

Antibiotics Sensitivity Patterns and Plasmid Profiles Analysis of Some Selected Bacteria Isolated from Septic Tank Sewage

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Abstract The antibiotics sensitivity patterns and plasmid profiles of some selected bacteria isolated from septic tank sewage in the region under study was evaluated. Three replicate samples from Locations A, B and C representing Agbor, Benin and Sapele regions of Delta and Edo States, Nigeria respectively was collected from both the influent tank (raw sewage) and the effluent chamber (semi treated sewage) between November 2018 and January 2019 for testing. A composite sample was formed from the three samples collected. Antibiotic resistance testing was done to determine the resistance profile of the isolates using Mueller-Hinton agar plates which were prepared and appropriately labelled. These plates were inoculated with standardized microbial broth cultures by spread plate technique and left to dry for 30 minutes. Commercially available antibiotics discs containing varying concentrations of various types of commonly used antibiotics were placed at adequate distances on each of the seeded agar plates and incubated for 12h. Plasmid DNA from all tested strains was obtained by using alkaline lysis method. Cells were grown overnight at 37°C in a nutrient broth in a 1.5 ml micro tube. The growth was harvested by centrifuging at 3,000 rpm for 10 minutes. The plasmid DNA was visualized by placing the gel in an ultraviolet transilluminator in a photo documentation system. The overall prevalence of antibiotic resistance was highest in Amoxicillin (90.5%) and least in Augmentin (40.5%). There was no significant difference ($p>0.01$) between antibiotic resistance in raw sewage and in the semi-treated sewage. *Escherichia coli* had the highest MAR index (0.9) while *Proteus* sp. had the lowest MAR index (0.4). Plasmid analysis of 9 bacterial isolates showed that 4 (44.4%) possessed plasmids each of molecular weight 23.1kb while 5 (56.6%) had no plasmid DNA.

Keywords: antibiotics sensitivity, plasmid profiles, bacteria, sewage, septic tank

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1. Introduction

Antibiotics are a group of naturally-occurring, semi-synthetic and/or chemically synthesized compounds with antimicrobial activity. They find wide application in human and veterinary medicine in the treatment and prevention of a number of diseases and as growth promoters in animal intensive industries [1]. The resistance to a broad range of antibiotics by microorganisms is a major concern in modern medicine. Antibiotic resistance can be defined as the survival of microorganisms despite exposure to antibiotics designed to kill them or to impede their growth. Antimicrobials themselves act as a selective pressure that allow the growth of resistant bacteria within a population and inhibits susceptible bacteria [2]. Clinical infections, diseases and death caused by resistant bacteria are increasing by the day [3]. Antibiotic resistance can be

established and propagated in human and animal digestive systems [4]. The spread of resistant strain within the gastro intestinal tract (GIT) is enabled by the co-occurrence of high levels of bacteria and sub-lethal doses of antibiotics. However, there are some uncertainties and speculation about environments such as sewage treatment plants (STPs) [5] and aquatic environments in general [6], which provide enabling conditions for the establishment and propagation of antibiotics resistant bacteria. The genetic structure of such microorganisms may be altered by the direct or indirect effect of waste water components leading to bacteria with high antibiotic resistance [7].

Effluent emanating from pharmaceutical manufacturing plants and waste water treatment facilities receiving wastewater from sewage, hospitals and veterinary clinic are some of the notable sources through which antibiotics are released into the environment [8]. Exposure to improperly treated waste water has several health consequences such exposure to disease, spread of antibiotics resistant bacteria and threat to aquatic lives

[9,10]. Several classes of antibiotics have been discovered in sewage and sewage treatment plants (STPs). These include β -lactams, trimethoprim, sulphonamides, fluoroquinolones, macrolides, and tetracycline [8,11,12]. Many of these antibiotics find their way into sewage unchanged as they are not completely metabolized during therapeutic use. One of the mobile genetic elements through which antibiotics resistance spreads in bacteria are plasmids-extrachromosomal DNA capable of replication independently of the chromosome [13].

Various researchers have reported bacteriophage isolated from sewage carrying various lactamase genes, an easy route for disseminating antibiotic resistance among STP bacterial communities [14]. Activated sludge tanks, trickling filters and others STPs are examples of biological reactors that concentrate bacteria in conditions designed to encourage their proliferation and activity. They reduce viable concentration of faecal bacteria such as coliforms and enterococci depending on the plant efficiency [15]. However, relatively high number of bacteria still remains in STP effluents, up to 10^3 cfu/ml⁻¹ *Enterococci* have been reported [16]. Some of these bacteria carry antibiotics resistance genes especially in the influent. About 40% of the cultivable *E. coli* strains isolated from sewage were resistant to one or more antibiotics and 9.8% were resistant to more than three antibiotics [17]. Various multi resistant plasmids harbouring transposons each carrying up to 10 different antibiotic resistance genes have been isolated from STPs [18].

Besides health fears, there is an increasing concern about the potential ecological impacts from both the presence of antibiotics and resistant bacteria in the environment. In this work, drug resistance pattern and plasmid profiles of some selected bacterial isolates from septic tank sewage were studied. The choice of antibiotics used in this study was informed by the fact that they are employed in the treatment of many ailments within the study area.

2. Materials and Methods

2.1. Sample Collection

Sewage effluent samples were collected from different septic tanks within three locations in Edo/ Delta States (Agbor, Benin and Sapele) in South - South region of Nigeria in November/December 2018 and January 2019 respectively; for microbiological analyses using 1 litre plastic bottles and 200 ml dark glass bottles. Samples were collected in triplicates according to the standard methods for examination of water and waste water [19] and stored in ice packs ($4 \pm 2^\circ\text{C}$) and transferred to the laboratory for analyses.

All three sample locations comprising the study area fall within the same geo-politico-cultural and climatic region with similar annual mean temperature and rainfall patterns. Despite these however, several inherent variations exist from one sample location to another. These locations were chosen for the possible impact of the slight variability in soil type - Sapele having whitish coastal soil texture being closest to the Delta; while Agbor and Benin have the red-yellow ferral soils; and socio-cultural effect on

the biomass composition and microbial community in the septic tank system. These septic tanks from the various locations (A, B and C) were chosen for their similarity in size and content. The only difference between the tanks was the number of persons served by the single tank and the life style of the users.

The microbial characterisation of each of these sample locations of both the influent and the effluent chamber were separately studied (not reported here), but for the purpose of studying the antibiotics sensitivity pattern of bacteria in the septic tank community, two composite samples of raw and semi treated sewage effluents from these three locations were made. That is, one composite raw sewage sample and one composite semi treated sewage sample respectively. Each of these composite samples was then divided into two equal portions of raw, and semi treated samples subsequently. One portion (half) of the composite raw sample, and another aliquot (half) of the semi treated sewage were each separately analysed for the composition of the microbial community as well as evaluate the antibiotic sensitivity pattern of the two sewage chambers. The remaining (half) portions of the raw and semi treated sewage composite samples were mixed together for the purpose of the antimicrobial sensitivity analysis of the septic tank sewage in the study area.

2.2. Antibiotics Sensitivity Testing

The respective purified bacterial isolates were transferred to sterile peptone water under aseptic conditions and incubated for about 10 h. The turbidities of the broth cultures were adjusted to match an opacity standard (Barium sulphate + Tetraoxosulphate (IV) acid solution). The resulting broth culture has a microbial cell density of about 10^6 cfu/ml. Mueller-Hinton agar plates were prepared and appropriately labelled. These plates were inoculated with the standardized microbial broth cultures by spread plate technique [20]. The inoculated plates were left to dry for 30 minutes. Commercially available antibiotics discs containing varying concentrations of various types of antibiotics (Augmentin 30 μg , Gentamycin 10 μg , Pefloxacin 30 μg , Tarivid 10 μg , Streptomycin 30 μg , Septrin 30 μg , Chloramphenicol 30 μg , Sparfloxacin 10 μg , Ciprofloxacin 10 μg and Amoxicillin 30 μg) were placed at adequate distances on each of the seeded agar plates with the aid of sterile forceps. The plates were incubated for 12 h. The resultant visible zones of inhibition were measured using a ruler; distances lesser than 14 mm were regarded as resistant (R), while distances which ranged from 14 mm to 17 mm were indicated as intermediate (I). Also, zones of inhibition greater than 17 mm were recorded as susceptible/sensitive (S) for the respective isolates [21].

The multiple antibiotic resistance (MAR) index of isolates was defined as a/b when 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed [22].

2.3. Plasmid Profile Analysis

Plasmid profile analysis (PP) is a method used to determine the number and size of plasmids in bacteria

isolates. Plasmid DNA from all tested strains was obtained by using alkaline lysis method [23]. Cells were grown overnight at 37°C in a nutrient broth in a 1.5 ml micro tube. The growth was harvested by centrifuging at 3,000 rpm for 10 minutes. For the electrophoresis, 0.8 % agarose was prepared by weighing 0.8 g of agarose powder with a weighing balance. The powder was mixed with 100 ml of electrophoresis buffer and then heated in a microwave oven until completely melted. Ethidium bromide was added to the gel at a final concentration 0.5 µg/ml to facilitate visualization of DNA after electrophoresis. The DNA was visualized by placing the gel in an ultraviolet transilluminator in a photo documentation system (Clinix Japan, Model 1570).

In the present study, the antibiotic susceptibility testing of bacterial isolates show that *Escherichia coli* of all the isolates tested had the highest multiple antibiotic resistances (MAR) index (resistance count) on the 10 antibiotics tested (0.9). *Proteus* sp. had the lowest MAR index (0.4) while the rest isolates had MAR indexes ranging from 0.5-0.7. *Bacillus subtilis* showed no resistance to the antibiotics used in the study (Figure 1a and b & Figure 2 and 3). Amoxicillin resistance was the highest (90.5 %) among isolates while Augmentin had the lowest resistance among isolates (40.5 %) (Table 1). There was no significant difference ($p > 0.01$) between antibiotics sensitivity in raw sewage and semi treated sewage in all antibiotics tested.

3. Results

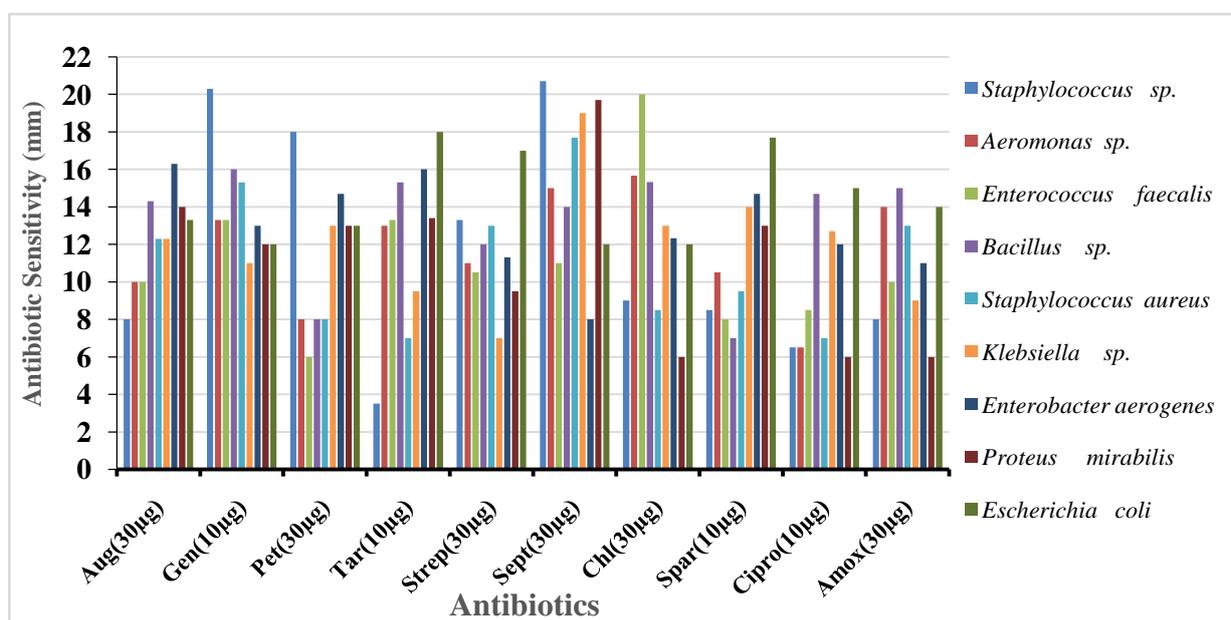


Figure 1a. Bacterial antibiotic susceptibility pattern in septic tank raw sewage sample

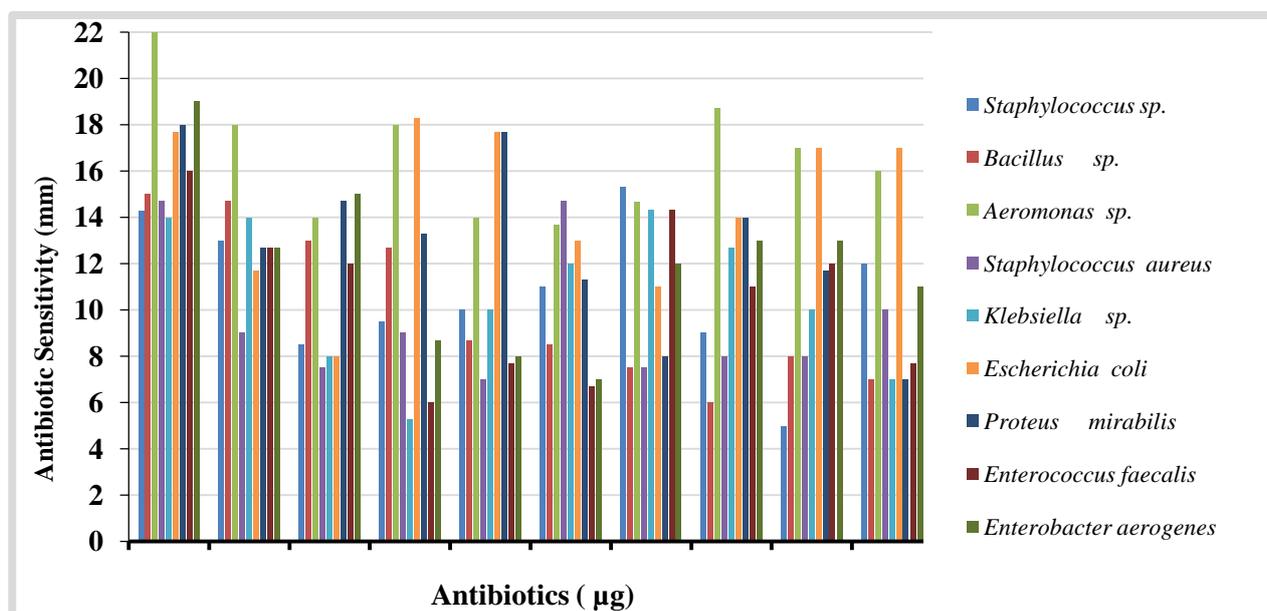


Figure 1b. Bacterial antibiotic susceptibility pattern in septic tank semi-treated sewage sample

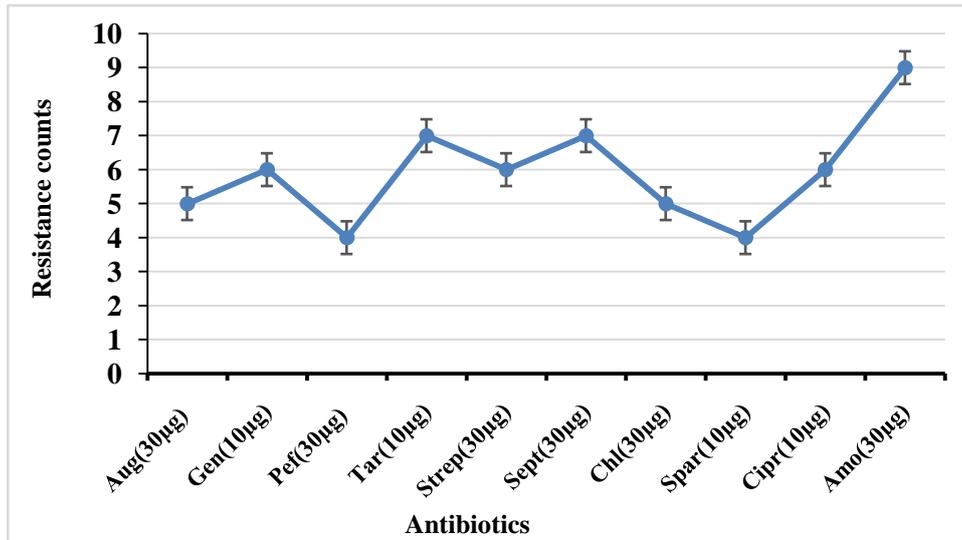


Figure 2. Resistance pattern of the various antibiotics used in the study

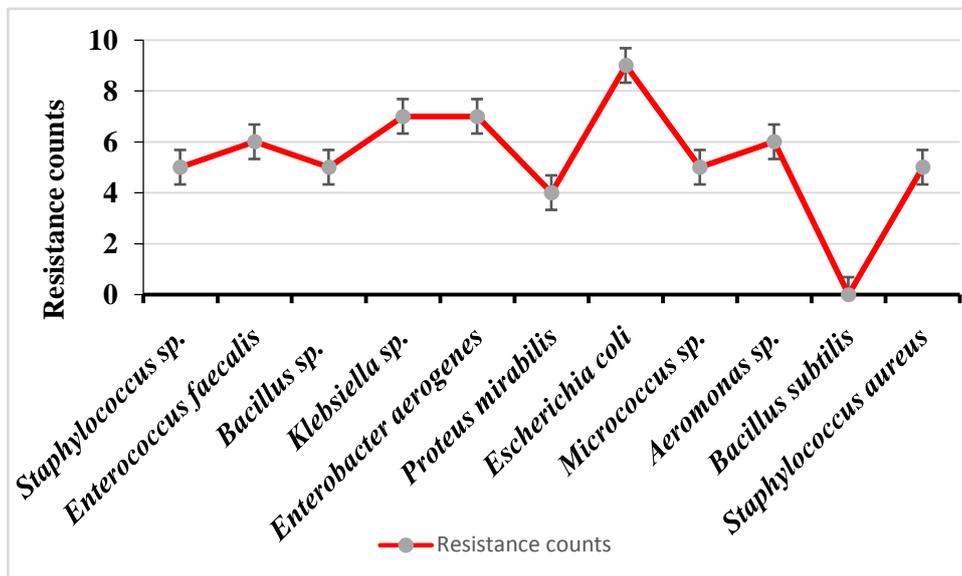


Figure 3. Antibiotics resistance pattern among bacterial isolates

Table 1. Antibiotics Sensitivity Testing of Bacterial Isolates from Septic tank

| Bacterial Isolates | Antibiotics | | | | | | | | | |
|--------------------|--------------|--------------|--------------|--------------|----------------|---------------|----------------|---------------|----------------|--------------|
| | Aug. (30 µg) | Gen. (10 µg) | Pef. (30 µg) | Tar. (10 µg) | Strep. (30 µg) | Sept. (30 µg) | Chlor. (30 µg) | Spar. (10 µg) | Cipro. (10 µg) | Amo. (30 µg) |
| Staphylococcus sp. | R | R | S | S | R | I | R | S | S | R |
| Enterococcus sp. | R | R | R | R | S | R | I | S | S | R |
| Bacillus sp. | S | R | I | R | R | R | S | S | S | R |
| Klebsiella sp. | S | R | R | R | R | R | S | R | R | R |
| Enterobacter sp. | S | I | I | R | R | R | R | R | R | R |
| Proteus sp. | R | S | S | R | I | I | S | R | R | S |
| Escherichia coli | R | R | R | R | I | R | R | R | R | R |
| Micrococcus sp. | S | I | I | S | R | R | R | S | R | R |
| Aeromonas sp. | S | I | R | R | R | R | S | S | R | R |

S = Sensitive (>17mm), R =Resistant (<14mm)
I=Intermediate (14-17mm)

The result of the plasmid DNA analysis of the bacterial isolates analysed shows positive band for 4 of the isolates with plasmids DNA of molecular weights 23,130 bp (Lane 3, 4, 6, and 9) representing 44.4 % of the isolates analysed (Figure 4).

Tentative isolates *Escherichia coli*, *Bacillus sp.*, *Enterobacter aerogenes* and *Klebsiella sp.* were reported as the plasmid carrying isolates. The other 5 isolates (56.5 %) showed no positive band for plasmid DNA.

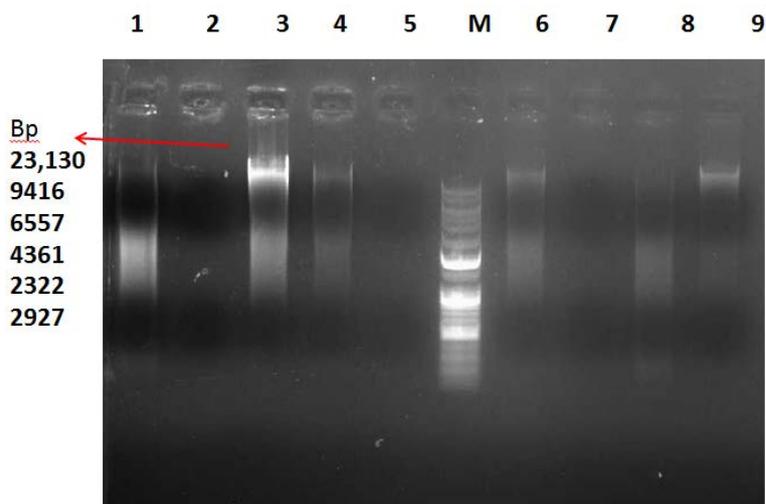


Figure 4. Gel Electrophoresis of Plasmid DNA of the Bacteria Isolates from Septic Tank sewage

4. Discussion

4.1. Antibiotic Susceptibility of the Bacterial Isolates from the Septic Tank Sewage

This study reveals that of the 9 isolates tested, *Escherichia coli* had the highest multiple antibiotics resistance (MAR) index (0.9) from the 10 antibiotic types the isolates were exposed to. *Proteus mirabilis* had the lowest MAR index (0.4) while the rest isolates had MAR indices ranging from 0.5 - 0.7. Amoxicillin resistance was highest (90.5 %) among the isolates while augmentin and pefloxacin had the lowest antibiotic resistance (40.5 and 41.0 %) respectively. There was no significant difference at $p > 0.01$ between antibiotic susceptibility in raw and semi-treated sewage in all the antibiotics tested. This result is similar to those obtained by [24] who reported 100 % resistance of their *E. coli* isolates to Amoxicillin and likewise by [25] who reported 81 % resistance of their *E. coli* isolates to tetracycline. Again, antibiotics sensitivity profiles of *E. coli*, *Vibrio* sp. and *Salmonella* sp. isolated from water sources in Oproama community in Niger Delta showed high sensitivity to ofloxacin, nalidixic acid and nitrofurantoin and high resistance to amoxicillin, augmentin, cotrimazole and tetracycline [26]. This again is consistent with results of this present study. The frequency of resistance, co-resistance and resistance genes are high and similar in coliforms from humans and their environment [27,28].

The resistance pattern of the bacterial isolates in this study also aligns with the work of [29] on β -lactamase producing Enterobacteriaceae; where they observed that the antibiotics most frequently involved in resistance were gentamycin, cotrimoxazole, tetracycline, tobramycin and chloramphenicol. Therefore, its multiple resistance to commonly used antibiotics constitutes a huge threat to man. The resistance of *E. coli* to most commonly used antibiotics such as tetracycline, streptomycin and ampicillin has been documented to be due to the acquisition of resistance plasmids [30]. [17] noted that 40% of the cultivable *E. coli* strains isolated from sewage were resistant to one or more antibiotics and 9.8% were resistant to at least three antibiotics or more. Generally, since the late 1990s several groups

of antibiotics have been reported in sewage and sewage treatment plants (STPs) including B-lactams, macrolides, sulphonamides, trimethoprim, aminoglycosides, fluoroquinolones, and tetracycline [8,11,12]. Most bacterial isolates except *Salmonella* and *Shigella* species are found to be resistant to commonly used antibiotics such as ampicillin, amoxicillin, ofloxacin and gentamicin [31]. Several of these antibiotics are not completely metabolized during therapeutic use and are therefore excreted into sewage unchanged. The effectiveness of antibiotics treatment of diseases will decline due to the development of antibiotic resistance in the environment [31].

It is evident from the result of this study that antibiotics resistance can thrive in waste water treatment facilities; therefore providing a route to drug resistant pathogens to enter the environment. Also, some workers have reported correlations between high concentrations of antibiotics in sewage and/or the environment and elevated levels of resistance in antibiotics by bacteria [6]. Although variable, antibiotic concentrations in STPs influents have been found to be between 0.1 - 1.0 mg/l, while secondary effluent levels were reported an order of magnitude lower. Similarly, resistance of bacteria species from hospital wastewaters to several antimicrobial agents have been reported [10]. However, they are not unanimous as to whether the relative rates of antibiotics resistance are on the increase during STP processes. Some researchers have also revealed minimal difference in bacterial resistance levels between raw and treated sewage [15] which is again consistent with findings of this study.

4.2. Plasmid Profiles of the Bacterial Isolates from the Septic Tank Sewage

Plasmid profiling of the bacterial isolates from the study shows that of the 9 isolates analysed, 4 revealed positive bands for plasmid DNA of molecular weights 23,130 bp (Lane 3, 4, 6 and 9) representing 44.4 % of the total isolates. Tentative isolates *Escherichia coli* (Lane 3), *Bacillus* sp. (Lane 4), *Enterobacter* sp. (Lane 6) and *Klebsiella* sp. (Lane 9) respectively were reported as the plasmid carrying isolates. The other 5 isolates (*Staphylococcus* sp. (Lane 1), *Enterococcus faecalis* (Lane 2),

Aeromonas sp. (Lane 5), *Micrococcus* sp. (Lane 7) and *Proteus mirabilis* (Lane 8) representing 55.5 % of the total isolates analysed showed no positive bands for plasmid DNA. The presence of plasmids however may not automatically confer antibiotic resistance in this study as such plasmids were not clearly delineated as resistance plasmids. This notwithstanding, drug resistant bacteria in the environment presents serious implications for public health.

Consequences include the possibility of spread of resistance among supposed sensitive organisms in the surrounding habitats through transfer of the resistance gene which leads to a change in the microbial composition of the environment [32]. The resistance of *E. coli* to some commonly used antibiotics such as ampicillin, tetracycline, ciprofloxacin and streptomycin has been linked to the acquisition of resistance plasmids [30]. Such antibiotic resistance has been discovered to be very common among growing children in Nigeria [33]. This observation is of great concern since exposure to antibiotics is common in adults and older children; hence the urgency in characterising these plasmids.

5. Conclusion

The prevalence of antibiotics resistance in septic tank sewage and the likelihood of this resistance being associated with plasmids have been reported. Bacterial species from the genera *Staphylococcus*, *Enterococcus*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Proteus*, *Escherichia*, *Micrococcus* and *Aeromonas* were isolated from the septic tank wastewater. Of the antibiotics tested against these isolates, *Escherichia coli* had the highest multiple antibiotic resistances (MAR) index while *Proteus* sp. had the lowest MAR index. The rest isolates had intermediate MAR indexes. Going by these findings therefore, we can conclude that both the raw and semi treated sewage septic tank system consist of a significant proportion of possibly resistant bacteria, with *E. coli* being the most resistant of them all. Though plasmids have been linked with the presence of antibiotics resistance in some bacteria, the presence of plasmids however may not automatically confer antibiotic resistance in this study as such plasmids were not clearly delineated as resistance plasmids.

Conflict of Interest

The authors have no conflicting interest.

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