

# Isolation and Identification of Resistant Bacteria from Petroleum Producing Vicinity by Gene Amplification and Sequencing

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## Authors' contributions

This work was carried out in collaboration among all authors. Author OO designed the study, author OA performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OA and OO managed the analyses of the study. Author OA managed the literature searches. All authors read and approved the final manuscript.

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## ABSTRACT

This investigation focuses on molecular identification of antibiotic resistant bacteria isolated from petroleum producing vicinity using 16S rRNA sequencing based technique. The bacterial 16S rRNA gene sequences were amplified using polymerase chain reaction, sequenced, characterized and compared by using primers which has been compared to national center for biotechnology information (NCBI) sequence database. The presence of the plasmid mediated antibiotic resistance determinants CTX-M and QNRB genes in the bacterial isolates were analyzed. A total of four bacterial isolates that were resistant to all the antibiotic agents used were identified molecularly. The BLAST results showed 100 % similarity and phylogenetic study indicated that the genes were evolutionarily related to *Morganella morganii*, *Pseudomonas xiamenensis*, *Chryseobacterium cucumeris* and *Staphylococcus sp.*, respectively. The genes obtained were submitted to the NCBI gene bank and were assigned accession number; MN094330, MN094331, MN094332 and MN094333, respectively. CTX-M and QNRB genes were however absent in the bacterial isolates. The result identified some peculiar abilities of the bacterial isolates to be resistant to antibiotics and suggests a correlation with resistance and

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hydrocarbon utilizing bacteria. The level of resistance could be as a result of the disinfection process during wastewater treatment procedure or the same adaptive mechanisms possessed by the isolates to control the hydrocarbon concentration in their cell. The study also clearly indicates that these wastewaters, when discharged into the environment directly may pose a risk for the spread of antibiotic resistant bacteria.

**Keywords:** 16s rRNA sequencing; blast; NCBI sequence database; molecular techniques; resistance.

## 1. INTRODUCTION

Technology based on PCR and DNA sequencing is considered a very sensitive tool for the precise identification of microorganisms [1]. To study bacterial phylogeny and taxonomy, the 16S rRNA gene sequences are very useful. With the gene presence in almost all bacteria, often existing as a multi gene family, or operons, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the 16S rRNA gene (1500bp) is large enough for informatics purposes [2]. With the use of 16S rRNA sequences, numerous bacterial genera and species have been reclassified and renamed; classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species have been facilitated [1].

Most recently, the environment is being recognized for its important role in the global spread of clinically relevant antibiotic resistance because environmental microorganisms are highly antibiotic resistant and harbor responsible genetic content. These organisms can enter the natural environment and share antibiotic resistance genes (ARGs) with indigenous bacteria through horizontal gene transfer, resulting in ARG migration through soil and aquatic environments, eventually altering the evolutionary direction of microbial communities in ecosystems [3]. Although antibiotic resistant bacteria and genes are found in different environments [3,4], most reported reservoirs of antibiotic resistance genes are from hospital [5], pharmaceutical [6,7], livestock farm [8], tannery wastewater [9]. In this present study, we isolated, amplified and molecularly characterized the antibiotic resistance of bacteria isolated from oil producing vicinity and also investigated the gene responsible for the resistance.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Samples

Samples were collected from the vicinity of an onshore oil production platforms located in

Ogba/Egbema/Ndoni local Government Area of Rivers State, Nigeria. The soil samples were collected at a depth of 0-15 cm with a clean auger into a sterile polythene bag. The oilfield wastewater samples were collected using 4 litre capacity sterile bottle. Samples collected were stored in an ice packed cooler.

100 g of the soil samples were enriched with various concentrations (10 %, 25 %, 50 % and 75 %) of oilfield wastewater and incubated in a rotary shaker. Samples were analyzed at 7 days interval.

### 2.2 Preparation of Enriched Soil Sample Inoculum

One gram (1 g) of the enriched soil samples was serially diluted onto 9 ml of sterile normal saline in a test tube to give an initial dilution of 1:10 ml ( $10^{-1}$  dilution). Subsequent dilutions were done up to  $10^{-3}$  dilution [10].

### 2.3 Isolation of Bacteria

Isolation of heterotrophic bacteria was done using Nutrient Agar by the spread plate technique as described by Prescott et al. [10]. Aliquots (0.1 ml) of serially diluted samples of  $10^{-2}$  dilution were spread plated onto dried sterile Nutrient Agar plates in duplicates. The plates were incubated at 37 °C for 24 hours. Representative colonies were selected based on their shapes, sizes, texture, opacity, chromogenesis, and were sub-cultured to purify them into pure isolates for characterization. The purified colonies represented the bacteria isolated from the enriched soil samples.

### 2.4 Antibiotic Sensitivity test

Ten milliliter (10 ml) of sterile Nutrient Broth was prepared; a loopful of the 18 hours old culture of the test organism on the Nutrient Agar plate was collected and inoculated into the broth to make a suspension. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard (suspension contains approximately  $1.5 \times 10^8$  cfu/ml), this was performed visually by using adequate light to visually compare the inoculum tube and the 0.5 McFarland standard

against a card with a white background and contrasting black line.

Fifteen minutes after adjusting the turbidity of the inoculum suspension, a sterile swab stick was dipped into the adjusted suspension, the dry surface of the Muller Hinton Agar was inoculated by streaking the swab over the entire sterile agar surface this was repeated twice to ensure even distribution of the inoculum. It was left for 15 minutes before applying the drug impregnated disk and incubated at 37 °C for 18-24 hours. After 24 hours, the plates were examined for inhibition and resistant categories according to the zone interpretation table [11]. The antibiotics and their different concentrations in the disc were; Ceftriaxone (30 µg), Erythromycin (10 µg), Gentamycin (10 µg), Ceftazidime (30 µg), Ofloxacin (10 µg), Augmentin (30 µg), Cefuroxime (10 µg), Ciprofloxacin (5 µg) and Nitrofurantion (100 µg). The presence of a growth inhibition zone larger than or equal to the established breakpoint diameter was an indication of susceptibility to that agent. The result interpretation was done using National Community for Clinical Laboratory Standards (NCCLS) zone interpretative criteria for susceptible (S) (diameter, ≥ 20 mm), resistant (R) (diameter, ≤ 17) or intermediate (I) (17-19 mm) [12].

## 2.5 Molecular Identification of Bacterial Isolates

Bacterial isolates that were resistant to the antibiotic sensitivity test were subjected to molecular identification using gene amplification and sequencing techniques. The 16S rRNA regions of the rRNA gene of the isolates were amplified after extraction and quantification of the DNA using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTACGACTT-3' primers on an ABI 9700 Applied Bio systems thermal cycler at a final volume of 40 ml for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5 µM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 °C for 5 minutes; denaturation at 95 °C for 30 seconds; annealing at 52 °C for 30 seconds; extension at 72 °C for 30 seconds for 35 cycles and final extension at 72 °C for 5 minutes. The product was resolved on a 1 % agarose gel at 130 V for 30 minutes and visualized on a blue light transilluminator.

The sequences of the isolates were done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye ® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were as follows 32 cycles of 96 °C for 10 seconds, 55 °C for 5 seconds and 60 °C for 4 minutes. The identified isolates were submitted to the Gene bank and accession numbers were assigned.

## 2.6 Determination of Plasmid mediated Antibiotic Resistance

CTX-M and QNRB genes from the isolates were amplified using the CTXM-F: 5'-CGCTTTGCGATGTGCAG-3' and CTXM-R: 5'-ACCGCGATATCGTTGGT-3', QNRB-F: 5'-GATCGTCAAAGCCAGAAAGG-3' and QNRB-R: 5'-CGATGCCTGGTAGTTGTCC-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 ml for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 µM and 50 ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 °C for 5 minutes, denaturation, 95 °C for 30 seconds; annealing 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72 °C for 5 minutes. The product was resolved on a 1 % agarose gel at 120 V for 25 minutes and visualized on a UV transilluminator for a 550 bp product size [13]. The primers were designed using the Primer-blast on NCBI and synthesized by Inqaba Biotechnology Ind, Pretoria, South Africa. The PCR reactions were controlled using an in-house *E. Coli* harbouring CTX-M and QNRA, B and S resistance gene.

## 3. RESULTS

Ten (10) discrete colonies identified according to their morphology were labeled 1-10 as shown in Tables 1 and 2. The Gram positive bacterium isolate numbered 1 in Table 1 were resistance to all the antibiotic agents used. The Gram negative labeled 5, 8 and 9 in Table 2 were resistance to all the antibiotic agents. A total of 4 bacteria were obtained as the most resistance bacteria.

The four (4) bacterial isolates that were all resistance to the antibiotic agents were subjected to molecular identification. Fig. 1 showed the

various bands in the agarose gel. The molecular ladder used had a base pair of 1000, while the bacteria labeled OA1 to OA4 showed 1500 base pair which represented the 16S rRNA gene bands.

showed 100 % relatedness to their relatives in the gene bank and were assigned accession numbers. The isolates labelled OA1, OA2, OA3 and OA4 were identified as *Morganella morganii* (MN094330), *Pseudomonas xiamenensis* (MN094331), *Chryseobacterium cucumeris* (MN094332) and *Staphylococcus* sp (MN094333), respectively.

Fig. 2 shows the phylogenetic tree and the evolutionary relationship of the individual isolates. The isolates labelled OA1 to OA4

**Table 1 Antibiotic sensitivity profile of gram positive bacteria isolated from soil treated with various concentrations of oilfield wastewater**

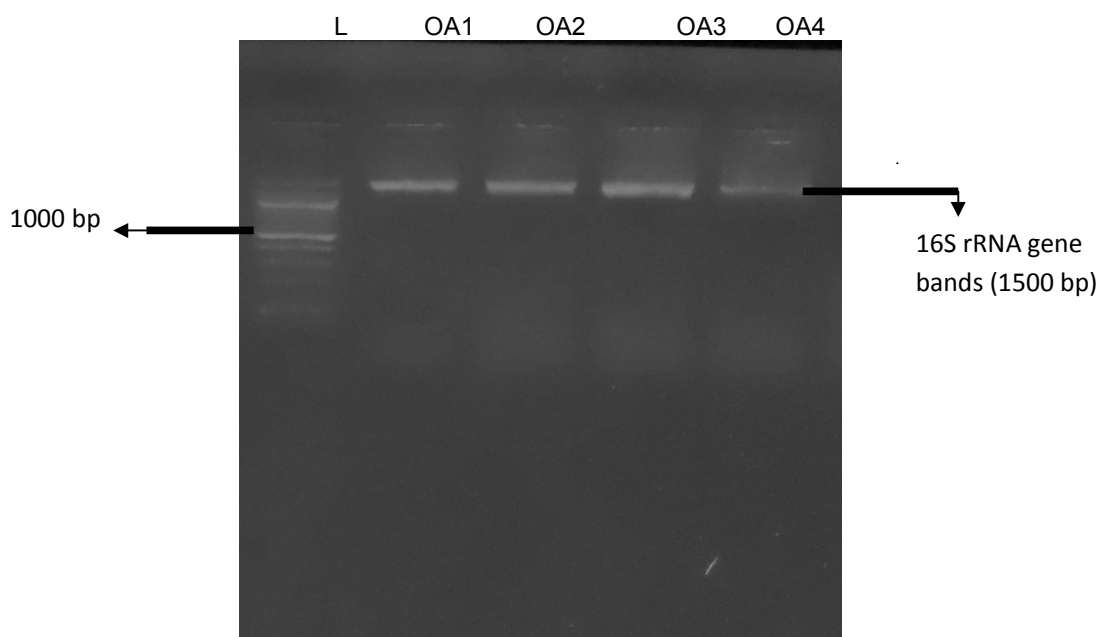
Bacterial Isolate	CTR (30 µg)	ERY (10 µg)	GEN (10 µg)	CAZ (30 µg)	OFL (10 µg)	AUG (30 µg)	CRX (10 µg)	CPR (5 µg)
1	R	R	R	R	R	R	R	R
2	S	S	R	S	R	S	S	R
3	R	S	S	S	R	S	R	R
4	S	R	S	R	S	R	S	R

KEY: R- Resistance, S- Sensitivity, I-Intermediate, CTR-Ceftriaxone, ERY-Erythromycin, GEN-Gentamycin, CAZ-Ceftazidime, OFL-Ofloxacin, AUG-Augumentin, CRX-Cefuroxime, CPR-Ciprofloxacin

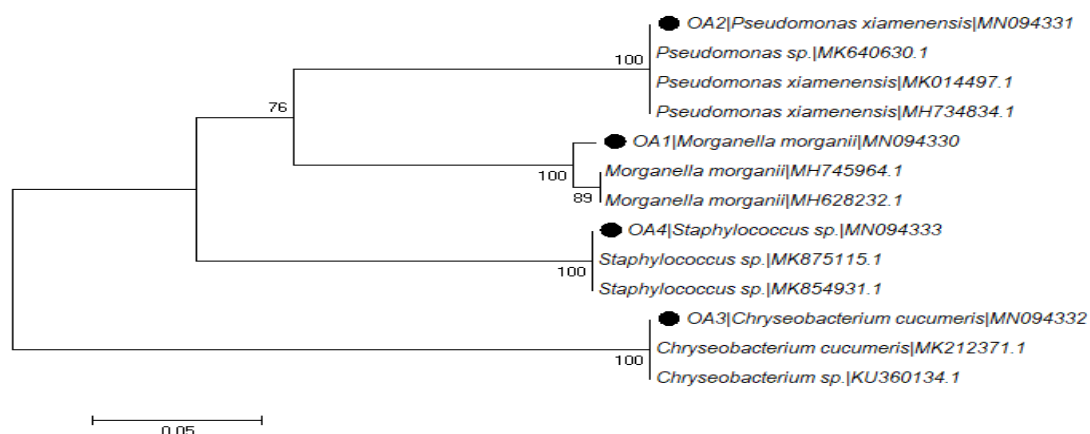
**Table 2 Antibiotic sensitivity profile of gram negative bacteria isolated from soil treated with various concentrations of oilfield wastewater**

Bacterial Isolate	NIT (100 µg)	AMP (30 µg)	GEN (10 µg)	CAZ (30 µg)	OFL (10 µg)	AUG (30 µg)	CRX (10 µg)	CPR (5 µg)
5	R	R	R	R	R	R	R	R
6	S	S	I	R	R	R	S	R
7	S	R	I	S	S	R	R	R
8	R	R	R	R	R	R	R	R
9	R	R	R	R	R	R	R	R
10	S	S	R	R	S	R	R	R

KEY: R- Resistance, S- Sensitivity, I-Intermediate, NIT-Nitrofurantoin, AMP-Ampicillin, GEN-Gentamycin, CAZ-Ceftazidime, OFL-Ofloxacin, AUG-Amoxycillin/Clavulanate, CRX-Cefuroxime, CPR-Ciprofloxacin



**Fig. 1. Agarose gel electrophoresis of the 16S rRNA gene of bacterial isolates**



**Fig. 2. Phylogenetic tree showing the evolutionary distance between the bacterial Isolates**

#### 4. DISCUSSION

Total of (ten) 10 bacteria were isolated from the soil samples treated with various concentration of oilfield wastewater. This shows that these organisms were able to survive the various concentrations of oilfield wastewater and its contents. All the isolates were tested for antimicrobial sensitivity while four (4) of the isolates were resistant to the entire antibiotic agent used. Other isolates also showed multiple drug resistance. The level of resistance among the isolates to the antibiotics suggests that the resistance genes could be widely present in the isolates found within the sample sites. This is in agreement with the findings of Rutgeron et al. [14], and Aleruchi et al. [15] that the elevated level of resistance found in the discharge vicinity is definitive evidence that selection for antibiotic resistance occurs in polluted environment. The research on antibiotics resistance in oilfield environmental samples is very limited. The bacteria that were most resistant were been identified using molecular methods. These methods are considered to be specific, sensitive, versatile and rapid for the identification of microorganisms at the species level [16]. Agarose gel electrophoresis showing the DNA bands of the individual isolates confirms the extraction of the DNA from the bacteria. Evolutionary relationship from the phylogenetic tree showed that the bacteria were 100 % related to their relatives in the gene bank. The bacteria identified include *Morganella morganii*, *Pseudomonas xiamenensis*, *Chryseobacterium cucumeris* and *Staphylococcus* sp. *Morganella morganii* is a known bacterium that is associated with infections in animals and with human animal bite wound infection, which suggests that they

may also cause zoonotic infectious diseases [17-21]. *Morganella morganii* is a well characterized opportunistic pathogen [22]. *Pseudomonads*, *Chryseobacterium* and *Staphylococcus* are well known and wide spread microorganisms that have been isolated from a variety of natural sources including soil, plants, waters and from clinical specimen. They are characterized by high level of metabolic diversity [23,24,25]. The bacteria were analyzed for their resistant gene using CTX-M and QNRB primers. According to Anjum [26], monitoring of resistance present in bacteria should not only be done from clinical samples but also in healthy humans, animals and the environment to have an understanding of the baseline levels of antimicrobial resistance present in these niches and the possible measures that can be taken to control its rise in the future. However the result did not show any positive resistance gene for CTX-M and QNRB in the bacteria. The reason could be that the primer used could not have targeted the gene the bacteria uses for antibiotic resistance. Another possible reason could be that the isolates were gotten from a non clinical environment, where the uses of antibiotics are not common. Isolates gotten from clinical environment are known to harbour resistant gene. Besides acquiring resistances to antibiotic, consistent stress on microbial cells could play important role to encourage acquiring resistance [27]. According to Wright [4], environmental resistome constituting large amount of resistant genes have the high potential to be transferred to pathogens and the evidences that at least some clinically relevant resistance genes have originated in environmental microbes have been reported. They may have used other form of mechanism described by Fernandez et al. [28]

and Yuan et al. [29], which could be intrinsic resistance. The authors described intrinsic resistance as the innate ability of a certain bacterial species to resist the activity of a particular antimicrobial agent through its inherent structural or functional characteristics and that the intrinsic insensitivity can be due to the lacking affinity of the drug for the bacterial target, inaccessibility of the drug into the bacterial cell or extrusion of the drug by chromosomally encoded molecules with active exportation activities. According to Cox and Wright [30], intrinsic resistance is maintained by a species independent of any antibiotic selective pressure and by definition, is not acquired by horizontal gene transfer (HGT). The risk of intrinsic resistance found in environmental microorganisms being transferred to pathogens is of major concern [30,31,32]. The positive control (in house *E. coli* strains) harboured a combination of CTXM and QNRB gene. Pre dominant CTXM and QNRB in *E.coli* have been reported by several researchers [33,34]. Plasmids with QNR genes have been found to co transfer CTXM genes. The association between QNRB like determinants and CTXM has been reported by Ruiz et al. [34].

The emergence and dissemination of the antibiotic resistance organisms and their respective genes and mobile elements in the environment could pose much higher risk to human health than previously expected [27].

## 5. CONCLUSION AND RECOMMENDATION

Bacteria associated with oilfield vicinity were isolated and identified molecularly. The bacteria demonstrated to have possessed high capability to withstand antibiotics which could be as a result of disinfection process in the wastewater treatment procedure or their constant exposure to toxicity from the environment. The level of resistance are of public health concern even though was from a non clinical environment.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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