GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Detection of antibiotic resistance in bacteria isolated from industrial sewage

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GSC Biological and Pharmaceutical Sciences, 2022, 18(02), 058–065

Publication history: Received on 09 September 2021; revised on 12 October 2021; accepted on 14 October 2021

Article DOI: https://doi.org/10.30574/gscbps.2022.18.2.0306

Abstract

Industrialization and indiscriminate discharge of sewage being implicated in environmental pollution is responsible for the release of micropollutants which over time is expected to alter the physiology and metabolic pathways of microbiota native to the environment. This study was undertaken to track antibiotic resistant genes in industrial wastewater. Heterotrophic bacterial count indicated a vast abundance of culturable bacteria (76-232.66× 10^4 Cfu/ml). Hydrocarbon utilizing bacteria count ranged between (91.66 - 111.6×10^4 Cfu/ml). Antibiotic resistant bacteria isolates from industrially impacted wastewater identified by Sanger's sequencing included *Bacillus lichenforminis* (KF737353.1), *Alkanindiges* sp. *5-0-9* (LT158291.1*)*, *Bacillus thuringiensis* (MK875170.1, MK517632.1 EU697392.1) *Bacillus altitudinis* (KY777585.1), *Bacillus cereus* (KR185830.1) and *Bacillus subtilis* (MK124647.1). This study infers that industrial effluent is a potent reservoir for antibiotic resistant bacteria of environmental and public health concerns.

Keywords: Antibiotic; Resistance; Bacteria; Sewage

1. Introduction

Sewage consists of substances either organic or inorganic which most often mediate into solution in the aqueous phase and due to their characteristic size are referred to as *'micropolllutants'.* Micropollutants implicated in sewage range from radioactive isotopes, metals, hydrocarbons, phenolic compounds, nitrogenous substances, phosphorus, sodium, aggregates of soil, fecal substance, microorganism amongst others [1,2]. Sewage frequently deviates from the natural balance with respect to composition and possess a degraded quality owing to introduction of xenotic substances. Since the advent of antibiotics and development of new generation of therapeutic agents over past decades and in recent times, there has been constant recovery of antibiotics in wastewater. Pharmaceutical waste, hospital waste and agricultural waste are contributing significantly to the swell in antibiotics levels in sewage [3-7]. Waste from these sources and products can trigger genomic modification for antibiotic resistance, as these eventually end up in the environment

Antibiotic resistance could occur either generally or specifically as a result of inadequate waste management practices owing to the fact that antibiotics cannot be completely metabolized by the human and animal body system [8] coupled with the incomplete decomposition of chemical or genomic altering agents in waste from industrial manufacturing/production processes. Thus, traces of antibiotics or therapeutic agents end up in the environment via solid or liquid routes. Waste discharged either from persons or industries containing antibiotics have the potential to effect mutagenesis, illicit resistance on resident microbial populations in their surrounding either through selective pressure or horizontal gene transfer [9, 10].

The proliferation of antibiotic-resistant bacteria and the occurrence of antibiotic resistance genes in wastewaters are notable factors that contribute to the decline in the potency of antibiotics interventions globally [3]. Incidence of

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antimicrobial resistance is on the increase and of course a significant concern for public health Resistance genes for commonly used antibiotics have been detected in wastewater [3, 4, 10, 11]. Overbye and Barrett [12] reported that in the US, approximately two million people were diagnosed of an antibiotic resistant infection of which ninety thousand were life threatening. This statistic throws a curious light on developing economies where antibiotic resistance can only be emphasized considering our social, economic and cultural practices.

Microorganisms in polluted sites engage different metabolic pathway in attempt to breakdown and digest available compounds for carbon and energy requirements. Therefore, if the microbial consortium or quorum is high, there is the tendency for resistance to develop as diverse and complex bacteria biofilms form and spontaneously populate with enhanced capacity to degrade these chemical substances (i.e. antibiotics) and make degradation products available for further degradation by other microbes within the consortium [13].

This study aimed to track the presence of antibiotic resistant genes posed by micropollutants in industrial wastewater in Port Harcourt.

2. Material and methods

2.1. Study Location

This study was conducted in Port Harcourt, the capital city of Rivers and the hub of the oil and gas industry in Nigeria. Wastewater samples were collected from 8 industrial establishments situated in Trans Amadi, an area which is a focal point for medium to large scale industrial and servicing outfits located in Port Harcourt, Rivers State, Nigeria. The high concentration of industries in this area of the city was responsible for the selection of the area for sample collection. The effluent points sampled and the industrial activities taking place there are: FLS1 - aluminium manufacturing, LFS2 - cement manufacturing, TAS3 - main canal receiving effluents from Trans Amadi Industrial Layout, HSS4 - heavy duty engine servicing, SMS5 - oil servicing industry, NRS6 - vegetable oil manufacturing, FAS7 - aluminium manufacturing, FIS8 - fish farming.

2.2. Collection of Samples

Wastewater samples were collected from eight (8) locations with sterile containers. Each sample bottle was rinsed with the appropriate sample before the final collection [14]. The samples were placed in an ice packed cooler and immediately transported to the laboratory for analyses within six (6) hours of collection.

2.3. Microbiological analyses of the waste water samples

2.3.1. Isolation of Pure Culture

Aliquots of appropriate dilutions were inoculated on surface dried appropriate medium in duplicates and spread with sterile glass spreader prior to incubation at 37^0 C for 24 hours. Discrete colonies were sub cultured on fresh nutrient agar (NA) plate in order to isolate pure cultures.

2.3.2. Enumeration of Total Heterotrophic Bacteria

Total heterotrophic bacteria (THB) count was determined by spread plate method. Bacteria colonies that appeared on the nutrient agar plates after incubation at 37°C for 24 hours were counted and the mean expressed as Cfu/ml.

2.3.3. Hydrocarbon Utilizing Bacterial Count

Hydrocarbon utilizing bacterial (HUB) count was determined following method described by Chikere and Ekwuabu [15]. Hydrocarbon impregnated sterile Whatman No.1 filter papers were placed on lids of inverted Bushnell-Haas agar plates and incubated for 14 days at 30°C. Bacterial colonies that appeared as hydrocarbon utilizing were counted and the mean expressed as Cfu/ml.

2.4. Coliform Bacterial Count

Total coliform count was determined using Endo-agar following 24 hours of incubation at 37°C.

2.5. DNA extraction

Bacterial deoxyribonucleic acid (DNA) was extracted from colonies sub-cultured on sterile Nutrient broth using NORGEN DNA extraction Kit (Model 24700, (NORGEN CANADA) following the manufacturer's instructions.

2.6. PCR Amplification of DNA Fragments

The PCR reaction was performed on the extracted DNA samples using universal degenerate primers 27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3 and 1492R reverse 5'GGTTACCTTGTTACGACTT 3' (De Santis *et al*., 2007).

2.7. Sequencing

Sanger Sequencing was done at Inqaba Biotech/West Africa. Sequences were blasted against reference sequences in the GenBank for identification.

2.8. Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed on surface nutrient agar containing bacteria isolates following the method described by Alharthi *et al*. (2016) using varying concentrations of nine antibiotics, amikacin (AN) 30 µg, amoxicillin (AMC) 30 mg, cefazolin (Cz) 30 µg, cefotaxime (CTX) 30 µg, erthromycin (E) 15 µg, gentamicin (GM) 10 µg, oxacillin (OX) 1 µg, tetracycline (Te) 30 µg and vancomycin (VA) 5 µg. After 48 hours of incubation at 28°C, zones of inhibition were measured.

3. Results

3.1. Microbiological Parameters of Wastewater

The total heterotrophic bacterial count (THBC) ranged from 76 – 105 Cfu/ml results for the wastewater samples are presented in Figure 1, Figure 2 shows result for coliform bacterial count (Cfu/ml) across the study locations, the results ranged from 40 – 239 Cfu/ml. Figure 3 shows the result for hydrocarbon utilizing bacteria count, which ranged from 92-112 Cfu/ml.

Table 1 shows the molecular identity of the antibiotic resistant isolates. The isolates were identified as *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis, Bacillus thuringiensis, Bacillus altitudinis, Bacillus licheniformis* and *Alkanindiges* sp. 5-0-9. Table 2 shows the isolates resistant to the tested antibiotics. All the isolates showed resistance to at least three antibiotics, with isolate 003 C showed resistance to all the antibiotics except ofloxacin. Figure 4 shows the phylogenetic tree of isolates.

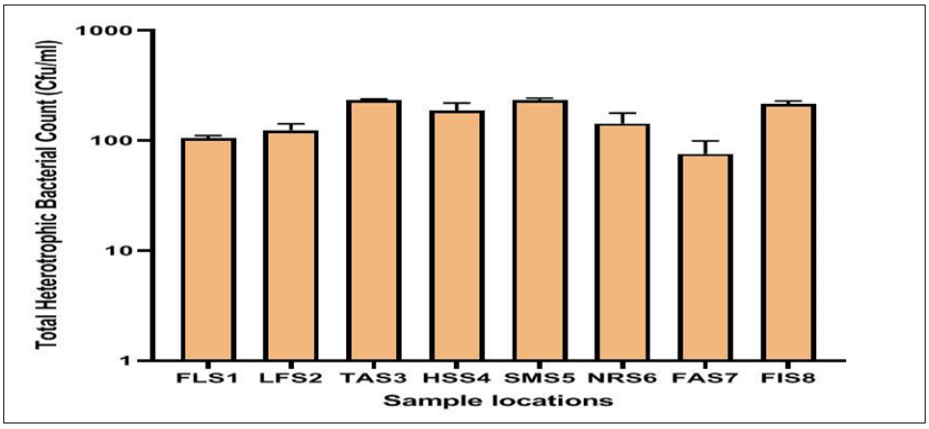

Figure 1 Total Heterotrophic Bacterial Count across the Study Locations

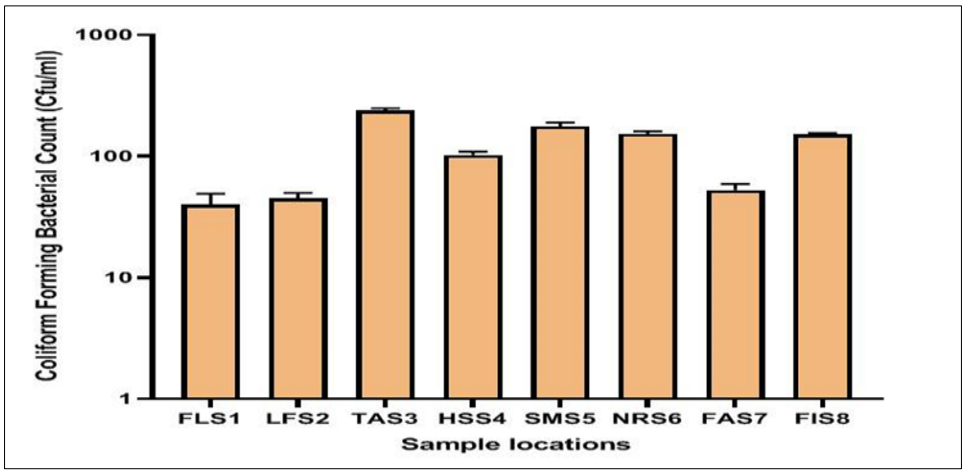

Figure 2 Coliform Forming Bacterial Count across the Study Locations

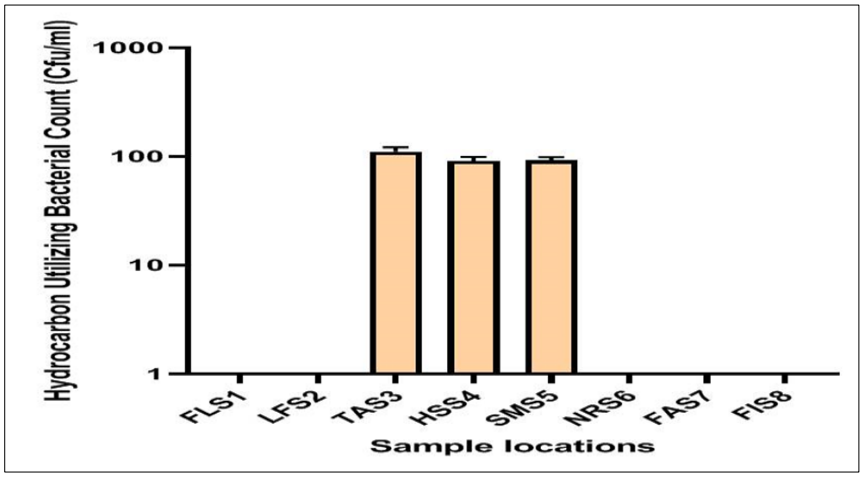

Figure 3 Hydrocarbon Utilizing Bacterial Count across the Study Locations

Table 1 Molecular Identification of Antibiotic Resistant Isolates

Isolate code	Similarity/E-score	Gene Bank Accession Number/	Identity Of Isolate Obtained
001 A	96.26/0.0	MK124647.1	*Bacillus subtilis*
001 C	KR185830.1	97.6/0.0	*Bacillus cereus*
003 A	86.58/00	KF737353.1	*Bacillus licheniformis*
003 B	87.7/00	MK875170.1	*Bacillus thuringiensis*
003 C	No significant similarity found. Too Short sequence		
004 A	95	KY777585.1	*Bacillus altitudinis*
007 A	86.58/00	KF737353.1	*Bacillus licheniformis*
007B	97.69/E97.43/00	KX507732.1 OrLT158291.1	uncultured bacterium or *Alkanindiges* sp. 5-0-9
008 A	97.38/00	MK517632.1	*Bacillus thuringiensis*
008 B	99.55/00	EU697392.1	*Bacillus thuringiensis*

Table 2 Antibiotics Sensitivity Result

Isolate code	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR	ERY	CXC	CTR
001 A	0	0	12	0	25	10	20	25	ND	ND	ND
001 B	7	7	15	0	18	0	20	20	ND	ND	ND
001 C	0	24	8	0	36	15	16	25	ND	ND	ND
002 A	4	0	15	ND	30	0	ND	ND	17	0	0
002 B	25	13	15	17	20	29	24	32	ND	ND	ND
002 C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
003 A	0	0	13	ND	18	0	ND	ND	0	0	0
003 B	0	0	13	0	22	0	0	29	ND	ND	ND
003 C	0	0	0	0	30	0	0	0	0	0	0
004 A	18	24	14	13	27	18	17	30	ND	ND	ND
004 B	25	0	19	15	28	20	0	35	ND	ND	ND
005 A	4	0	28	0	32	12	28	24	ND	ND	ND
005 B	10	15	11	0	26	0	0	31	ND	ND	ND
006 A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
006 B	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
007 A	0	0	17	0	0	0	0	35	ND	ND	ND
007 B	0	0	0	0	22	0	0	0	ND	ND	ND
007 C	7	7	15	0	18	0	20	20	ND	ND	ND
007 D	0	0	9	ND	28	0	ND	ND	0	0	0
008 A	0	0	15	0	30	8	17	20	ND	ND	ND
008 B	0	0	10	ND	38	0	ND	ND	10	0	0

KEY: ND= Not Determine; NC = Not clear; AUG= Augmentin; NIT: Nitrofurantion; CPR: Ciprofloxacin; CAZ: Cetazidime; GEN: Gentamicin; CXM: Cefixime; OFL: Ofloxacin; CTR: Cftriaxone; ERY: Erythromycin; CXC: Cloxacillin; CRX: Cefuroxime

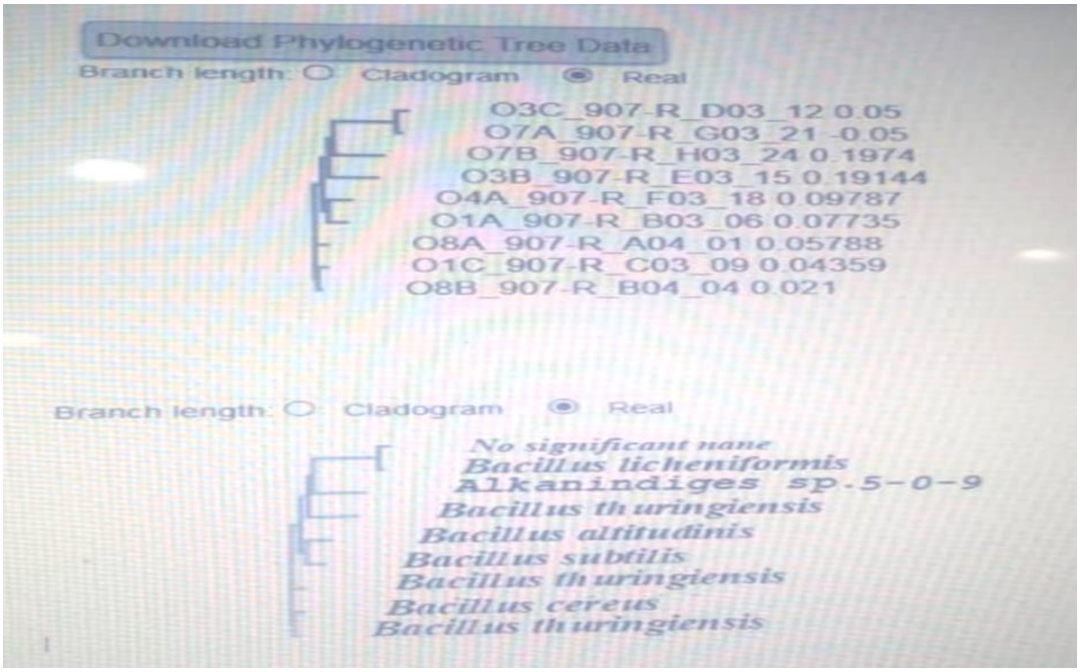

Figure 4 Phylogenetic tree of isolates

4. Discussion

This study examined the microbiological parameters of effluents from industries located at Trans Amadi Industrial Layout, Port Harcourt, Rivers State, with the aim to ascertain the incidence of antibiotics among the bacterial isolates. Effluent sample SMS5 (effluent from oil servicing company) had the highest THBC (236 cfu/ml). It could be that the effluent discharged is rich in various organic components that support the growth and abundance of diverse bacteria species, including hydrocarbon utilizing bacteria. Effluent sample NRS6, from a vegetable oil manufacturing site had the least THBC (76 cfu/ml). There was significant difference ($p < 0.05$) in the THBC across sampling sites, as well as between the highest and the least count. The range of THBC in the effluent samples is relatively low compared to range of $9.66\pm0.41x10^5$-$7.99\pm0.23x10^6$ cfu/ml reported by Egesi *et al*. [15] in their study of abattoir waste discharged into water bodies in Owerri, Nigeria, and also THBC of 4.0×10^3 - 6.7×10^5cfu/ml reported by Sule *et al*. [17] in their study of wastewater from a fish pond in Ilorin.

Result for the coliform forming bacterial count shows that sample TAS3 from the main canal receiving effluents from Transamadi Industrial Layout had the highest value (238 cfu/ml) while wastewater sample FLS1 from an aluminium manufacturing company had the least (40 cfu/ml). Coliforms were detected in all the waste water samples. Presence of coliforms gives a picture of the sanitary quality of the wastewater. Evidently the sampled wastewater contained faecal matter, an indication that the companies either discharged their sewages alongside their wastewater or there was seepage of sewage into the effluent discharge pipes. Expectedly, sample TAS3 exhibited higher coliform count than the other sites including the fish farm because its waste streams are numerous, including runoff from soil. All the samples had coliform count higher than the NESREA permissible limit of 5000/L. Significant difference ($p < 0.05$) was observed in coliform count across the sampling points. Sule *et al*. [16] and Egesi *et al*. [16] recorded a much higher coliform count of ranging from $1.78\pm0.29x10^5$-$2.16\pm0.15x10^6$ MPN/100 ml and $0 - 2x10^4$ cfu/ml respectively in their studies of the microbiological parameters of effluent in major cities in Nigeria.

Hydrocarbon utilizing bacteria were detected in samples TAS3, HSS4 and HSS4 which are samples from the main canal receiving effluents from Transamadi Industrial Layout, heavy duty engine servicing company and oil servicing industry respectively. By nature of the industries and the services they render, it is not surprising to find HUB in their wastewater. The presence of HUB in the wastewater is an indication of possible presence of hydrocarbon pollutant, which could suggest that these companies contribute to the hydrocarbon pollution of water bodies and their wastewater should be treated before discharge into the environment.

Twenty bacterial isolates with distinct morphological and microscopic characteristics where isolated from the eight sampling sites. Among the twenty bacterial isolates, only ten demonstrated resistance to the antibiotics tested. Gram reaction indicated that most of these organisms are gram positive rods of which eight were identified as species of *Bacillus* (*Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis, Bacillus thuringiensis, Bacillus altitudinis, Bacillus licheniformis* and *Alkaligenes* sp. 5-0-9). *Bacillus* species are common in the environment and this report supports the ubiquitous distribution of the genus. Becker *et al*. [18] similarly recovered members of *Bacillaceae* among the dominant bacterial families in oil refinery wastewater. The regulatory requirement for microbiological standard for wastewater to be discharged into a receiving water body is that it should not contain pathogens. It is known that some members of the genus *Bacillus* are pathogenic [19]. *Bacillus cereus* is a common food and water-borne pathogen [20, 21]. It is also an opportunistic pathogen that can cause infections in neonates and immunocompromised persons [22]. Although the isolate *Bacillus thuringiensis* is an opportunistic pathogens, their virulence could be boosted by the resistant genes they harbor.

Bacterial species under study exhibited varied responses to the antibiotic agents used which may be owing to adaption in their cellular metabolism and or physiology. Antibiogram results indicated that most of the gram positive cocci were sensitive to antimicrobial agents while gram positive rods indicated resistance. Wastewater are discharged into water ways and this medium serve as vehicle for the conveyance of the antibiotic resistant bacteria and genes in the environment [23, 24].

5. Conclusion

Bacterial species under study exhibited marked resistance to varied number of antibiotic agents which may be owing to adaption in their cellular metabolism. Also, identified *Bacillus* species suggests opportunistic pathogens which in normal situations pose self-limiting symptoms except in immunocompromised host. Except of course the R-genes confers on them increased or enhanced pathogenicity Vis-a –Vis virulence and thus, a cause for concern. This study reports the presence of antibiotic resistant bacteria in wastewater from industrial establishments in Port Harcourt,

Nigeria. Owing to the rising incidence of antibiotics resistance, wastewater should be adequately treated before discharge into the environment.

Compliance with ethical standards

Acknowledgments

Needs Assessment for Staff Development University of Agriculture, Makurdi. Benue State Nigeria.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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