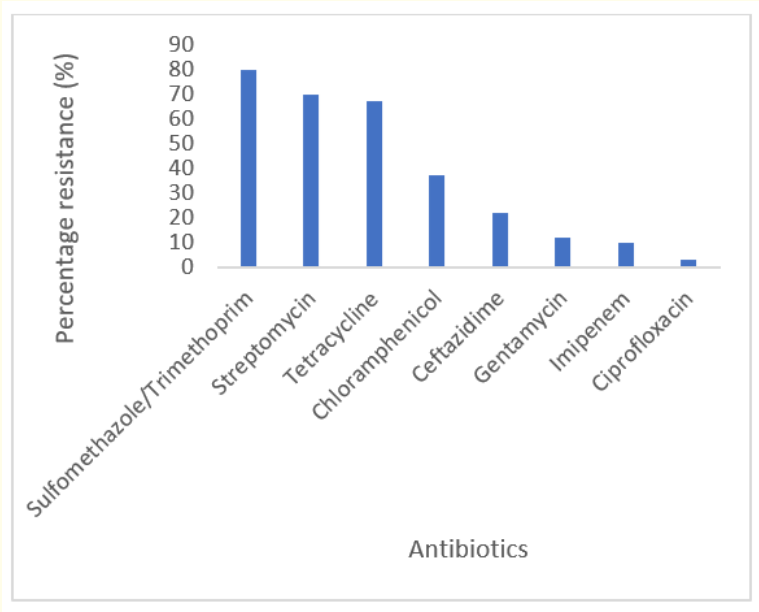

Figure 2: *Percentage resistance of the isolates to the antibiotics*

Determination of multiple antibiotics resistance index

The multiple antibiotics index (MAR) was calculated using the formula, MAR = a/b where "a" is total number of antibiotics shown resistance by an individual isolate/organism and "b" is the total number of antibiotic used in the study [19]. The MAR exhibited by the isolates ranged from 0.25 - 0.75.

Phenotypic resistance pattern	Percentage resistance	MAR Index	Identified organism (isolate code)
IPM-SXT-S	37.5	0.38	*E. coli* (L1A)
TET-IPM-C-SXT-S	50	0.50	*Klebsiella* sp. (L1B)
TET-C-SXT-S	50	0.50	*Proteus* sp. (LIC)
TET-SXT-S	37.5	0.38	*Enterobacter* sp. (L1D)
CAZ-CN-TET-SXT-S	62.5	0.63	*Enterobacter* sp. (L2A), *E. coli* (L2C, L2D, M2D, M4A, O6B)
SXT-S	25	0.25	*Enterobacter* sp. (L3B), *Klebsiella* sp. (M4B, T4B), *Serratia* sp.(M4C)
C-SXT-S	37.5	0.38	*E. coli* (L3A, O3B), *Enterobacter* sp. (M2B), *Klebsiella* sp. (O3C)

SXT	12.5	0.13	*E. coli* (L3C)
TET-S	25	0.25	*Proteus* sp. (T2A, O2D)
TET-C-SXT	37.5	0.38	*Serratia* sp. (L4A), *Proteus* sp. (M5A), *E. coli* (O2B), Klebsiella sp. (O5B)
CN-C-S	37.5	0.38	*Serratia* sp. (L4C)
CN-SXT-S	37.5	0.38	*Klebsiella* sp.(M1A), *E. coli* (M1C)
CN-SXT	25	0.25	*Klebsiella* sp. (M1B)
CN-C-SXT-S	50	0.50	*E. coli* (M1D)
CAZ-CIP-TET-C-SXT-S	75	0.75	*Enterobacter s*p. (M2C)
CAZ-CIP-SXT-S	50	0.50	*Proteus* sp. (M3B)
CAZ-TET-C-SXT-S	62.5	0.63	*Klebsiella* sp. (M3D)
CN-C-SXT	37.5	0.38	*E. coli* (T2C)
CAZ-CN-TET-SXT	50	0.5	*Enterobacter* sp (T1A)
CN-TET-C-SXT	50	0.5	*Klebsiella* sp.(T3A)
CAZ-TET-C-SXT	50	0.5	*Proteus* sp. (T3B), *Klebsiella* sp. (O5C)
CAZ-CIP-CN-TET-C-SXT	62.5	0.63	*Enterobacter* sp. (T3C)
CAZ-TET-SXT-S	50	0.5	*Proteus* sp. (T4B), *Enterobacter* sp. (O6A)
IPM-SXT	25	0.25	*Klebsiella* sp. (T4D)
CN-TET-IPM-C-SXT	62.5	0.63	*E. coli* (O1A)
TET-SXT	25	0.25	*Proteus* sp. (O2A)
CIP-CN-TET-SXT-S	62.5	0.63	*Proteus* sp. (O2C)
CIP-S	25	0.25	*Klebsiella* sp. (O3A)
TET-C	25	0.25	*E. coli* (O5A)

Table 4: *Antibiotic susceptibility profile of Enterobacteriaceae from Oluyoro hospital wastewater*
Legend: CAZ: Ceftazidime; CIP: Ciprofloxacin; C: Chloramphenicol; Imp: Imipenem; CN: Gentamycin; S: Streptomycin; SXT: Sulfamethoxazole; TET: Tetracycline.

Discussion

The isolates recovered from the different sampling points belonged to seven genera; *E. coli, Klebsiella* sp., *Proteus* sp., *Enterobacter* sp., *Salmonella* sp., *Serratia* sp. and *Providencia* sp. This agrees with the works of [20-22] who reported the presence of these genera in wastewaters. *E. coli* had the highest frequency of occurrence, this concurs with the findings of [23,24] who reported high prevalence of *E. coli* in hospital wastewaters. The results of the microbiological analyses of the wastewaters showed variations in the wastewater quality, this is in agreement with the works of [20] and [25] who reported variations in the microbiological qualities of wastewaters in Benin city and Minna. The result of the total bacteria count of the Wastewater samples showed variations and several inconsistencies in the microbial load of the different samples collected at different period of time. These variations might be due to the effect of certain environmental factors such as the availability of rainfall and anthropogenic activities. From the sampling periods, it was observed that high bacterial load was obtained during the rainy season as compared to the dry season this agrees with the work of [22]. The sewer drains, sampling point OPD recorded a very high bacterial load for every sampling. This may be as a result of high level of faecal contaminants present in the wastewater obtained from the sampling point. The lowest bacteria count was observed at the maternity ward. This might be as a result of

the presence of low organic matter concentration giving rise to low viability of the bacteria present. [26] noted that the presence of high bacterial load indicates a very high level of pollution and a reflection of the level of organic matter present.

The total bacteria count ranged between 1.56×10^7 and 1.65×10^9 cfu/ml. This is incongruous with the findings of [20] in University of Benin teaching hospital who reported total bacteria count of 3.3×10^3 - 2.5×10^{11} cfu/ml, this might be due to the difference in geographical locations of study sites, and the level of contamination present in the samples.

The physico-chemical parameters of the wastewaters showed several inconsistency with WHO standard for wastewaters. The temperature values of the wastewater samples varied significantly amongst the different sampling points, though within the WHO permissible limit for temperatures of < 35ºC [27]. The highest turbidity was observed in sampling point OPD which was recorded to be 34.73NTU which is far above the WHO permissible limit. The turbidity values obtained from these results indicates the level of pollution present in the samples. The turbidity values obtained from this observation was similar to the values of 16-35NTU reported by [20] and this may be related to apparent level of pollution going on in the hospital environment. The pH range fell within the WHO permissible limit of pH values for wastewater. This agrees with the findings of [20] who obtained a pH range between 6.5 - 7.5. Since the total dissolved solids and electrical conductivity values are indices of salinity hazard in water [28], the total dissolved solids present in the wastewaters showed slight variation amongst the sampling points but still fell within the permissible values of the World Health Organisation. The Biochemical Oxygen Demand (BOD) values of all sampling points were well above the permissible limit of 30mg/l set by WHO, indicating the rise in the pollutant level of all the samples [26]. This agrees with the findings of [20]. The Chemical Oxygen Demand (COD) values were all within the permissible limit of 600mg/l set by WHO for wastewater. The dissolved oxygen (DO) value of all sampling points were within the permissible limit of 10mg/l according WHO standard values. If wastewater remains untreated the level of dissolved oxygen are bound to reduce and might end up getting depleted, when this happens, this untreated wastewater might eventually find its way to the environment posing significant health hazards to humans and animals [29].

The results of the antibiotic susceptibility profile revealed that more than 95% of the test isolates were multidrug resistant with the range of resistance between 1 - 6 antibiotics were observed with the phenotypic resistance pattern TET-C-SXT-S occurring frequently. The data obtained from this present study showed that 22% of the isolates were resistant to Ceftazidime, 3% to Ciprofloxacin, 37% to Chloramphenicol, 10% to Imipenem, 12% to Gentamycin 70% to Streptomycin, 80% Sulfamethoxazole and 67% to Tetracycline.

The high percentage level of resistance recorded against Sulfamethoxazole, Streptomycin and Tetracycline in this study agrees with the findings of [30] and [31] who reported a high level of resistance against Streptomycin, Tetracycline and Chloramphenicol; this slightly disagrees with the findings of [32] who reported low resistance to chloramphenicol's and tetracyclines. *Enterobacteriaceae* are of utmost concern because these organisms are inherently resistant to many hydrophobic antibiotics [22]. The prevalence of multiple antibiotic resistant enterics was high, this was observed in *E. coli, Klebsiella* sp. and *Enterobacter* sp. displaying multiple antibiotic resistance to five different classes of antibiotics. This study is in coherence with the work of [23] who found high prevalence of multidrug resistant gram-negative bacteria isolated from hospital sewage; and the findings of [33] who reported *E. coli, Klebsiella* sp. and *Enterobacter* sp. showing multiple antibiotic resistance to different classes of antibiotics.

The highest MAR recorded against six different classes of antibiotics with phenotypic resistance patterns CAZ-CIP-TET-C-SXT-S and CAZ-CIP-CN-TET-C-SXT was exhibited by *Enterobacter* sp. (M2C) and *Enterobacter* sp. (T3C) with a MAR index of 0.75 and 0.63 respectively. Two major intrinsic mechanisms were reported to confer bacterial resistance to multiple antimicrobial drug classes: mutations in outer membrane porins resulting in reduced permeability to antimicrobials; and over expression of multidrug efflux pumps, which tend to pump out antibiotics before they (the antibiotics) have the opportunity of acting on their target [34,35]. In addition [34] observed that multiple antibiotic resistant bacterial strains may also arise due to unrelated mechanisms accumulating sequentially in an organism.

Since antibiotic resistant strains can transfer resistance to formerly sensitive strains, maintenance of antibiotic resistance among these organisms may serve as a possible reservoir for antibiotic resistance encoding genes [36]. The rise in frequency of drug resistant isolates supports the view that wide spread use of antibiotics results in the selection of resistant strains carrying plasmid encoding resistance [37]. These resistant strains may spread into different ecological niches, including normal intestinal flora leading to a further increase in the number of multiple antibiotic resistant bacteria.

Enterobacter and *Klebsiella* resistance to Carbapenems (Imipenem) might be as a result of their ability to produce an enzyme called carbapenemase which inhibits the activity of Carbapenems coupled with their environment giving rise to transfer of resistant traits to other micro-organisms. Several studies have shown the transfer of resistance plasmid between coliform bacteria in stagnant areas of sewage systems [38] multi drug resistant organisms are naturally fitter than the non-resistant micro-organisms and they can survive in a very harsh environment. Low resistance to ciprofloxacin a Fluoroquinolone and Imipenem a Carbapenem can be attributed to their mode of action by inhibiting DNA replication, while Carbapenem act by inhibiting peptidoglycan synthesis. While resistance to them might be as a result of the test isolates producing carbapenemase and production of extended spectrum beta lactamases as well as acquiring resistant plasmids from the environment which result in Fluoroquinolone and carbapenem resistance [39].

Conclusion

The high prevalence of Multiple Antibiotics Resistant (MAR) *Enterobacteriaceae* obtained from these sewers presents the environment as a high risk source. Waste-waters can serve as a direct or indirect source of disease to both humans and animals by conveying virulent multiple antibiotics resistant pathogens. Measures should therefore be in place to avert the likelihood of an epidemic.

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