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Plasmid Carriage and Antibiotics Susceptibility of Cultivable Bacteria Isolated from Hospital Wastewater

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Authors' contributions

This work was carried out in collaboration between all authors. Author MPU designed the study. Authors MPU and SCUN wrote the protocol. Author MPU analyzed the study and wrote the first draft of the manuscript. Authors MPU and SIU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Careless discharge of wastewater is another source of which the environmental health is continuously being threatened. Hospital wastewater contains clinically relevant strains. Study was undertaken on the prevalence of antibiotics resistant bacteria (ARB) in hospital wastewater and to determine the presence of plasmid and its association with antibiotic resistance expression among the bacteria using standard microbiological techniques and alkaline lysis method, respectively. Antibiotic susceptibility was determined by use of the disk diffusion method. Total heterotrophic count of 1.36 x 10^7 and 2.0 x 10^6 CFU/mL were obtained, *Escherichia coli* count gave 2.1 x 10^5 and 1.6 x 10^6 CFU/mL and Staphylococcal count gave 1.4 x 10^6 and 1.2 x 10^5 CFU/mL. Species of *Staphylococcus, Pseudomonas, Klebsiella, Bacillus, Escherichia, Acinetobacter* and *Salmonella,* were identified. Based on a differential staining process, the bacteria were tested against some commonly used antibiotics. A high profile of multi-antibiotics resistance was displayed among the bacteria. The percentage of resistance to the antibiotics were COT - 15%, AUG - 15%, GEN - 11%,

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TET - 10%, OFL - 3%, AMX - 10%, NIT - 5%, NAL - 6%, CXC - 10%, CHL - 6%, ERY - 3% and STR - 6%. Approximately 58% of the bacteria carried plasmids. Plasmid band size of 23.1 kbp was common among the bacteria. Resistance was observed in bacteria with plasmids as well as in those without plasmids. On curing, the organisms showed reduced resistance to the antibiotics while *Pseudomonas aeruginosa* and *Bacillus* sp. maintained their resistant pattern. Hospital wastewater contains loads of antibiotic resistant bacteria whose resistance in part are carried in plasmid. It contributes to environmental contamination with clinically important strains of microorganisms. The practice of releasing hospital wastewater into the environments should be strongly monitored and prohibited as the environment has a lot to do in our functioning and metabolic efficiency and therefore should be kept clean.

Keywords: Hospital wastewater; antibiotics susceptibility; microbial resistance; plasmid carriage.

1. INTRODUCTION

Wastewater is any water whose quality has degraded. In homes, industries and several other establishments, such water is discharged to a sewage system and contains countless number of microorganisms from diverse sources including sanitary wastes, dishes washings, laundering, processing/manufacturing, etc. Exposure to improperly treated wastewater has several health consequences such as exposure to diseases, spread of antibiotics resistant bacteria, threat to aquatic lives, etc. Wastewaters vary widely in composition, volume and flow depending on the source. In the hospitals, water has several uses and is discharged to one point. Studies have reported the presence of disinfectants, antibiotics, anesthetics, radioactive elements, static cytotoxic agents, other chemicals, hazardous materials, heavy metals, Adsorbable Organic Halogens (AOX), iodised X-ray contrast media and solvents in hospital wastewater [1-3]. These compounds are used for treatment, medical diagnostics, disinfection, and research [2] purposes. These and non-metabolized drugs excreted from patients ruin the physical, chemical, and biological quality of hospital wastewater. Hospital wastewater is normally discharged directly, without pre-treatment to sewers [4]. The microflora of hospital wastewater according to [2] comprises of saprophytic bacteria from the atmosphere, soil, medical devices, and water employed in the hospital practice. The pathogenic agents comprised of resident and community introduced strains [5] mainly from patients' excreta. The genetic structure of such microorganisms may be altered by the direct or indirect effect of wastewater components leading to bacteria with high antibiotic resistance [6].

Alexander Fleming warned against the misuse of antibiotics which could result in selection for resistant bacteria [7]. Development of antibiotic resistance consequentially threatens the effective prevention and treatments of infectious diseases resulting in prolong illness, increased health care costs and sometimes deaths. Rivers defined antibiotic resistance as the survival of microorganisms despite exposure to antibiotics designed to kill them or to impede their growth [8]. Resistance to antimicrobial agents in microorganisms is on the increase worldwide and is greatly responsible for substantial cases of therapeutic failures [9]. Wide application of antimicrobial agents in clinical settings for treatment of infectious disease can lead to the development and evolution of antibiotic resistant bacteria [9,10,3,11]. Thus, hospital wastewater contains larger numbers of resistant organisms [2] and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria. Hospital wastewater could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria [12]. The emergence and spread of antimicrobial resistance are complex problems driven by numerous interconnected factors. Antibiotic resistance is not only found in pathogenic bacteria but also in environmental organisms inhabiting terrestrial and aquatic habitats. However, higher numbers of resistant bacteria occur in polluted habitats [12].

This study was designed to (1) assess the antimicrobial resistance profile of bacteria from hospital wastewater in Lagos State, Nigeria against commonly used antimicrobial agents and (2) determine the presence of plasmid and its role in antibiotics resistance among the bacteria.

2. MATERIALS AND METHODS

2.1 Collection of Sample

Wastewater was collected from the University of Lagos Medical Centre into a sterile sample bottle

using a sterile syringe. The microbial and plasmid analyses were carried out, respectively in the Microbiology and Biochemistry laboratory of the Nigerian Institute of Medical Research, Yaba, Lagos State.

2.2 Microbial Analysis

2.2.1 Isolation

The sample was agitated for even distribution of the bacterial cells. The bacterial load of the wastewater was determined by performing a ten-fold serial dilution of the sample in test tubes containing sterile distilled water as described by Torimiro et al. [13] using the spread plate method. One milliter aliquot of 10^{-3} and 10^{-4} were aseptically transferred onto nutrient agar, manitol salt and eosin methylene blue medium plates in duplicates for the growth of heterotrophic bacteria, *Staphyloccocus* and *Escherichia coli*, respectively. The media were products of Oxoid, Unipath, United Kingdom. The inocula were spread using a sterile glass spreader. The plates were incubated aerobically at 37°C for 48 hrs. A separate nutrient agar was inoculated with the sample and was incubated anaerobically under 5-10% carbon dioxide atmosphere. After incubation, plates with colonies of 30-300 in number were selected, counted and recorded. The average of each dilution was calculated and the population obtained by multiplying the average counts by the dilution factor.

2.2.2 Purification

Discrete colonies from each plate were picked using a flamed-sterile wire loop and purified by sub-utilizing on nutrient agar plates. They were incubated aerobically and anaerobically for 24 hrs at 37°C.

2.2.3 Identification

Pure colonies were examined for the cultural/colonial morphology e.g. shapes pigmentation, etc. Gram staining reaction and different biochemical tests such as catalase, oxidase, citrate, motility, indole, urease, starch hydrolysis, sugar fermentation, Voges-Proskauer, coagulase, etc. were carried out on the colonies as described by Cowan and Steel [14].

2.3 Antibiotics Susceptibility Test

Antibiotics susceptibility of the isolates were determined against twelve commonly used antimicrobial agents using disk diffusion method described by Kirby-Bauer [15] following the CLSI assessment criteria of 2011. Bacterial inocula of 24 hr. old cultures of each isolate on Mueller Hinton (Oxoid, Unipath, United Kingdom) broth were prepared by adjusting the concentration to match that of 0.5 Macfarland standards, an equivalent of 1.0 x 10^8 CFU/ml bacterial density according to Cheesbrough [16]. Each bacterial suspension was evenly spread on Mueller Hinton agar plates (MHA). An antibiotics multidisc (Lab M, England) containing Chloramphenicol (10 µg), Ofloxacin (5 µg), Gentamicin (10 µg), Nitrofurantoin (30 µg), Co-trimoxazole (25 µg), Tetracycline (10 µg), Streptomycin (10 µg), Amoxicillin (10 µg), Nalidixic acid (30 µg), Augmentin (30 µg), Erythromycin (5 µg), Cloxacillin (5 µg) was placed on each of the inoculated MHA plates. Incubation was at 37°C for 24 hours. After incubation, the diameter of the Zones of inhibition was measured in millimeters and interpreted based on the CLSI [17].

2.4 Plasmid Screening

The alkaline lysis method described by Kado and Liu [18] was used to determine the presence of Plasmid in the isolates. Electrophoresis was carried out in Tris Acetate ETDA buffer containing ethidium bromide at 30 mA (70 V) for four hours. Plasmid bands were viewed on a U/V transilluminator and the photographs taken using a SonyDSC-T20 camera. Films were exposed for 90 seconds and later developed. Plasmid sizes were estimated from a standard curve drawn of the molecular sizes of the Hind III digested λ DNA ladder against their migration distance. Isolates with plasmid were cured by growing them on Mueller Hinton medium containing sodium dodecyl sulphate (SDS). Plates were incubated at 37°C for 48 hrs. The cells were harvested after 48 hrs. The plasmid profile analysis and antibiotics susceptibility of the cured isolates were repeated.

3. RESULTS AND DISCUSSION

3.1 Results

Microbial analyses of hospital wastewater by culturing revealed a load of total heterotrophic, *Escherichia coli*, and Staphylococcal counts with multi-drugs resistance ability.

3.2 Discussion

Environmental contamination with harmful substances from industrial processes and

medical services require urgent and adequate attention. This is because the quality of any environment has a high social and health influence on the inhabitants. Wastes become potential hazards, when not properly treated, releasing pathogens together with nutrients and compounds that keep on their proliferation. The level of growth of microorganisms on the culture plates following serial dilution reflected the level of contamination as well as the sanitary condition of the hospital wastewater. Table 1 shows high loads of heterotrophic bacteria, *Escherichia coli* and Staphylococcal counts from the hospital wastewater. Species of *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Clostridium*, *Pseudomonas*, *Proteus*, *Escherichia* and *Staphyloccocus,* etc*.* were identified in the wastewater (Table 2). Similar composition of organisms has been reported in the study of hospital wastewater by Akin [6] and Katouli et al. [3].

Table 1. Bacterial load of hospital wastewater

Bacteria	Load (CFU/mL)	
	10^3	10^4
Total heterotrophic plate count	1.36×10^7	2.0×10^6
Escherichia coli plate count	2.1×10^5	1.6×10^6
Staphylococcal count	1.4×10^6	1.2×10^5

The antimicrobial resistance nature of these organisms as seen on Table 3 is quite scary. The least number of drugs resisted was three by *B. subtilis* and *E. coli.* Among the pathogens, *Staphyloccocus aureus* (COT CHL TET ERY GEN AUG CXC) and *K. pneumoniae* (COT GEN AUG CXC AMX NIT NAL) resisted more of the drugs. Some Gram-positive bacteria in this study, also displayed increased resistance as observed in the resistance profile of *Staph. aureus* - COT CHL TET ERY GEN AUG CXC and *Bacillus* sp. - COT CHL TET ERY AUG STR CXC. This ability is known among the Gram-negative organisms and Russell et al. [19] in their study described this property as a function of their cell wall structure and the possession of enzymes that inactivate antibiotics. This observation indicates the extent of resistance among the bacterial population and loss of effectiveness of the antimicrobial agents. Resistance to several antimicrobial agents in bacteria species from hospital wastewaters have been reported in studies by Fekadu et al. [2]; Iweriebor et al. [9]; and Diwan et al. [20] as well as in studies by Martins et al. [21] and Lister et al. [22]. Multi-drug resistant forms of *Pseudomonas aeruginosa* are reported to be a major source of nosocomial infections and they are known to persist for extended periods when discharged in the environment [23]. Fig. 1 presents the percentage susceptibility of the bacteria species to the antibiotics. Highest resistance was observed for Co-trimoxazole (15%) and Augmentin (15%) while the least resistance was seen recorded for Ofloxacin (3%) and erythromycin 3%. Ofloxacin (18%) led Gentamicin (16%) with high sensitivity indicating the drugs' potency. Ofloxacin have been reported to display higher potency by Adedeji et al. [24].

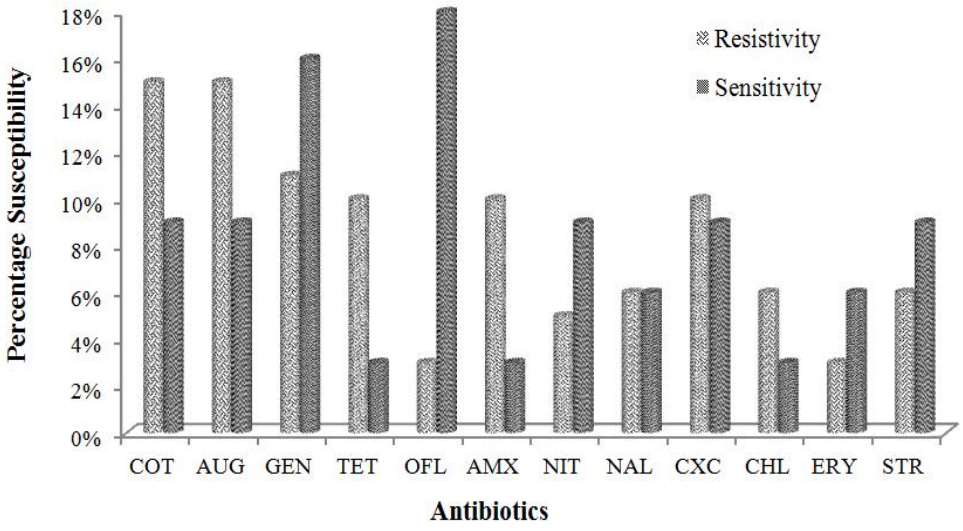

Fig. 1. Percentage susceptibility of the bacteria species from hospital wastewater

Table 2. Morphological and biochemical characteristics of the bacteria species isolated from hospital wastewater

Bacteria species	Colony morphology	Gram reaction / Shape	Urease	Motility	Citrate	Methyl red	Indole	Voges proskauer	Oxidase	Catalase	Hydrogen sulfide	Coagulase	Glucose	Lactose	Sucrose
Acinetobacter lwoffii	Flat, moist and yellowish	Gram -ve Rods	-	+	-	-	-	-	-	+	-	-	a	a	-
Bacillus subtilis	Circular, flat, hard and creamy pigment	Gram +ve Rods	-	+	-	-	-	+	-	-	-	+	a	-	a
Salmonella typhi	Smooth, raised, entire and cream pigment	Gram -ve Rods	-	+	+	+	-	-	-	+	+	-	ag	ag	ag
Escherichia coli	Smooth, circular, and greenish (metallic) sheen	Gram -ve Rods	-	-	-	+	+	-	-	+	-	-	ag	ag	ag
Staphyloccocus aureus	Shiny, rough, circular and yellow	Gram +ve Cocci	-	-	-	-	-	-	-	+	-	+	a	a	a
Bacillus sp.	Circular, flat, brittle and creamy	Gram +ve Rods	-	+	+	-	-	+	+	+	-	-	g	g	g
Pseudomonas aeruginosa	round, convex, regular, Moist and creamy	Gram -ve Rods	-	+	-	-	-	-	+	+	-	-	a	-	-
Klebsiella pneumoniae	Rough, raised, lobate and creamy	Gram -ve Rods	+	+	+	-	-	+	-	+	-	-	ag	ag	ag
Enterobacter aerogenes	Circular, raised, entire and pink	Gram -ve Rods		-	+	-	-	+	-	+	-		nd	nd	nd
Proteus vulgaris	Flat, viscous, opaque and milky	Gram -ve Rods	-	+	+	+	-	+	-	-	-	-	g	g	ag
Clostridium sp.	Rough, flat, entire and milky	Gram +ve Rods	+	+	+	+		-	-	-	+	-	a	-	a
Shigella sp.	Smooth, raised, entire and creamy	Gram -ve Rods	-	+	-	-		+	-	+	+	-	ag	ag	ag

Key: +ve/+ =positive; -ve/- = negative; nd = not detected; ag = acid and gas; a = acid; g = gas

Table 3. Plasmid and antibiogram profile of bacteria species Isolated from hospital wastewater

a. Bacterial species	b. Plasmid pattern (kbp)	Phenotypic Susceptibility Pattern			
		Resistance Pattern (≤12 mm)		Sensitivity Pattern (≥12 mm)	
		c. Before Plasmid Curing	d. After Plasmid Curing	e. Before Plasmid Curing	f. After Plasmid Curing
A. lwoffii		COT GEN AUG TET OFL AMX NIT NAL		OFL	
B. subtilis		COT TET CXC		CHL ERY GEN AUG STR	COT TET CXC CHL ERY GEN AUG STR
S. typhi	23.1, 19.5	COT GEN CXC AMX NAL		AUG OFL NIT	COT GEN CXC AMX NAL AUG OFL NIT
E. coli		GEN CXC AMX		COT AUG OFL NIT NAL	GEN CXC AMX COT AUG OFL NIT NAL
S. aureus	19.5	COT CHL TET ERY GEN AUG CXC		STR	COT CHL TET ERY GEN AUG CXC STR
Bacillus sp.		COT CHL TET ERY AUG STR CXC	COT	GEN	CHL TET ERY GEN AUG STR CXC
P. aeruginosa	22, 19.5	COT AUG TET AMX	COT AUG TET AMX	GEN OFL	GEN OFL
K. pneumoniae	23.1	COT GEN AUG CXC AMX NIT NAL		OFL	COT GEN AUG CXC AMX NIT NAL OFL
E. aerogenes	23.1	AUG CXC OFL AMX		COT GEN STR NIT NAL	AUG CXC OFL AMX COT GEN STR NIT NAL
P. vulgaris	23.1	GEN AUG STR NIT NAL		COT CXC OFL AMX	GEN AUG STR NIT NAL COT CXC OFL AMX
Clostridium sp.	23.1	COT CHL TET AUG STR		ERY GEN CXC	COT CHL TET AUG STR ERY GEN CXC
Shigella sp.		COT CHL GEN AUG STR		TET ERY CXC	COT CHL GEN AUG STR TET ERY CXC

Key: R = Resistant (≤12 mm), S = Sensitive (≥12 mm), COT = Co-trimoxazole (25µg), CHL= Chloramphenicol (10 µg), AUG = Augmentin (30 µg), CXC = Cloxacillin (5 µg), AMX= Amoxicillin (10 µg), ERY = Erythromycin (5 µg), GEN = Gentamicin (10 µg), STR = Streptomycin (10 µg), OFL = Ofloxacin (5 µg), NAL = Nalidixic acid (30 µg), NIT = Nitrofurantoin (30 µg), TET = Tetracycline (10 µg)

Molecular analysis of the bacteria in this study revealed the presence of the mobile genetic element, plasmids (Fig. 2). The presence and position of the plasmid fragments (bands) after gel electrophoresis was obvious in *Salmonella typhi, Pseudomonas aeruginosa* and *Staphyloccocus aureus* contained in lane 6, 9, and 10 of the figure, respectively. *S. typhi* showed plasmid sizes 23.1 kb and 19.5 kb with the resistance pattern COT GEN CXC AMX NAL. *P. aeruginosa* had 22 kb and 19.5 kb sizes of plasmids with a resistance pattern of COT AUG TET AMX. The bacteria, with or without plasmids showed no particular susceptibility pattern to the antibiotics (Table 3). In other words, among the isolates with muti-antibiotics resistance (MAR) pattern, 41.7% had no plasmid. Also, no particular molecular size plasmid could be associated with any particular antimicrobial resistance. This indicates that resistance to (various) antimicrobial agents were not associated with the presence of plasmids. For further verification, the isolates were cured of the plasmids and antibiotics susceptibility repeated. Surprisingly, the isolates were cured phenotypically (Table 3-f) and genotypically (Fig. 3) as was seen in their antibiotic sensitivity pattern (where their zones of clearence by the antibiotics exceeded 12 mm) and plasmid profile, respectively. The susceptibility profile of the bacteria however showed increased sensitivity compared to before the isolates were rid of the plasmids indicating plasmid-mediated antibiotics resistance. *Bacillus* sp. and *P. aeruginosa* however, still maintained their resistance to COT and COT AUG TET AMX, respectively (Table 3-d) indicating that their resistance gene is not contained in plasmid and therefore not linked to plasmid but rather could be a mutational process which together with acquired resistance hinder the effectiveness of therapeutic agents and the treatment of clinical conditions. *P. aeruginosa*, responsible for pneumonia infection in humans, in a report by Lister et al. [22] is known to coregulate different resistance mechanisms to overcome antibacterial challenges.

The presence of drugs resistant bacteria in the environment therefore 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/factor which leads to a change in the microbial composition of the environment; ingestion of the resistant organisms by animals or humans through food chain; among others. This study reveals that hospital wastewater contains loads of antibiotic resistant bacteria whose resistance in part are carried in plasmid. Hospital wastewater contributes to environmental contamination with harmful deposits that threaten the lives in the receiving ecosystem due to pathogens.

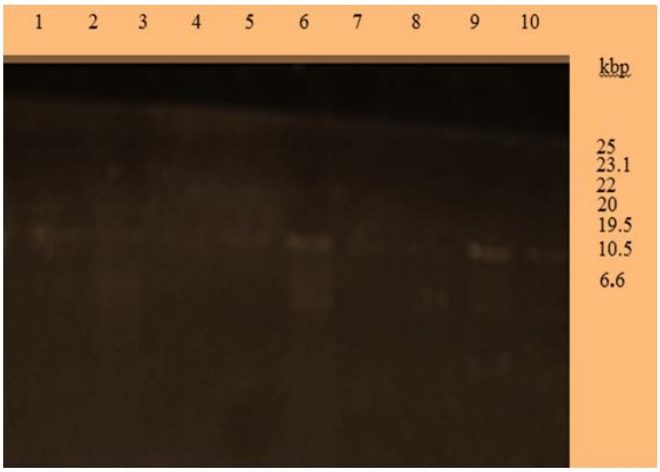

Fig. 2. Gel Image of Plasmid bands in bacteria species from hospital wastewaters

Lane 1 - Proteus vulgaris; Lane 2 - Clostridium sp.; Lane 3 - Klebsiella pneumoniae; Lane 4 - Enterobacter aerogenes; Lane 5 - Salmonella typhi; Lane 6 - Shigella sp.; Lane 7 - internal control; Lane 8 - Escherichia coli; Lane 9 - Pseudomonas aeruginosa; Lane 10 - Staphyloccocus aureus

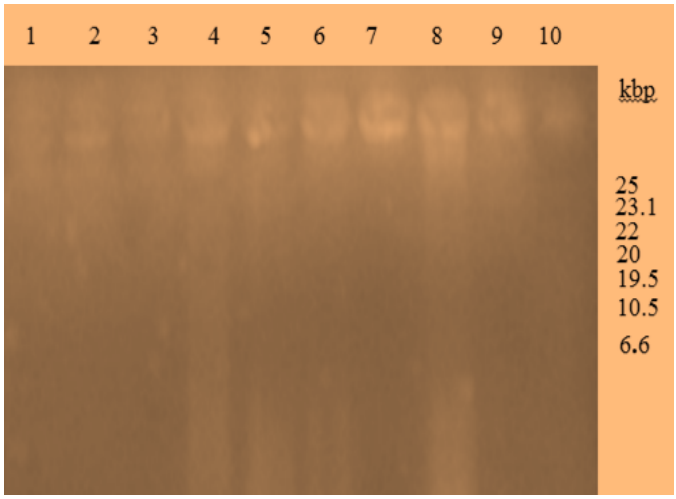

Fig. 3. Gel Image of plasmid profile of cured bacteria from hospital wastewater

Lane 1 - Proteus vulgaris; Lane 2 - Clostridium sp.; Lane 3 - Klebsiella pneumoniae; Lane 4 - Enterobacter aerogenes; Lane 5 - Salmonella typhi; Lane 6 - Shigella sp.; Lane 7 - internal control; Lane 8- Escherichia coli; Lane 9 - Pseudomonas aeruginosa; Lane 10 - Staphyloccocus aureus

4. CONCLUSION

A high load of pathogenic bacteria are contained in hospital wastewater. Bacterial species from the genera *Acinetobacter*, *Bacillus, Salmonella, Escherichia, Staphyloccocus*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Proteus*, *Clostridium* and *Shigella* were isolated from the wastewater. Of the entire antibacterial agents tested against these isolates, the least resistance was observed for ofloxacin which also displayed the highest sensitivity followed by gentamicin. Augmentin and Co-trimoxazole were the most highly resisted of the antibiotics. The bacteria species carried mobile genetic element, plasmid, of sizes 23.1, 22 and 19.5 kbp. *Bacillus* and *Pseudomonas* species displayed more than one plasmid bands and both retained their antibacterial resistance profile even after they were rid of the plasmid. The release of these pathogens into the surrounding habitats is unhealthy with serious environmental consequences which could be suffered by both plants and animals including humans. Our health depends on the quality of the environment. The practice of releasing hospital wastewater and other polluted substances into the environments should be strongly prohibited.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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