



# **Antibiotic Susceptibility of Resistant *Pseudomonas* Species Generated Through the Use of Differential and Selective Media for Isolation of *Pseudomonas* from Environmental Samples**

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## **Authors' contributions**

*This work was carried out in collaboration between both authors. Author PLP designed the study, carried out literature searches, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author WQC carried out the laboratory work and managed the analyses in the study. Both authors read and approved the final manuscript.*

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## **ABSTRACT**

Water and soil samples were cultured on Cetrinide agar, Kings B agar, and nutrient agar supplemented with 50 µg.mL<sup>-1</sup> chloramphenicol for the isolation of greenish pigment producing *Pseudomonas* species. Greenish pigment producing colonies that grew on the media were subjected to microscopic examination and selected physicochemical/biochemical tests to confirm that they belong to the *Pseudomonas* genus. Isolates confirmed to belong to the *Pseudomonas* genus were subjected to antibiotic susceptibility testing to various antibiotics using the disc diffusion method. The antibiotic sensitivity testing showed that two of the *Pseudomonas* isolates from nutrient agar plates supplemented with chloramphenicol showed resistance to Ampiclox, Rifampicin, and Norfloxacin. Also, one of the isolates showed resistance to Gentamycin, Amoxicillin, and Amoxicillin clavulanate. Two of the isolates from Kings B medium showed resistance to Ampiclox; another isolate showed resistance to Rifampicin, and one of the isolate that

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showed resistance to Ampiclox also showed resistance to Rifampicin, Chloramphenicol and Norfloxacin. Only one isolate from Cetrimide agar showed resistance to Ofloxacin and Nalidixic acid. Based on the EUCAST Breakpoint Table for *P. aeruginosa*, 12 out of the 14 *Pseudomonas* isolates (85.7%) were susceptible to Levofloxacin and Gentamycin, while 8 (57.1%) of the isolates were susceptible to Ciprofloxacin. The results indicate that *Pseudomonas* species isolated from the environment through the use of differential and selective media can become resistant to some antibiotics, and that Levofloxacin and Gentamycin could be used in the treatment of infections caused by antibiotic resistant *Pseudomonas*.

**Keywords:** *Cetrimide agar; Kings B agar; nutrient agar supplemented with chloramphenicol; greenish pigment producing Pseudomonas species; antibiotic resistance.*

## 1. INTRODUCTION

Certain species of *Pseudomonas* are of environmental and industrial importance due to their diverse metabolic capabilities and ability to produce extracellular materials. *Pseudomonas* species can be used in industrial processes such as enhanced oil recovery [1,2]. They can also be used in clean up of polluted environments due to their ability to degrade wide range of compounds [3,4,5].

Isolation of *Pseudomonas* species from the environment, plants, and clinical specimens are often achieved with the use of differential or selective media. These media are able to differentiate between colonies of *Pseudomonas* species and colonies of other bacteria species based on the ability of some species of *Pseudomonas* to produce greenish pigments. *P. aeruginosa* produces bluish green or yellowish green pigments [6,7], depending on the strain. Bluish green pigment production has been regarded as unique to *P. aeruginosa* [8]. Some other strains of *P. aeruginosa* can produce reddish brown or brown pigment [9]. *P. fluorescens* and *P. putida* produce yellowish green pigments [8,10,11]. Pigment production by *Pseudomonas* species occur in the presence of antibiotics [12,13], in the presence of certain quantities of sulphate and magnesium salts [14], or at low iron concentrations [11]. These facts are made use of in the preparation of culture media for the isolation of *Pseudomonas* species. Culture media that have been used in the differential and selective isolation of greenish pigment producing *Pseudomonas* species include cetrimide broth and Pseudocel agar [15], modified MacConkey agar developed by Daly et al. [7], Tech agar, *Pseudomonas* agar P and *Pseudomonas* agar F [16], *Pseudomonas* CN selective agar and *Pseudomonas* chromogenic medium [17], asparagine broth enriched with  $K_2HPO_4$  and  $MgSO_4 \cdot 7H_2O$  [18], and nutrient agar supplemented with chloramphenicol [13].

Growth of *Pseudomonas* species in the presence of substances that stimulates them to produce greenish pigments may lead to the development of antibiotic resistance in them. For instance, a greenish pigment producing *Pseudomonas* species isolated from media incorporated with antimicrobial agents for the cultivation of algae was reported to be resistant to fourteen antibiotics [19]. Information regarding the susceptibility patterns of *Pseudomonas* species isolated through the use of differential and selective media will be important to clinicians in selecting an appropriate antibiotic regimen for the timely treatment of infections caused by the bacteria in case they escape from the laboratory. Early treatment with known effective antibiotic(s) will reduce the probability of the emergence of resistant mutants. The aim of this study is thus to determine the antibiotic susceptibility profile of greenish pigment producing *Pseudomonas* species isolated from environmental samples that have become antibiotic resistant as a result of using differential and selective media in their isolation.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Samples for Isolation of *Pseudomonas* Species

Water and soil samples were used for the study. The water samples were collected from the Eagle island river located behind the Rivers State University, Nigeria; the samples were collected at about 2 m from the shoreline at a depth of 0 – 30 cm using 500 mL sterile water sampling bottles. The soil samples were collected from a crude oil polluted site in Bodo community, Gokhana LGA, Rivers State; the samples were collected with the aid of disinfected hand trowel and wide mouth amber bottles of about 50 mL capacity. The samples were taken to the Microbiology laboratory of the Rivers State University for bacteriological analysis.

## 2.2 Isolation of *Pseudomonas* Species Using Differential and Selective Media

The differential and selective media used in culturing the samples for isolation of greenish pigment producing *Pseudomonas* species include Cetrimide agar, Kings B agar, and nutrient agar supplemented with 50 µg.mL<sup>-1</sup> chloramphenicol (N50C). The media were all prepared according to the manufactures instructions. In preparation of N50C, sterilised molten nutrient agar was allowed to cool to about 50°C before addition of a predetermined volume of a 1000 µg.mL<sup>-1</sup> stock solution of the antibiotic so as to achieve the targeted concentration (50 µg.mL<sup>-1</sup>). The predetermined volume was derived using the equation  $M_1V_1 = M_2V_2$  [20], Where  $M_1$  is the concentration of the antibiotic stock solution,  $V_1$  is the volume of the antibiotic stock solution required,  $M_2$  is the antibiotic concentration specified for the nutrient agar medium, and  $V_2$  is the volume of the nutrient agar medium to be prepared.

The samples were serially diluted through a tenfold serial dilution process to 10<sup>-4</sup>. About 0.1 mL of the different dilutions was then plated separately on plates of Cetrimide agar, Kings B agar, and N50C. Inoculated plates were incubated at ambient temperatures (28 – 32°C) for a minimum of 48 hours. After incubation, greenish pigment producing colonies that grew on the plates were sub-cultured unto sterile nutrient agar plates from whence there stock cultures were prepared.

## 2.3 Identification of Greenish Pigment Producing Isolates

The greenish pigment producing isolates were subjected to the Gram staining and microscopic examination, and the following physicochemical/biochemical tests: catalase, oxidase, motility, citrate utilisation, indole production, Methyl Red-Vogues Proskauer (MR-VP), blood haemolysis, casein hydrolysis, lecithinase production, and fermentation tests using glucose, lactose, maltose, sucrose, mannitol, xylose, starch, and glycerol.

## 2.4 Antibiotic Susceptibility Testing of the Greenish Pigment Producing Isolates

The susceptibility of the greenish pigment producing isolates to various antibiotics was determined using the disc diffusion method. The antibiotics used and the quantities in the disc are

as follows: Ciprofloxacin – 10 µg, Chloramphenicol – 30 µg, Erythromycin – 30 µg, Levofloxacin – 20 µg, Gentamycin – 10 µg, Ampiclox – 20 µg, Rifampicin – 20 µg, Amoxicillin – 20 µg, Streptomycin – 30 µg, Norfloxacin – 10 µg, Septrin – 30 µg, Ampicillin – 30 µg, Cefalexin – 10 µg, Ofloxacin – 10 µg, Nalidixic acid – 30 µg, and Amoxicillin clavulanate – 30 µg.

Broth cultures of the isolates were first prepared by separately inoculating a colony of the isolates into 10 mL sterile nutrient broths, which were then incubated at ambient temperature (28 – 32°C) for about 18 hours. After incubation, the broth cultures were adjusted to 0.5 McFarland turbidity standard through absorbance measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set at 600 nm and addition of sterile normal saline. The adjusted broth cultures were then plated separately, in duplicate, onto sterile nutrient agar plates with the aid of sterile swab sticks. Antibiotic discs (Optudisc; Optun laboratories Nigeria, Ltd) were then placed on the inoculated plates with the aid of sterile forceps. The plates were incubated at 37°C for 24 hours, after which the zones of inhibition around the discs were measured and compared with EUCAST Breakpoint Table [21].

## 3. RESULTS

### 3.1 Greenish Pigment Producing Bacterial Population as Assessed Using the Differential and Selective Media

The bacterial population of the samples as assessed using the differential and selective media is shown in Table 1a – 1c. From the Tables it can be seen that the highest proportion of bacterial population producing greenish pigment was obtained with the use of Cetrimide agar.

**Table 1a. Bacterial population as assessed using Cetrimide agar**

SC	TBP (CFU/mL)	GPB (CFU/mL)	PBG (%)
RG	$5.10 \times 10^4$	$3.90 \times 10^4$	76.47
S1	$1.07 \times 10^4$	$1.07 \times 10^4$	100.00
S2	$3.60 \times 10^4$	$3.00 \times 10^4$	83.33

SC – sample code, TBP – total bacterial population, GPB – greenish pigment producing bacterial population, PBG – percentage of bacterial population producing greenish pigment,  $PBG = \frac{GPB \times 100}{TBP}$

**Table 1b. Bacterial population as assessed using Kings B agar**

SC	TBP (CFU/mL)	GPB (CFU/mL)	PBG (%)
RG	$1.02 \times 10^5$	$4.00 \times 10^3$	3.92
S1	$1.33 \times 10^8$	$3.00 \times 10^6$	2.26
S2	$1.15 \times 10^8$	$2.50 \times 10^6$	2.17

**Table 1c. Bacterial population as assessed using N50C**

SC	TRP (CFU/mL)	GPB (CFU/mL)	PBG (%)
RG	$1.40 \times 10^3$	$2.0 \times 10^2$	14.29
S1	$4.44 \times 10^5$	$5.0 \times 10^4$	11.26
S2	$4.60 \times 10^5$	$1.5 \times 10^4$	3.26

TRP – total chloramphenicol resistant bacteria,

$$PBG = \left( \frac{GPB \times 100}{TRP} \right)$$

### 3.2 Identification of the Greenish Pigment Producing Bacteria

A total of fourteen greenish pigment producing bacterial (GPPB) isolates were selected from the three different media used (Cetrimide, Kings B, and nutrient agar supplemented with chloramphenicol), and coded according to the

names of the media used for their isolation as follows: CM1, CM2, CM3, CM4, CM5, K1, K2, K3, K4, K5, CH1, CH2, CH3, and CH4. All the isolates were Gram negative rods. Results generated from the physicochemical/biochemical tests carried out on the isolates is presented in Table 2a – 2b. From the Table it can be seen that all the isolates exhibited almost the same reaction pattern.

### 3.3 Susceptibility of the *Pseudomonas* Isolates to Various Antibiotics

Inhibition of growth of the selected *Pseudomonas* isolates by various antibiotics is presented in Fig. 1a – 1d. From the figures it can be deduced that the isolates from plates of N50C were less susceptible to some of the antibiotics than the other isolates from Cetrimide or Kings B agar medium.

## 4. DISCUSSION

In sourcing for *Pseudomonas* species, samples or specimens are cultured on certain differential or selective media. Culturing samples on these differential or selective media for the isolation of *Pseudomonas* species can lead to the

**Table 2a. Physicochemical/biochemical characteristics of selected GPPB isolated through the use of Kings B and N50C**

	K1	K2	K3	K4	K5	CH1	CH2	CH3	CH4
Ctl	+	+	+	+	+	+	+	+	+
Oxd	+	+	+	+	+	+	+	+	+
Mtl	+	+	+	+	+	+	+	+	+
CtU	+	+	+	+	+	+	+	+	+
Ind	-	-	-	-	-	+	-	-	-
VP	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-
HBA	β-H	β-H	β-H	β-H	β-H	β-H	β-H	β-H	β-H
CsH	+	+	+	+	+	+	+	+	+
LcP	-	+	+	+	+	+	+	+	+
GluF	A	A	A	A	A	A	A	A	0
LtF	0	0	0	0	0	0	0	0	0
XsF	0	A	A	A	A	A	0	0	A
MalF	0	0	0	0	0	0	0	0	0
MntF	0	A	A	A	A	A	0	0	A
SucF	0	0	0	0	0	0	0	0	0
StaF	A	0	0	0	0	A	0	0	0
GlyF	A	A	A	A	A	A	A	0	0

GPPB - greenish pigment producing bacteria, Ctl – catalase, Oxd – oxidase, Mtl – motility, CtU – citrate utilisation, Ind – indole, VP – Vogues Proskauer, MR – Methyl red, HBA – haemolysis on blood agar, β-H – beta haemolysis, CsH – Casein hydrolysis, LcP - lecithinase production, GluF – Glucose fermentation, LtF – Lactose fermentation, XsF – Xylose fermentation, MalF – Maltose fermentation, MntF – Mannitol fermentation, SucF – Sucrose fermentation, StaF – Starch fermentation, GlyF – Glycerol fermentation, A – only acid produced, 0 – no reaction

emergence of multidrug resistant *Pseudomonas*. Knowing the antibiotics that are still effective against such multidrug resistant *Pseudomonas* species will aid clinicians in making empirical decisions in the treatment of infections caused by such bacteria.

**Table 2b. Physicochemical/biochemical characteristics of selected GPPB isolated through the use of Cetrimide agar**

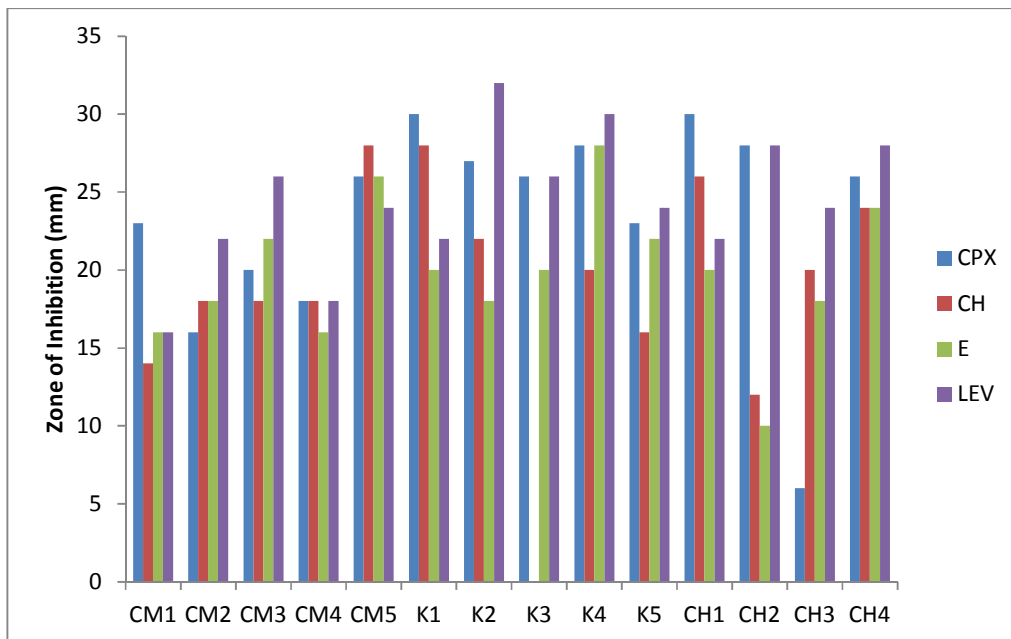
	CM1	CM2	CM3	CM4	CM5
Ctl	+	+	+	+	+
Oxd	+	+	+	+	+
Mtl	+	+	+	+	+
CtU	+	+	+	+	+
Ind	-	-	-	-	-
VP	-	-	-	-	-
MR	-	-	-	-	-
HBA	β-H	β-H	β-H	β-H	β-H
CsH	+	+	+	+	+
LcP	+	+	-	+	+
GluF	A	A	A	A	A
LtF	0	0	0	0	0
XsF	A	A	0	A	A
MalF	0	0	0	0	0
MntF	A	A	0	A	A
SucF	0	0	0	0	0
StaF	0	0	A	0	0
GlyF	A	A	A	A	A

In this research, the efficacy of sixteen antibiotics against *Pseudomonas* species isolated through the use of Cetrimide agar, Kings B agar medium, and nutrient agar supplemented with 50 µg/mL chloramphenicol was investigated. Greenish pigment producing colonies that grew on these media were initially suspected to be *Pseudomonas* species. The greenish pigment producing isolates were confirmed to belong to the *Pseudomonas* genus from the results obtained from the microscopic examination and physicochemical/biochemical tests. They were all positive for catalase, oxidase, motility, citrate utilisation, and casein hydrolysis; negative to Vogues Proskauer, Methyl red, lactose fermentation, maltose fermentation, and sucrose fermentation tests; and they all produced beta haemolysis on blood agar. These reaction patterns are exhibited by many species of *Pseudomonas* as confirmed from selected literature and text books [3,22,23,24].

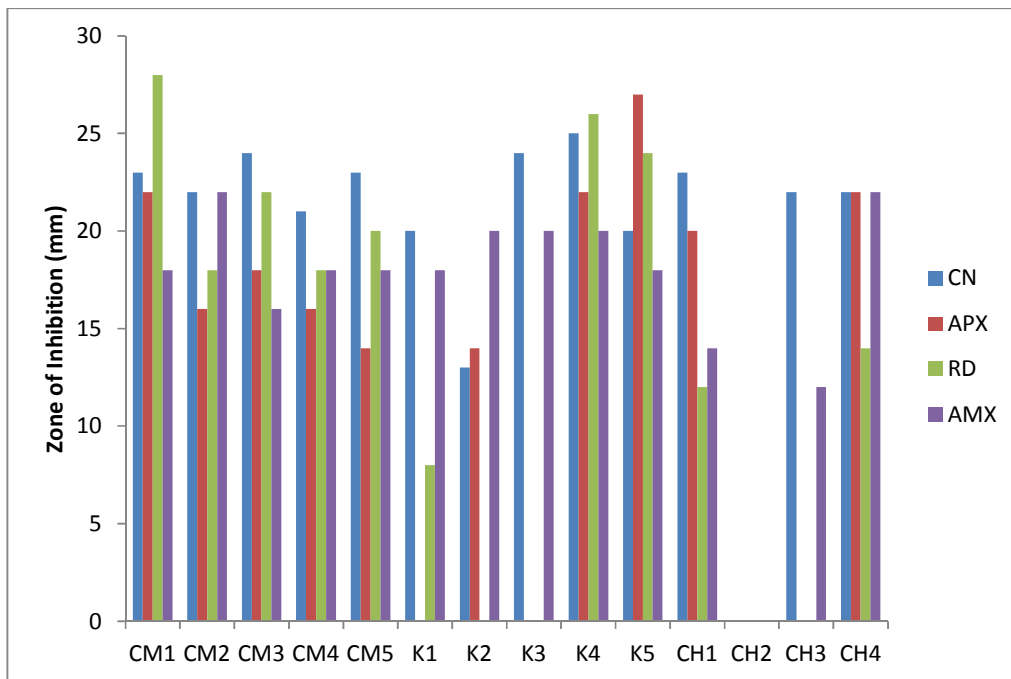
The results of the antibiotic sensitivity testing carried out on the various *Pseudomonas* isolates (Fig. 1a – 1d) showed that two isolates (CH2 and CH3) from nutrient agar plates

supplemented with chloramphenicol showed resistance to Ampiclox, Rifampicin, and Norfloxacin; there were no zones of inhibition for the antibiotics against the isolates. The isolate CH2 also showed resistance to Gentamycin, Amoxicillin, and Amoxicillin clavulanate. Two of the isolates (K1 and K3) from Kings B medium showed resistance to Ampiclox; another isolate (K2) showed resistance to Rifampicin. Also, isolate K3 showed resistance to Rifampicin, Chloramphenicol and Norfloxacin. Only one isolate (CM3) from Cetrimide agar showed resistance to Ofloxacin and Nalidixic acid. From these observations, it can be seen that *Pseudomonas* species isolated from the environment using a differential or selective media expresses some level of resistance to some antibiotics. This is in agreement with the work of Peekate and Abu [19] in which it was shown that culturing samples on a special medium supplemented with Chloramphenicol and Nystatin for the selective isolation of algal cells led to the emergence of antibiotic resistant bacteria of which a species of *Pseudomonas* was among them. In another related study, culturing samples in media having sub-lethal concentration of antibiotics has been shown to elicit multidrug resistance in *Pseudomonas* species and other bacteria species [25].

The selective media Cetrimide (or Pseudosel) agar contains 0.03% of the inhibitory agent Cetrimide (cetyl trimethyl ammonium bromide). Addition of this inhibitory agent at 0.03% to Mueller-Hinton agar has been shown to make *Pseudomonas aeruginosa* succumb readily to several antimicrobial agents [26]. In this research, only one isolate (CM3) from Cetrimide agar showed resistance to Ofloxacin and Nalidixic acid. It may thus be agreed that culturing samples on Cetrimide agar for the isolation of *Pseudomonas* do not readily lead to the generation of resistance in *Pseudomonas*. However, this is not so with Kings B medium; three of the isolates from Kings B medium showed resistance to one or more antibiotics. One of the isolate (K3) showed resistance to four antibiotics inclusive of broad spectrum antibiotics. Kings B medium employs the fact that some species of *Pseudomonas* produces pyoverdine (a yellowish green fluorescent siderophores) in iron deficient environment [27]. Production of pyoverdine/siderophores endows *Pseudomonas* species with some level of virulence [14]. This may in turn make the bacterium resistant to some antibiotics, as shown in this study.



**Fig. 1a. Susceptibility of the isolates to Ciprofloxacin (CPX), Chloramphenicol (CH), Erythromycin (E), and Levofloxacin (LEV)**



**Fig. 1b. Susceptibility of the isolates to Gentamycin (CN), Ampiclox (APX), Rifampicin (RD), and Amoxicillin (AMX)**

The antibiotic(s) having the highest zone of inhibition against CM3 was Levofloxacin (26 mm), against K1 was Ciprofloxacin (30 mm), against K2 was Levofloxacin (32 mm), against K3 were Ciprofloxacin, Levofloxacin, and Septrin (26 mm), against CH2 were Ciprofloxacin and

Levofloxacin (28 mm), and against CH3 was Ampicilin (28 mm). Comparing the zones of inhibition of these antibiotics against the isolates

with EUCAST (European Committee on Antimicrobial Susceptibility Testing) Breakpoint Table for *Pseudomonas aeruginosa* confirms that

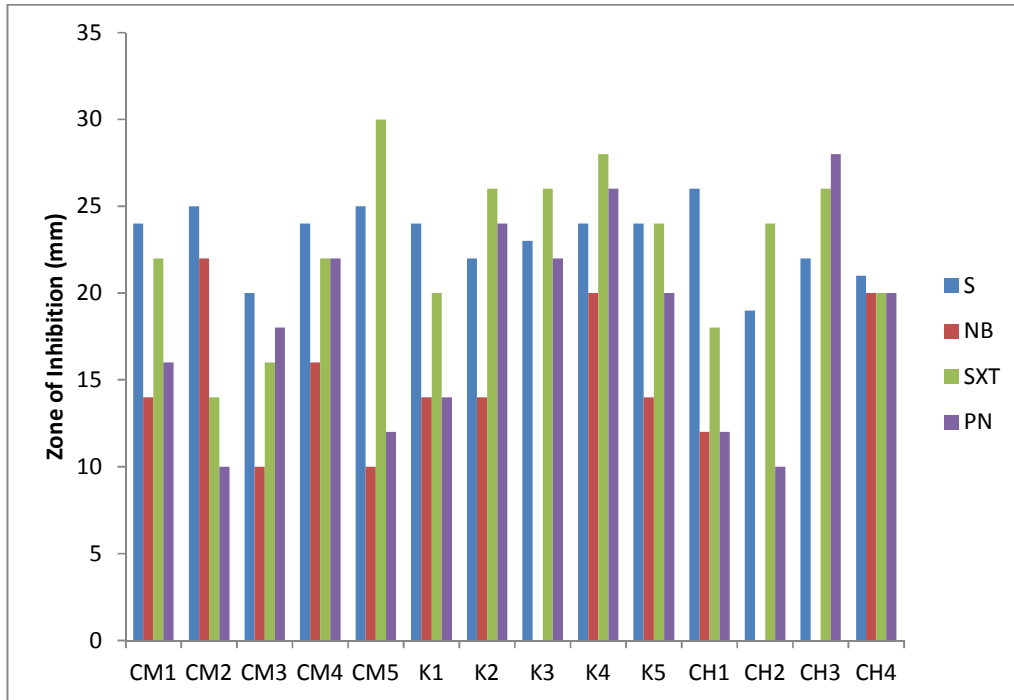


Fig. 1c. Susceptibility of the isolates to Streptomycin (S), Norfloxacin (NB), Seprin (SXT), and Ampicilin (PN)

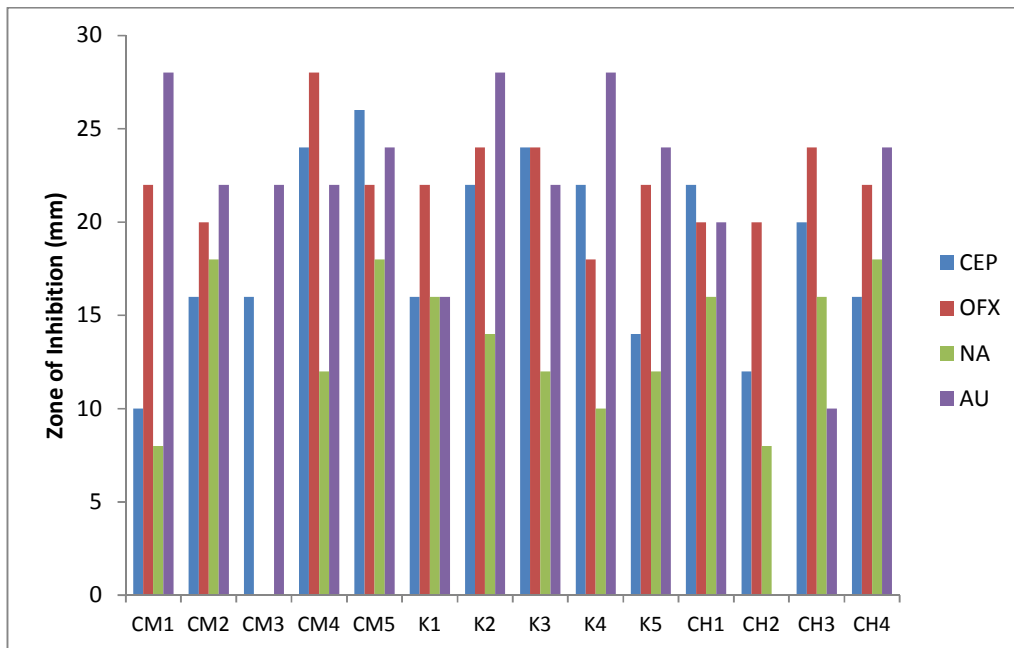


Fig. 1d. Susceptibility of the isolates to Cefalexin (CEP), Ofloxacin (OFX), Nalidixic acid (NA), and Amoxicillin clavulanate (AU)

these isolates were susceptible to the antibiotics. Based on the EUCAST Breakpoint Table for *P. aeruginosa*, 12 out of the 14 *Pseudomonas* isolates (85.7%) were susceptible to Levofloxacin and Gentamycin, while 8 (57.1%) of the isolates were susceptible to Ciprofloxacin. This is an indication that Levofloxacin and Gentamycin could be used in the treatment of infections caused by antibiotic resistant *Pseudomonas*. Levofloxacin has been shown to have an excellent bactericidal activity against *P. aeruginosa* [28]. On the other hand Gentamycin have been demonstrated to suppress the re-growth of *P. aeruginosa* after reducing its population [29]. The suppression was shown to occur in a concentration dependent manner, and a thrice daily dosing against the pathogen was recommended for this antibiotic.

In this study it has been shown that *Pseudomonas* species isolated from the environment through the use of differential and selective media can become resistant to some antibiotics. It is also shown in this work that Levofloxacin and Gentamycin is effective against such *Pseudomonas* species isolated using such media.

## 5. CONCLUSION

In this work it has been shown that Levofloxacin and Gentamycin are effective against *Pseudomonas* species isolated from environmental samples through the use of differential and selective media. This implies that Levofloxacin or Gentamycin could be used in the treatment of infections caused by antibiotic resistant *Pseudomonas* species generated from the use of differential and selective media in their isolation. Future work can be carried out to determine the minimum inhibitory concentration of Levofloxacin and Gentamycin against resistant isolates of *Pseudomonas* and other bacterial species.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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