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ANTIBIOTIC RESISTANCE PROFILE OF WASTEWATER ISOLATES OBTAINED FROM UNIVERSITY OF CALABAR TEACHING HOSPITAL AND GENERAL HOSPITAL CALABAR, NIGERIA.

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Abstract
The antibiotic resistance profile of bacterial isolates obtained from the wastewaters of the University of Calabar Teaching Hospital (UCTH) and the General Hospital Calabar (GHC), Cross River State, Nigeria, was determined using the disc-diffusion method. A total of 125 bacterial isolates from both hospitals` wastewater comprising of the following genera: *Escherichia*; *Salmonella*; *Shigella*; *Klebsiella; Pseudomonas*; *Streptococcus*; *Bacillus*; *Staphylococcus* and *Proteus*, were tested for their antibiotic resistance capability. Data obtained showed that all the isolates from both hospitals had multiple antibiotic resistance (MAR). Over fifty five percent of the isolates from UCTH and 12.5% of the isolates from GHC exhibited resistance to the antibiotics tested (amoxycilin, gentamycin, augumetin, chloramphenicol, erythromycin, tetracycline, ciprofloxacin, streptomycin, and cotrimoxazole). Amongst the UCTH isolates, 5 different antibiotic patterns were observed ranging from 6-12 MAR combinations while 8 different antibiotic resistance patterns ranging from 4-12 MAR combinations were obtained from the GHC isolates. All the UCTH isolates were resistant to the antibiotics commonly used in the hospital (amoxicillin, augumentin, chloramphenicol, gentamycin, erythromycin, tetracycline, ciprofloxacin, streptomycin and cotrimaxazole) except *Escherichia coli* and *Pseudomonas* which are sensitive to ciprofloxacin. The same trend was obtained for the GHC isolates for the commonly used antibiotics (chloramphenicol, erythromycin, tetracycline, streptomycin, cotrimaxazole) in GHC. The lowest resistance of 25% was obtained for ofloxacin which was one of the antibiotics rarely used in both hospitals. Results obtained show the important public health hazard associated with the discharge of untreated hospital wastewater into the environment.
KEY WORDS: antibiotics, bacterial isolates, resistance, hospital wastewater.

Introduction

A myriad of substances such as antibiotics, synthetic drugs, radionuclide, and solvents, etc. are used in hospitals for medical treatment, diagnostics, disinfection and research. After application, many non-metabolized drugs are excreted by the patients and enter the wastewater. Antibiotics in these wastewater may be present at levels that could not only alter the ecology of the environment but also give rise to antibiotic resistance (Raloof, 1998).

Acquired resistance to antibiotics may arise by cellular mutation or by acquisition of genetic elements in the form of plasmids or transposons (Russel and Path, 2001). The occurrence of strongly selective environments such as hospitals, promotes, not only the growth of resistant bacteria, but also leads to an increase in the frequency of resistance bacterial genes and genetic elements such as plasmids.

Hospital wastewater, even if it is treated, may contain pathogenic drug-resistant bacteria, which constitute the most dangerous single risk factor for dissemination of pathogenic and drug-resistant organisms in the environment (Diab *et al*., 2008). These resistant bacterial species may be transmitted to humans and farm animals causing infection that cannot be treated by conventional antibiotics (Khachatourians, 1998). Hence, the hospital wastewater with its high content of multidrug resistant bacteria and the presence of enteric pathogens could pose a grave problem for the community receiving such wastewaters (Cabrera *et al*. 2004; Chitris, 2004; Somwang *et al*., 2005).

Often times in Nigeria, the untreated wastes of hospital and urban sewage systems are released into rivers, lakes and other surface waters which also serve as sources of drinking water for local communities and poor homes and could also help sources of water for the water treatment plants that provide drinking water for the cities. Despite this practice, few characterizations of hospital wastewaters have been carried out in Nigeria to determine the antibiogram of the microbial diversity inherent in such discharges. The aim of this research therefore was to determine the antibiotic resistance profile of bacteria in wastewaters coming out of hospital drains in Calabar. The study was carried out on the wastewaters of the University of Calabar Teaching Hospital (UCTH), and the General Hospital Calabar (GHC). Both hospitals are public health centers located in Calabar, Cross River State, Nigeria, that provide hospital services in a broad category of illnesses and injuries.

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Materials and methods

Collection of samples

Wastewater samples were collected from UCTH and GHC both located in Calabar Metropolis, Nigeria. The sampling was performed according to the protocol of Nunez and Moretton (2007). Samples were collected over a 10h period taking samples every 2h for 16 days using sterile 500ml sample bottles. The same volume (250ml) of each partial sample was mixed at the end of the day to obtain the composite sample used for microbiological analyses. A total of 76 samples were collected from both hospital and all samples were preserved in an icebox until transported to the laboratory.

Isolation of total culturable heterotrophic bacteria (TCHB).

Isolates of TCHB were obtained through 10-fold serial dilution of the wastewater samples in sterile physiological saline and pour plating of 0.1ml aliquots in duplicates on Nutrient Agar (Difco Lab). Discrete colonies that developed after incubation at 30^{o}C for 48h were subcultured to obtain pure cultures which were stored at 4^{0}C and used subsequently for microscopic characterization and biochemical analyses.

Isolation of enteric bacteria

One ml aliquots of appropriately diluted samples were plated out (pour plate) in duplicates on MacConkey agar to screen for coliform bacteria. To screen for *Salmonella* spp. and *Shigella* spp., the samples were enriched in Selenite F broth for 8h and then pour plated on *Salmonella-Shigella* agar and incubated for 24-48h. The samples were also enriched on alkaline peptone water (pH 8.3) and then plated out (pour plate) on thioglycholate citrate bile salt sucrose (TCBS) agar, to isolate *Vibrio* species. Pure cultures of isolates were stored on nutrient agar slants at 4^{0}C for characterization and identification (Krieg and Holt, 1994).

Characterization and identification of isolates

The isolates were characterized and identified following biochemical procedures as described in Bergey`s Manual of Systemic Bacteriology (Krieg and Holt, 1994).

Antibiotic resistance studies

The standardized disc diffusion method (Bauer et al., 1999) and the zone-size interpretation chart of Kirby-bauer (1966) was used for the *in vitro* determination of the bacterial sensitivity to the various antibiotics selected, selection of antibiotics was based on their usage in the hospitals. The antibiotics used were; amoxycilin (25 μg/ml), gentamycin (10μg/ml), augumetin (30μg/ml), chloramphenicol (30μg/ml), erythromycin (10μg/ml), tetracycline (30μg/ml), ciprofloxacin (10μg/ml), streptomycin

(10µg/ml), and cotrimoxazole (25µg/ml). Ofloxacin (5µg/ml), ceftriazone (25µg/ml), and pefloxacin (5µg/ml) which are rarely used antibiotics in both hospitals were also included in this study.

Commercially prepared paper discs impregnated with the various antibiotics were mounted on Muller-Hinton agar plates (Lab M) containing pure cultures of the various isolates. Each plate was inoculated with 200µl of bacterial suspensions containing 10^7 to 10^8 cells obtained using basic 0.5 Mcfarland solution (NCCLS, 1990). A total of 125 randomly selected isolates were tested and results obtained were classified as resistant or sensitive. Inhibition zone diameters were measured after 18-24h of incubation.

Results

The composite total bacterial counts from UCTH and GHC wastewater samples were $7.80 \pm 3.6 \times 10^6$ and $9.50 \pm 4.5 \times 10^6$ CFU/ml for TCHB, and $3.30 \pm 2.1 \times 10^6$ and $4.20 \pm 2.9 \times 10^6$ CFU/ml for coliforms respectively (Table 1). The percentage of coliforms to total bacteria is 42.31% and 44.21% respectively. A total of 125 isolates comprising of 65 from UCTH and 60 from GHC were identified based on their colonial morphology and their biochemical characteristics. They belonged to the following bacterial genera: *Escherichia*; *Salmonella*; *Shigella*; *Klebsiella; Pseudomonas*; *Streptococcus*; *Bacillus*; *Staphylococcus* and *Proteus*. All these bacterial genera except *Proteus* were isolated from both hospital wastewaters. *Proteus* was isolated from only UCTH wastewater samples. Tables 2 and 3 show the frequency of isolation of these bacterial genera

Table 1: Total bacterial and coliform counts of UCTH and GHC wastewaters

Location	Total bacterial Counts (CFU/ml	Total Coliform counts (CFU/ml	% Coliform
UCTH	$7.80 \pm 3.6 \times 10^6$	$3.30 \pm 2.1 \times 10^6$	42.31
GHC	$9.50 \pm 4.5 \times 10^6$	$4.20 \pm 2.9 \times 10^6$	44.21

*Composite mean value of triplicate counts.

Table 2: Frequency of isolation of the different bacterial genera from the wastewaters of UCTH.

Bacteria genera	Total number of samples	No. of positive samples	Frequency of isolation (%)
Salmonella	76	37	48.68
Shigella	76	57	75.00
Klebsiella	76	38	50.00
Escherichia	76	76	100.00
Pseudomonas	76	40	52.63
Streptococcus	76	76	100.00
Bacillus	76	39	51.32
Staphylococcus	76	37	48.68
Proteus	76	57	75.00

Table 3: Frequency of isolation of the different bacterial genera from the wastewater of GHC

Bacteria genera	Total number of samples	No. of positive samples	Frequency of isolation (%)
Salmonella	76	28	36.84
Shigella	76	42	55.26
Klebsiella	76	32	42.11
Escherichia	76	76	100.00
Pseudomonas	76	36	47.37
Streptococcus	76	66	86.84
Bacillus	76	42	55.26
Staphylococcus	76	51	67.11

Resistance patterns of isolates from both hospital wastewaters showed that all the isolates demonstrated multiple antibiotic resistance (MAR). Five genera (*Salmonella*, *Klebsiella,* *Bacillus*, *Staphylococcus* and *Proteus*) from the UCTH wastewater samples showed resistance to all the antibiotics tested (Table 4). Only one genus (*Salmonella*) isolated from the GHC wastewater samples showed resistance to all the tested antibiotics (Table 4). None of the bacteria isolated from the wastewater samples of both hospital demonstrated single antibiotic resistance to all the antibiotics tested.

Table 4: Antibacterial susceptibility profile of bacteria isolated from UCTH wastewater

Antibiotics tested	Disc potency (µg/ml)	*Salmonella*	*Shigella*	*Klebsiella*	*Escherichia*	*Pseudomonas*	*Streptococcus*	*Staphylococcus*	*Bacillus*	*Proteus*	% Resistance of all organisms
Amoxycillin	25	R	R	R	R	R	R	R	R	R	100
Augumentin	30	R	R	R	R	R	R	R	R	R	100
Chloramphenicol	30	R	R	R	R	R	R	R	R	R	100
Gentamycin	10	R	R	R	R	R	R	R	R	R	100
Erythromycin	5	R	R	R	R	R	R	R	R	R	100
Tetracycline	30	R	R	R	R	R	R	R	R	R	100
Ciprofloxacin	10	R	R	R	S	S	R	R	R	R	77.8
Streptomycin	10	R	R	R	R	R	R	R	R	R	100
Cotrimaxozole	25	R	R	R	R	R	R	R	R	R	100
Ofloxacin	5	R	S	R	S	R	S	R	R	R	66.7
Pefloxacin	5	R	R	R	R	R	S	R	R	R	88.9
Ceftriazone	30	R	S	R	S	R	R	R	R	R	77.8
% Resistance of single organism		100	83.3	100	75.0	91.7	83.3	100	100	100	

Table 4: Antibacterial susceptibility profile of bacteria isolated from GHC wastewater

Antibiotics tested	Disc potency (μg/ml)	*Salmonella*	*Shigella.*	*Klebsiella*	*Escherichia*	*Pseudomonas*	*Streptococcus*	*Staphylococcus*	*Bacillus.*	% Resistance of all organisms
Amoxycillin	25	R	R	R	R	S	R	R	R	87.5
Augumentin	30	R	R	R	R	S	R	R	R	87.5
Chloramphenicol	30	R	R	R	R	R	R	R	R	100
Gentamycin	10	R	R	R	S	R	R	S	R	75.0
Erythromycin	5	R	R	R	R	R	R	R	R	100
Tetracycline	30	R	R	R	R	R	R	R	R	100
Ciprofloxacin	10	R	R	R	S	R	R	S	S	62.5
Streptomycin	10	R	R	R	R	R	R	R	R	100
Cotrimaxozole	25	R	R	R	R	R	R	R	R	100
Ofloxacin	5	R	S	S	S	S	S	R	S	25.0
Pefloxacin	5	R	R	R	S	R	S	R	R	75.0
Ceftriazone	30	R	S	R	S	R	R	R	R	75.0
% Resistance of single organism		100	88.3	91.7	58.3	75.0	83.3	83.3	83.3	

All the isolates from the UCTH wastewater samples showed resistant to all the antibiotics (amoxycillin, gentamycin, augmentin, chloramphenicol, erythromycin, tetracycline, streptomycin and cotrimaxazole) commonly used in the hospital except Ciprofloxacin. *Escherichia coli* and *Pseudomonas* from UCTH and *Escherichia* alone from GHC were sensitive to Ciprofloxacin (Tables 4 & 5). More organisms - *Shigella*, *Escherichia* and *Streptococcus* showed sensitivity to Ofloxacin (one of the less used antibiotics). *Shigella* and *Escherichia* were sensitive to ceftriazone, another not frequently used antibiotic. Only *Streptococcus* in UCTH and *Streptococcus* and *Escherichia* in UCTH showed sensitivity to Pefloxacin – third rarely used antibiotic employed. Data obtained from the GHC wastewater analyses showed that only *Salmonella* had developed resistance to all twelve antibiotics used in this study (Table 5). It was also found that all the bacterial isolates from the GHC had developed resistance to some of the antibiotics (chloramphenicol, erythromycin, streptomycin, tetracycline and cotrimaxazole) commonly in the hospital. Only *Salmonella* and *Staphyloc occus* showed resistance to ofloxacin which was also rarely used in that hospital.

Discussion

The TCHB and coliform counts in the hospital wastewater samples investigated were similar to counts reported for San Martin Hospital, Argentina by Nunez and Moretton (2007). Similar results were also obtained for the Porto Alegre hospital de Clinicas wastewater, Brazil (Ortolan, 1999) and the

University of Ilorin Teaching Hospital wastewater (Olayemi and Opaleye, 1990). The counts obtained for the bacterial groups at the two studied sites indicated high pollution level. The safe discharge level of such wastewaters should not exceed 100CFU/ml for TCHB and 30CFU/ml for coliforms (EPA Guidelines, 2003). Out of the 7.80±3.6 x 10^6 CFU/ml total bacteria count obtained in the UCTH wastewater samples, 42.31% were coliforms while 44.21% of the 9.50±4.5 x 10^6 CFU/ml of total bacteria count obtained from GHC were coliforms (Table 1). *Escherichia coli* and *Streptococcus* spp were the most frequently isolated bacteria (Table 2). All the isolates had multiple antibiotic resistance, with most of the isolates from both hospital samples (Tables 3 & 4) exhibiting resistance to at least 6 antibiotics. Resistance to all of 9 commonly used antibiotics was recorded for 7 genera in UCTH namely: *Salmonella*, *Klebsiella, Shigella, Streptococcus, Bacillus*, *Staphylococcus* and *Proteus* (Table 4), whereas 4 genera (*Salmonella*, *Shigella*, *Klebsiella* and *Streptococcus*) showed resistance to the same nine antibiotics in GHC (Table 5). Previous reports have implicated *E. coli* strains as the main carrier of antimicrobial resistance in faecal flora, as resistance in other species was rare especially in the absence of antimicrobial selection (Osterblad, et al. 2000; Guardaasi, *et al*., 1998). Results obtained in this study however have shown that other genera can acquire multiple antibiotic resistance capacity and could surpass *E. coli* in exhibiting resistance to tested antibiotics. The ability/predisposition to develop resistance under conditions of antibiotic selective pressure (e.g. hospital environments) might be responsible for the acquisition of the antibiotic resistance capacity of these bacteria. The spectrum of resistance exhibited when compared with *Escherichia* indicates that the multidrug resistance genes responsible for the unique characteristics observed might also be domiciled in some of these bacterial genera.

The highest prevalence of resistance among the bacterial isolates was shown for the most commonly used antibiotics in both hospitals (Tables 3 & 4). This result thus lays credence to a previous report by Islam *et al*. (2008) which claims that resistance development was directly related to the use of antibiotics.

It is widely acknowledged that the selection and dissemination of antibiotic resistant bacteria in nature should be curtailed in order to maintain the ecological balance that favours the predominance of a susceptible bacterial flora in the environment and to ensure effective treatment against infectious diseases in humans and farm animals. Thus, the indiscriminate use of antibiotics and the discharge of untreated antibiotic-laden hospital effluents into the environment may disrupt the microbial balance in nature in favour of resistant bacteria. This paper highlights the important public health hazard that may be associated with the indiscriminate discharge of hospital effluent into the environment without adequate treatment.

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