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Molecular Detection of Virulence Genes Associated With Multi- Drug Resistant Pseudomonas Aeruginosa Isolated from Clinical and Environmental Samples within Maiduguri

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Abstract: ***Pseudomonas aeruginosa is an ubiquitous opportunistic pathogen having numerous virulence factors and the ability to acquire multidrug resistant traits. This study aimed to determine the presence of the virulence genes algD and lasB in multidrug resistant Pseudomonas aeruginosa isolated from clinical and environmental samples within Maiduguri. A total of 200 samples were collected from four sources namely; Farm soil at root region of groundnut, abattoir waste water, commercial boreholes and urine of urinary tract infected patients. All positive growths on cetrimide agar were further identified biochemically. Antibiotic profile of confirmed Pseudomonas aeruginosa was determined using Kirby-Baeur disc diffusion method. The genes algD and lasB were detected in the multidrug resistant isolates. The result also showed that 90.90%, 81.81% and 81.81% of the urine isolates were resistant to nalidixic acid, chloramphenicol and tetracycline respectively while highest sensitivity was observed against amikacin at 81.81%. Highest resistance among environmental samples was observed in abattoir wastewater with 95.24% resistance to chloramphenicol while least resistance was observed in borehole isolates with 0.00% resistance to Gentamicin, Amikaicin, Meropenem and Aztreonam. According to the findings of this study also, there is no significant difference in habouring***

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virulence genes among the isolates of the different sources. All the multidrug resistant isolates were found to habour both algD and LasB genes. In conclusion, P. aeruginosa was found in all of the studied sources and widespread of algD and LasB genes in the multidrug resistant isolates from all the sources.

Keywords: Pseudomonas Aeruginosa, Virulence, Multi Drug Resistance, Genes.

1. INTRODUCTION

Pseudomonas aeruginosa, a widely distributed bacterium is gram negative and belongs to the Pseudomonadaceae family. It has the ability to thrive in various settings and habitats (Silby et al., 2011). Compared to other bacteria such as Escherichia coli, Mycobacterium tuberculosis, and Bacillus subtilis, the size of the genome of P. aeruginosa is quite larger, ranging from 5.5 to 7 million base pairs (mbp). Within this genome, a significant portion is dedicated to encoding regulatory enzymes that play crucial roles in transportation, metabolism, and organic compounds removal. This expanded coding capacity of the P. aeruginosa genome grants it a greater degree of metabolic flexibility and the tendency to adapt well to the flactuations in its environment (Stover et al., 2000; Klockgether et al., 2011). Pseudomonas aeruginosa which is widely known as an opportunistic pathogen, is recognized as the predominant bacterium associated with hospital- acquired infections (Barbier et al., 2013). It has a minimal impact on people who are in good health, but it poses a significant risk of sickness and death for individuals with weakened immune systems (Sadikot et al., 2005). P. aeruginosa is capable of producing alginate (algD), and exopolysacccharide, and elastase B (LasB) enzyme when exposed to specific environmental conditions. Empirical treatment options for unconfirmed cases of P. aeruginosa infection include both single- drug therapy and combination therapy. This approach to treatment has been shown to decrease the death rate in patients suffering from severe P. aeruginosa infections. (El Solh and Alhajhusain, 2009; Park et al., 2012). Nevertheless, tackling P. aeruginosa infections becomes increasingly difficult because of the tendency of the bacterium to develop resistance against numerous existing drugs (Lister et al., 2009).

2. RELATED WORKS

Based on the isolation of Pseudomonas spp, many studies conducted within the study location (Borno State), North eastern States (Adamawa, Bauchi, Taraba, Yobe and Gombe States) and other regions (North central; Jos, Niger and Kwara States and North west; Kaduna, Kano, Katsina and Sokoto States) have all reported the isolation of Pseudomonas spp in diverse settings to include but not only clinical settings (from patients' specimens ranging from urine, stool and other swab samples to hospital surfaces and equipments such as door handles, bed surfaces, surgical blades, hospital water supplies and waste water outlets) and different environmental sources such as but not restricted to boreholes, municipal water supplies, and wastewater outlets, abbatoir wastewater, farm soil and other community sources (Kerr and Snelling, 2009). Many studies have demonstrated how the improper management of abattoir waste leads to the contamination of residential areas. The detrimental

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effects of these waste effluents on the surrounding environment are often underestimated, causing pollution both locally and in neighboring regions (Akinro et al., 2009). According to a research conducted by Chika et al. (2016), of the 360 samples collected and analysed from abattoir in Abakaliki, 147 (40.8%) positive isolates for Pseudomonas aeruginosa has been found. Other research on the resistance of P. aeruginosa to gentamacin revealed 14.3% by Iroha et al (2015) are resistant to gentamacin. Pseudomonas aeruginosa from urine demonstrated 90.90% resistance to nalidixic acid which corresponds to the 88% resistance to nalidixic acid obtained by Isichei-Ukah and Enabulele, (2018) in Benin City. The clinical isolates sensitivity to imipenem (91%), amikacin (78%), ceftazidime (75%) and meropenem (72%) from the findings of Isichei-Ukah and Enabulele, (2018) in Benin City closely agree to the result of this study that showed 72%, 82%, 55% and 82% resistance respectively. According to the findings of this study there is low resistance to ciprofloxacin (27.27%) which tallies slightly with the findings of Okon et al. (2009) conducted in the University of Maiduguri Teaching Hospital resulting in 30.2% resistance to ciprofloxacin. Based on the presence of AlgaD, Wa'ad (2011) in his research revealed the presence of algD gene in all of the Psueudomonas aeruginosa tested. This finding is also in line with the findings of Lomholt et al. (2001) in 141 Pseudomonas aeruginosa which showed apr and LasB genes were universally present and also agrees to the work of Mitov et al. (2010) that portrayed vast dessimination of algD and LasB genes among 202 Pseudomonas aeruginosa taken from cystic fibrosis. Lanotte et al. (2004) also found widespread of these genes from clinical samples.

3. METHODOLOGY

The Study Area

The study was carried out in Maiduguri, the capital and biggest town in Borno State, in North Eastern Nigeria. The town is located along the seasonal Ngadda River that fades into the Firki swamps at regions close to Lake Chad (Encyclopedia Britanica, 2019) with the total area of 105.5 km^2 and of 320 m elevation. The city has the Coordinates of 11 ^{o}50'N 13 ^{o}09'E (Britanica, 2019).

Sample Size Determination

Using the prevalence rate of 2.1% reported by Okon et al. (2009), in a research carried out on prevalence of resistant P. aeruginosa in urine at University of Maiduguri Teaching Hospital, the sample size was calculated employing the formular below

$$n = \frac{t^2 \times P(1-P)}{D^2}$$

Where n= sample size
t = 1.96 for Confidence level at 95% (1.96^2=3.8416)
P = Prevalence rate at 3% (this is to be converted by dividing the p value by 100. =2.1/100=0.021) and
D = 0.05 for Marginal error at 5% (D^2=0.0025).
The sample size for urine sample was obtained as 31.59, but rounded up to 50 for better

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precision.

Samples Collection

For soil sample**,** fifty different soil samples at root regions of leguminous plant (Ground nut) were collected from 5 randomly selected farmlands (10 samples from each source): Dala Alemderi, Bulumkuttu Abuja, Bakassi, Ngomari and Bintu Sugar. Five grams of soil samples were collected in a sterile polythene bag.

For abattoir waste water sample, samples were taken on a weekly basis inside 50 mL sterile universal containers. The waste water samples were taken at different points in the abattoir like the drainages and still water within the environment of the abattoir, and containers were labeled accordingly. The samples were taken between March and July, 2022. The collected samples were taken inside a container containing cold ice.

For borehole water samples, total of 50 water samples were taken at 5 commercial boreholes at Moduganari, Bulumkutu Yan Doya, Dala Alemderi, Abuja Sheraton and Kulollori. The taps were allowed to flow freely for few minutess before the samples were collected in a sterile container in the morning, afternoon and evening. The collected samples were taken in cold ice.

For urine sample collection, a total of 50 urine samples were collected from patients of urinary tract infections at Fatima Ali Sheriff Hospital Maiduguri. The collected samples were properly labeled and placed inside sterilized vials on cold ice pack.

All samples were taken to the Microbiology Laboratory, Department of Microbiology, Faculty of Science, University of Maiduguri.

Identification of Pseudomonas Aerugunosa

The cultured samples were observed for growth on Centrimide agar and their characteristic morphological appearance on Blood and MacConkey agar. Gram stain was conducted using standard protocol to further confirm P. aeruginosa as gram negative bacilli. The biochemical Tests like oxidase, catalase, indole, coagulase, Triple sugar iron agar tests were conducted and growth at 42^{o}C together with the release of blue phenazine pigment (pyocyanin) on cetrimide agar were observed for further confirmation of P. aeruginosa.

Gram Staining

A suspension of the sample was smeared on a greese-free slide. The smear was air dried and passed over heat to fix it. Crystal Violet was poured then allowed to stay for 30 seconds and washed off using water. Gram's iodine was flooded for 1 minute and then rinsed using water. The smear was then washed with 95% alcohol for about 10-20 seconds and rinsed with water. Finally, the smear was overloaded with a counter stain safranin for 1 minute and rinsed with water. The slide was then bloated, air dried and viewed under a microscope using oil emersion objective and the results were recorded (CMP, 2016).

4. RESULTS AND DISCUSSION

Isolation and Identification of Pseudomonas Aeruginosa from Clinical and Environmental Samples

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According to this research, the result revealed P. aeruginosa to have a characteristic rod shape appearance and is gram negative after gram staining and viewing under the microscope. On nutrient agar plates, the organism appeared as large, opaque and smooth colonies with flat edges and raised centre with fruity odour. The colonies from urine appeared mucoid and larger than environmental isolates. On Blood agar, P. aeruginosa exhibit β hemolysis expressed as clear zones around the colonies with metallic sheen. The organism formed colorless colonies on MacConkey agar, indicating that the organism is a non-lactose fermenter.

Out of the total number of 200 samples collected (50 from each of Urine, Borehole water, Abattoir waste water, and farm soil), abattoir waste water showed the highest number of positive samples 31(62%) on Pseudomonas selective agar (Cetrimide), while borehole water samples recorded the least 12 (24%).

Moreover, samples from the abbatoir wastewater recorded the highest occurrence of P. aeruginosa isolates 21(42%) following biochemical tests in which the organisms were motile, catalase, citrate and oxidase positive but urease, indole, methyl red and Voges Proskauer negative and form alkaline slant and alkaline base with no gas or hydrogen sulphide production on Triple Sugar Iron agar. Urine has the total of 19 (38%) Pseudomonas spp based on growth on cetrimide agar with only 11 (22%) biochemically confirmed as P. aeruginosa. Chi square comparing the values obtained showed that there is no significant difference in isolation rate of Pseudomonas spp and that of P. aeruginosa among the clinical and environmental samples (Table 1.1).

Table 1.1: Distribution of P.Aeruginosa From Clinical And Environmental Samples Within Maiduguri

Sample	**Samples Collected**	**Positive Sample (%)**	**X^2 (P-value)**	**Positive P. aeruginosa (%)**	**X^2 (P-value)**
Urine	50	19 (38)	9.856 (0.0198)	11 (22)	9.151 (0.0274)
Borehole water	50	12 (24)		8 (16)	
Abattoir Waste water	50	31 (62)		21 (42)	
Farm soil	50	23 (46)		11 (22)	
Total	200	85		51	

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	Samples											
	Urine (n=11) (%)			Borehole water (n=8) (%)			Abattoir waste water (n=21) (%)			Farm soil (n=11) (%)		
Ant	S	I	R	S	I	R	S	I	R	S	I	R
CN	8(72.72	1(9.09)	2(18.18)	7(87.50)	1(12.50)	0(0.00)	11(52.38)	7(33.33)	3(14.30)	8(72.72)	2(18.18)	1(9.09)
AM	9(81.81)	1(9.09)	1(9.09)	6(75.00)	2(25.00)	0(0.00)	15(71.42)	5(23.81)	1(4.76)	8(72.72)	1(9.09)	2(18.18)
CP	8(72.72)	0(0.00)	3(27.27)	5(62.50)	1(12.50)	2(25.00)	17(80.95)	1(4.76)	3(14.39)	10(90.09)	0(0.00)	1(9.90)
LV	7(63.64)	2(18.18)	2(18.18)	5(62.50)	2(25.00)	1(12.50)	12(57.14)	8(38.10)	1(4.76)	5(45.45)	2(18.18)	4(36.36)
CPZ	6(54.55)	0(0.00)	5(45.45)	6(75.00)	1(12.50)	1(12.50)	6(28.57)	4(19.05)	11(52.38)	6(54.55)	2(18.18)	3(27.27)
CEF	6(54.55)	4(36.36)	1(9.09)	6(75.00)	1(12.50)	1(12.50)	17(80.95)	3(14.29)	1(4.76)	7(63.64)	1(9.09)	3(27.27)
IMP	8(72.72)	1(9.09)	2(18.18)	5(62.50)	2(25.00)	1(12.50)	16(76.19)	2(9.52)	3(14.29)	9(81.81)	2(18.18)	0(0.00)
ME	9(81.81)	0(0.00)	2(18.18)	6(75.0)	2(25.00)	0(0.00)	12(57.14)	4(19.05)	5(23.81)	10(90.90)	0(0.00)	1(9.09)
AZT	8(72.72)	1(9.09)	2(18.18)	6(75.00)	2(25.00)	0(0.00)	5(23.81)	4(19.05)	12(57.14)	4(36.36)	4(36.36)	3(27.27)
TE	2(18.18)	0(0.00)	9(81.81)	5(62.50)	1(12.50)	2(25.00)	8(38.10)	2(9.52)	11(52.38)	8(72.72)	0(0.00)	3(27.27)
CH	0(0.00)	2(18.18)	9(81.81)	6(75.00)	1(12.50)	1(12.50)	1(4.76)	0(0.00)	20(95.24)	8(72.72)	0(0.00)	3(27.27)
NA	0(0.00)	1(9.09)	10(90.90)	4(50.00)	0(0.00)	4(50.00)	5(23.81)	4(19.05)	12(57.14)	7(63.63)	2(18.18)	2(18.18)

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Distribution of Resistance of P. aeruginosa among Clinical and Environmental Isolates

The distribution of antibiotic resistance among clinical and environmental isolates of P. aeruginosa showed varying level of resistance among the test isolate based on their sources. The clinical isolate was observed to exhibit the peak level of resistance to Nalidixic acid, followed byTetracycline and Chloramphenicol with a value of 10(90.90%), 9(81.81%) and 9(81.81%) respectively. Moreso, the lowest level of resistance in the clinical sample was observed in Amikacin and Cefepime having 1(9.09%) and 1(9.09%) respectively.

Among samples collected from the environment varying pattern of resistance was also observed. The highest level of resistance was observed in Nalidixic acid 4(50.00%), Cloromphenicol 20(95.24%) and 10(90.90%) from samples from Borehole water, Abattoir waste water and Farm soil respectively. No resistance to Gentamicin and Amikacin was observed in Borehole water, while in sample from Farm soil, Imepenem recorded no resistance 0(0.00) (Table 1.2)

CN: Gentamycin AM: Amikacin CP: Ciprofloxacin LV: Levofloxacin
CPZ: Ceftazidime
CEF: Cefepime IMP: Imepenem ME: Meropenem AZT: Aztreonam
TE: Tetracycline
CH: Chloramphenicol NA: Nalidixic Acid

Detection of algD and LasB Genes in Multidrug Resistant Pseudomonas Aeruginosa Isolated from Clinical and Environmental Samples

The gel electropherisis image for the detection of algD and LasB genes of Pseudomonas aeruginosa isolates from environmental samples within Maiduguri showed that the 3 samples Alemderi soil sample 2 (AL2), Abattoir drainage liquid sample 2 (DL2) and Abattoir drainage liquid sample 5 (DL5) are all positive for both algD and LasB genes as the gel image showed band of molecular size 1310 bp and 300 bp respectively.

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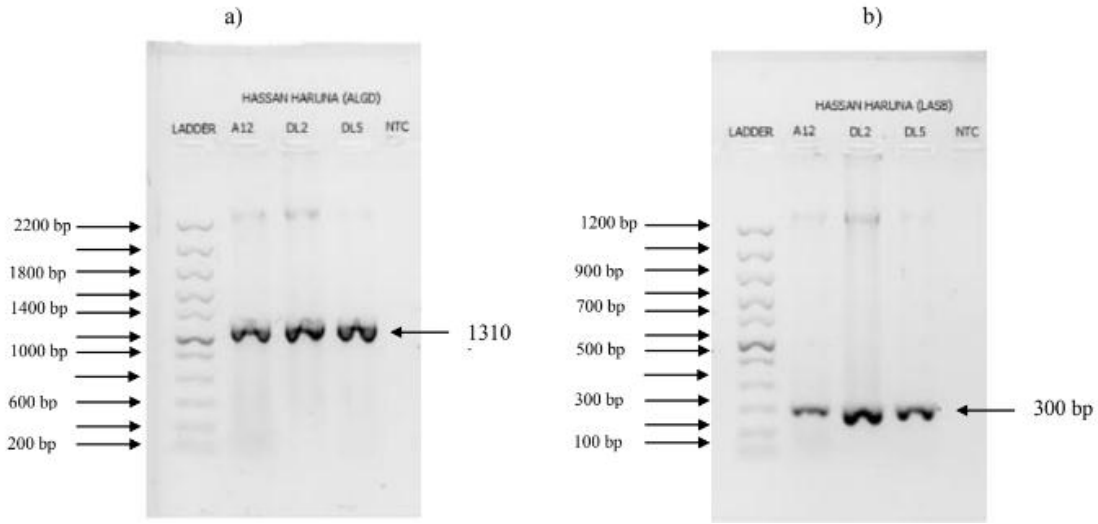
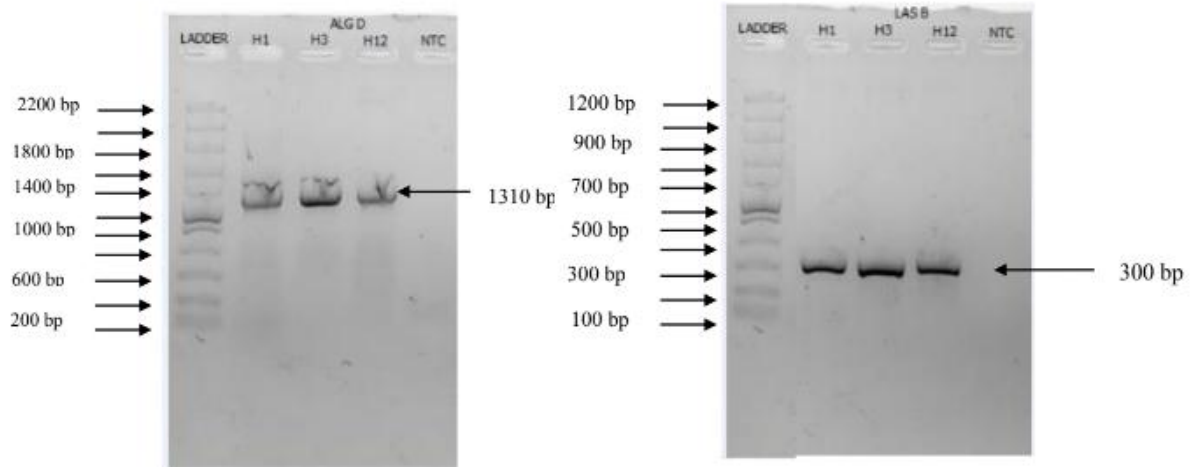
Key: AL2: Alemderi Soil Sample 2, DL2: Drainage Liquid Sample 2, DL5: Drainage Liquid



Sample 5

Figure 1.2: Agarose gel electrophoresis image for the detection of algD and LasB genes **of** Pseudomonas aeruginosa isolates from urine samples within Maiduguri

a) **b)**



Key: H1: Human Urine sample 1, H3: Human Urine Sample 3, H12: Human Urine Sample 12.

5. DISCUSSION

The result of identification of Pseudomonas aeruginosa from this study showed that the organism has a distinctive characteristic appearances on different growth medium: (Smooth,

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non-lactose fermenting colonies with leafy margin on MacConkey Agar, Yellow-green mucoid colonies and β-haemolysis with metallic sheen on Cetrimide and Blood agars respectively). The organism was also observed to be Gram negative rod, and the biochemical properties are all in conformity with documented literatures on the P. aeruginosa properties, biochemical and morphological characteristics. Table 1.1 showed that Pseudomonas spp was found to be largely isolated from the abattoir waste water 31 (62%) and the least isolation rate was observed in borehole water samples 12 (24%). Additionally, the highest and lowest isolation rate of P. aeruginosa 21 (42%) and 8 (16%) was found to be abattoir waste water and borehole water samples respectively.

Pseudomonas aeruginosa, a versatile microorganism, is known for occupying numerous habitats and is commonly found in aquatic environments. Its prevalence has been assessed to gauge the potential risks it poses to human health (Mena and Gerba 2009). Pseudomonas aeruginosa was as well detected in soil, even though, its dissemination and sufficiency has not been richly understood.

The relatively low resistance of the soil isolates depicted by the findings of this study could be due to limited exposure of the isolates to antibiotics and the moderate resistance observed may result from chemical fertilization among others.

According to the findings of this study as shown in Table 1.2, 14.29% were resistant to ciprofloxacin while 14.3% were resistant to gentamicin.

Table 1.1 also showed that, out of the 50 Borehole water samples used in this study, 8 Pseudomonas aeruginosa have been isolated. Based on the outcome of this study, Pseudomonas aeruginosa from urine demonstrated 90.90% resistance to nalidixic acid.

The result of this research revealed highest resistance against Nalidixic acid (91%), Chloramphenicol (81.81%) and Tetracycline (81.81%). Moreso, the findings of this study showed low resistance to ciprofloxacin (27.27%) and low resistance to Amikacin (9.09%) as shown by Table 1.2 which also revealed low sensitivity to Chloramphenicol (0.00%). Response to gentamicin in this study revealed 18.18% resistance.

The implication of Multidrug resistant P. aeruginosa in clinical cases is that it increases the hospitalization burden through prolonging hospital stay and resulting in secondary diseases. In the environment, dessimination of P. aeruginosa resistant to many antibiotics poses health threat to immunocompromised individuals particularly and the general population at large.

The PCR detection of algD gene result showed that all of the isolates assayed habour this gene, as gel electrophoresis revealed DNA bands of 1310 base pair molecular size (Figure 1.1a and 1.2a) and also showed that all the isolates assayed for LasB gene were positive as gel electrophoresis revealed bands that has 300 base pairs molecular size as shown in Figure 1.1b and 1.2b for both environmental and clinical samples respectively

6. CONCLUSION

At the end of this research work, it was discovered that 21 (42%), 8 (16%), 11 (12%) and 11 (22%) P. aeruginosa were isolated from abattoir waste water, borehole water, farm soil and urine samples of patients of urinary tract infection respectively. The organism is relatively resistant to commonly used antibiotics due to exposure or indiscriminate use of these drugs. The algD and LasB genes are widely spread among Pseudomonas aeruginosa regardless of

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the habitat or source of the isolate.

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