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Detection of Exoenzyme Effectors and Determination The MIC of Antibiotics for Pseudomonas Aeruginosa Isolated from Ear Infections Patients in Basrah Province, Iraq

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Abstract:

Ear infections are a common health problem, the most common microorganism which cause of infection is *Pseudomonas aeruginosa*. The study aimed to detection for bacterium ability of virulence factors production, molecular detection on that Exoenzyme effectors. The results of *P. aeruginosa* is the most type at18.62%. This bacterium was had a high level of resistance to the Ticarcillin antibiotic at 73.69%, were 10% of isolates (MDR), 42% (XDR) and 5% (PDR), production was each of the biofilm at 89.47%, protease enzyme at 79.94%, and Metallo beta-lactamase at 47.36%. Presence of exoenzyme genes (exoS:57.89%, exoT: 84.21%, exoU: 73.68% and exoY: 89.47%).

Keywords: Ear infection, Pseudomonas aeruginosa, Virulence factor, Exoenzyme.

Introduction:

Ear infection is an inflammation effect the individual of all ages, but children get them more often than adults(Getaneh *et al*., 2021). Research indicates that 60–80% of children experience recurrent ear infections during their early years of life. Ear infection often develops shortly after a cold for infants. However, an ear infection in an adult may indicate a more serious condition. An abrupt outflow of green or yellow fluid from the ear may indicate an injured eardrum (Jamal *et a*l., 2022). There are two types of ear infections: otitis externa and otitis media, which are typically brought on by microorganism and arise when fluid accumulates behind the eardrum. An ear infection can affect anyone

(Wiegand *et al*., 2019). The use of antibiotics, and advancing age are all play a role in the development of this illness (Almuhayawi *et al*., 2023).

Pseudomonas aeruginosa is one important cause to ear infections in addition to the presence causes of other microorganisms(Sathe et al., 2023). Are worldwide, they are found in soil, vegetation's, water, plants, and animals with a predilection to moist environments (Aujoulat et al., 2012). P. aeruginosa is a Gram negative, rod-shaped bacterium and one of the most common opportunistic pathogens to human with the ability of causing a wide range of infections types, including pneumonia, otitis externa, otitis media, wound infection, burn infection keratitis, and bloodstream infection (Kocsis et al., 2021).

Pseudomonas aeruginosa is using of intrinsic and acquired resistance mechanisms to resistance most antibiotics(Mahdi Alhamdani & Al-Luaibi, 2020). On the other hand, adaptive antibiotic resistance of P. aeruginosa is a characterized mechanism to bacteria, which includes biofilm formation and production of multidrug tolerant persisted cells (Bassetti et al., 2018).

The emergence and spread of antimicrobial resistant strains of *P. aeruginosa* gives it resistance to many drug classes, multidrug resistance(MDR) is antimicrobial resistance shown by a species of microorganism to at least one antimicrobial drug in three or more antimicrobial categories, Extensively drug-resistant (XDR) is the non-susceptibility of one bacteria species to all antimicrobial agents except in two or less antimicrobial categories. Pandrug-resistant (PDR) is the non-susceptibility of bacteria to all antimicrobial agents in all antimicrobial categories. Which belong to these families of antibiotic aminoglycosides, carbapenems, fluoroquinolones and penicillins/cephalosporins (Magiorakos *et al*., 2012).

Virulence factors enable pathogenic microorganisms to colonize host cells ultimately resulting in tissue damage as well as local and systemic inflammation(Qin *et al*., 2022). These factors are important for pathogens to establish an infection and span a wide range. Some selective media were used to identify the ability of some bacterial isolates to produce some virulence factors and the ability to analyze some of the substances present in these media(Nies *et al*., 2021). *P. aeruginosa* is a highly adaptable organism. It can grow on a wide variety of substrates and alter its properties in response to changes in the environment.

The aim of the study is to isolate and identify *P. aeruginosa* from ear infection specimens, as well as determine the antibiotics susceptibility test and MIC value against *P. aeruginosa* isolates, detection the ability of *P. aeruginosa* isolates on the virulence factors production and molecular detection of the Exoenzyme effectors which include four effectors (ExoY, ExoU, ExoS and ExoT) using specific primers.

Materials and methods:

Collection samples:

A total of 102 ear swabs were collected from male and female patients of different age groups who attended to an ear, nose, and throat consultant (ENT), in the hospitals of Basrah Province. Samples were incubated in the laboratory for twenty-four hours at 37°C. With the patients' permission, the doctor took the swabs, and the patient's data was then entered.

Identification of bacterial isolates:

The specimens were cultured on blood agar, MacConkey agar, and subcultures on cetrimide agar then were incubated for twenty-four hours at 37°C. Bacterial isolates were identified by looking at the morphological characteristics to grow colonies, such as their color and shape. As well as ability isolates for fermentation of lactose sugar, the odor these bacteria release, their ability to hemolysis, and their ability to produce bluish-green dye.

Identification by Biochemical tests, which includes the oxidase test is used to identify for ability bacteria that generate cytochrome c oxidase. The catalase test is used to determine if bacteria have the catalase enzyme. Citrate utilization test used to detect the bacterial isolate ability to utilize citrate as its carbon and energy source.

Detection of virulence factors:

Congo red agar used to detect the capacity of P. aeruginosa to produce biofilms. There were three classes of biofilm formation: weak, intermediate, and strong on agar. The black-colored colonies produce a strong biofilm, colonies which gave the white color are intermediate to biofilms forming and colonies which gave pink color are weak to biofilm produce, wheare colonies which appear a red color are non-producing to biofilms.

Skim milk agar was used to detect the ability of bacterial isolates to produce protease enzyme. After incubated, when a transparent edge appears around the colonies growing in the medium, this is an indicator of the ability of P. aeruginosa to produce the protease enzyme.

The Imipenem-EDTA combined disk test method was used to detected for able of *P. aeruginosa* isolates to produce metal beta-lactamase enzymes, two disks of Imipenem antibiotic were placed on the surface of the bacterial culture, to one of the discs added 10 microliters of EDTA , then the plates were incubated in the incubator at 37 °C for 24 hours , results were recorded by measuring the diameter of the inhibition zone around each disc, If the increase in the diameter of the inhibition zone around the Imipenem disc to which EDTA of it added is equal to or greater than 7 mm from the diameter of the inhibition zone around the Imipenem disc alone, then this indicates a positive test.

Genotyping of P. aeruginosa isolates:

Bacterial genomic DNA was extracted by using the Biocomma ® Nucleic acid extraction kit, according to the manufacturer's instructions. By using a thermocycler or PCR (polymerase chain reaction) technique while using specific primers. The mixture of PCR reaction in a total volume of 25 µl, the PCR reaction using to detected for the presence of the Exoenzyme genes (S, T, U and Y) were performed according to.

Sequence analysis of Exoenzyme genes:

The amplified fragments of Exoenzyme genes regions were sent for sequencing to (Macrogen company, Korea). Next, the Basic Local Alignment Search Tool (BLAST) was used to compare the homology of *exo* genes region with the counterpart region deposited in the public database at the Gene Bank (figure 1).

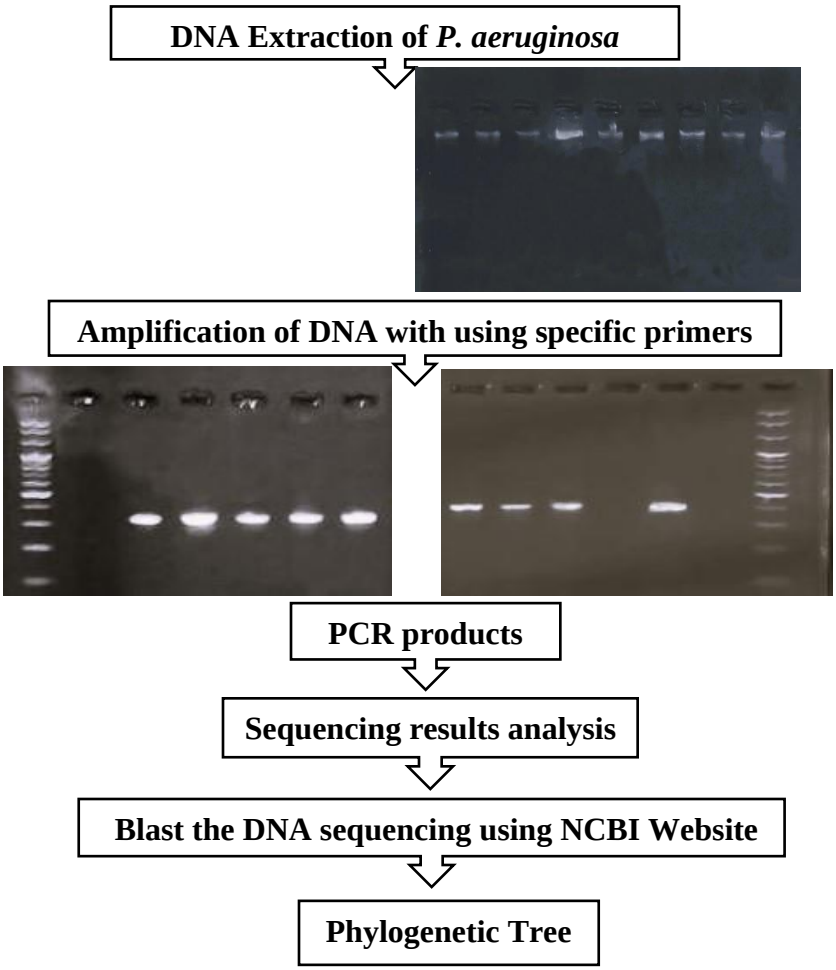

Figure 1: A schematic diagram representing the steps in molecular identification on Exoenzyme genes of the *P. aeruginosa* isolates. The genomic DNA was obtained from the purified bacteria isolates; the DNA was amplified using specific primers. The PCR products were then sent for sequencing analysis. Bioinformatics software was utilized to analysis the results and to identify the Exoenzyme genes to *P. aeruginosa* isolates by alignment them with the public database on the NCBI website.

Results and Discussion:

Demography of patients:

A total of 102 ear swabs were collected from patients attending to ear, nose and throat consultant ENT, from 61 males and 41 females, from different ages. After making the final diagnosis to specimen, 19 \ 102 (18.62%) isolates were identification as P. aeruginosa from ear infection patients. This study was compatible with many local and international studies, which showed that P. aeruginosa is one of the most common bacteria that cause an ear infection. According to these findings.

Identification methods:

P. aeruginosa is a Gram-negative and an important life-threatening nosocomial pathogens that are responsible for several dangerous illnesses and constitute a major risk to human health(Qin et al., 2022).

Traditional methods:

microscopical identification:

P. aeruginosa is Rod-shaped cell, Gram-negative and typically measure 0.5 to 0.8 µm in width and 1.5 to 3.0 µm in length, they can be found single or in pairs.

Plate based method:

Morphological characteristics of *P. aeruginosa* on MacConkey agar show flat, smooth, non-lactose fermenting colonies with regular edge(Forbes *et al*., 2007). In other hand *P. aeruginosa* colonies in blood agar showed the ability to produce hemolysin of type beta-hemolysis. To confirm the diagnosis of bacteria, was used Cetrimide Agar, which is a special medium for identifying *P. aeruginosa*, gave grow colonies on medium a blue-green dye (Al-Kabi *et al*., 2022).

Biochemical identification methods:

Table 1 shows the results of different biochemical testes subjected to *P. aeruginosa* isolates as further confirmation.

Table 1: Number of the biochemical tests for Identification P. aeruginosa.

TYPE OF TESTS	RESULT OF TEST
Gram stain	-
Catalase test	+
Oxidase test	+
Citrate utilization	+
Lactose fermentation	-
H_2S production	-
β-hemolysis	+
Kliglar	+

+ positive, - negative

This corresponds to aforementioned(Tadesse & Alem, 2006).

Bacterial confirmation by Vitek® 2 compact:

The VITEK ® 2 automated microbiology system was used in the identification of microorganisms. After biochemical and Gram staining tests, to confirm the identified bacteria. The isolates were streaked onto the Cetrimide agar and incubated at 37 °C for 24 hours to obtain pure colonies, with the use of identification gram negative bacteria (ID-GNB) cards according to the manufacturer's instructions(Ramzan *et al*., 2023). VITEK 2 system was used to confirm the identification of *P. aeruginosa isolates*.

Antibiotics sensitivity test using Vitek2 system and determine (MIC):

Vitek2 device was used to determine the sensitivity and resistance of P. aeruginosa to antibiotics. The minimum inhibition concentration (MIC) of each antibiotic was measure against 19 isolates of P. aeruginosa.

Table 2: Minimum inhibition concentrations (MIC) to antibiotics against *P. aeruginosa* isolates.

Antibiotic / No	TI	TIM	PRL	TZP	CAZ	FEP	IMP	MEM	AK	CN	TOB	CIP
1	64 R*	64 S	64 S	8 S	8 S	4 S	≤0.25 S	≤0.25 S	>16 S	8 I	4 S	≤0.25 S
2	32 S	32 S	≤4 S	≤4 S	4 S	2 S	2 S	0.5 S	≤2 S	≤1 S	≤1 S	≤0.25 S
3	16 S	16 S	≤4 S	≤4 S	2 S	≤1 S	2 S	1 S	≤2 S	≤1 S	≤1 S	≤0.25 S
4	≥ 128 R	≥128 R	≥128 R	≥128 R	≥64 R	≥64 R	≥ 16 R	≥16 R	≥64 R	8 I	≥16 R	≥ 4 R
5	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	4 *I	≥64 R	≥16 R	≥16 R	≥64 R	≥16 R	≥16 R	≥4 R
6	64 S	64 S	≥128 R	64 S	8 *I	32 R	2 S	4 S	4 S	2 S	≤1 S	≤0.25 S
7	32 R*	32 S	64 S	8 S	4 S	4 S	2 S	0.5 S	≤2 S	2 S	≤1 S	≤0.25 S
8	32 S	16 S	≤4 S	≤4 S	4 S	8 S	2 S	≤0.25 S	4 S	4 S	≤1 S	1 S
9	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	≥ 64 R	≥ 64 R	≥ 16 R	≥ 16 R	8 I	≥ 16 R	≥ 16 R	≥ 4 R
10	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	4 S	≥ 64 R	≥ 16 R	≥ 16 R	≥ 64 R	≥ 16 R	≥ 16 R	≥ 4 R
11	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	≥ 64 R	≥ 64 R	≥ 16 R	≥ 16 R	≥ 64 R	≥ 16 R	≥ 16 R	≥ 4 R
12	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	4 *I	≥ 64 R	≥ 16 R	≥ 16 R	≥ 64 R	≥ 16 R	≥ 16 R	≥4 R
13	>32 S	16 S	≤4 S	8 S	2 S	≤1 S	2 S	1 S	≤2 S	≤1 S	≤1 S	≤0.25 S
14	≥ 128 R	≥ 128 R	≥ 128 R	>128 R	≥ 64 R	≥ 64 R	≥ 16 R	≥ 16 R	>16 S	>4 S	≥ 16 R	≥ 16 R
15	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	4 *I	≥ 64 R	≥ 16 R	≥ 16 R	≥ 64 R	≥ 16 R	≥ 16 R	≥ 4 R
16	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	16 I	8 *I	2 S	0.5 S	≤2 S	≥ 16 R	≥ 16 S	≥ 4 R
17	≥ 128 R	≥ 128 R	≥ 128 R	≥ 128 R	4 *I	≥ 64 R	8 I	≥ 16 R	≥ 64 R	≥ 16 R	≥ 16 R	≥ 14 R
18	≥ 128 R	≥ 128 R	≥ 128 R	≤4 S	4 S	≤1 S	≤0.25 S	≤0.25 S	8 S	≤1 S	≤1 S	≤0.25 S
19	≥ 128 R	≥ 128 R	≥ 128 R	16 S	16 S	8 S	2 S	4 S	16 S	≥ 16 R	≥ 16 R	≥ 4 R

***S, sensitive to antibiotic; *R, resistant to antibiotic; *I, Intermediate.**

The result showed that some isolates resistance to many of antibiotics with high value of MIC as seen in (figure 2) 4 isolates were sensitive to all antibiotics used in the AST card at Vitek 2 system, while one isolate was seen resistant to all antibiotics. The result of the statistical analysis of antibiotics resistance were significant p<0.000. The bacteria showed a high resistance rate to antibiotics for both Ticarcillin at 73.69%, and Piperacillin at 68% , the result agrees with(Goli *et al*., 2016).

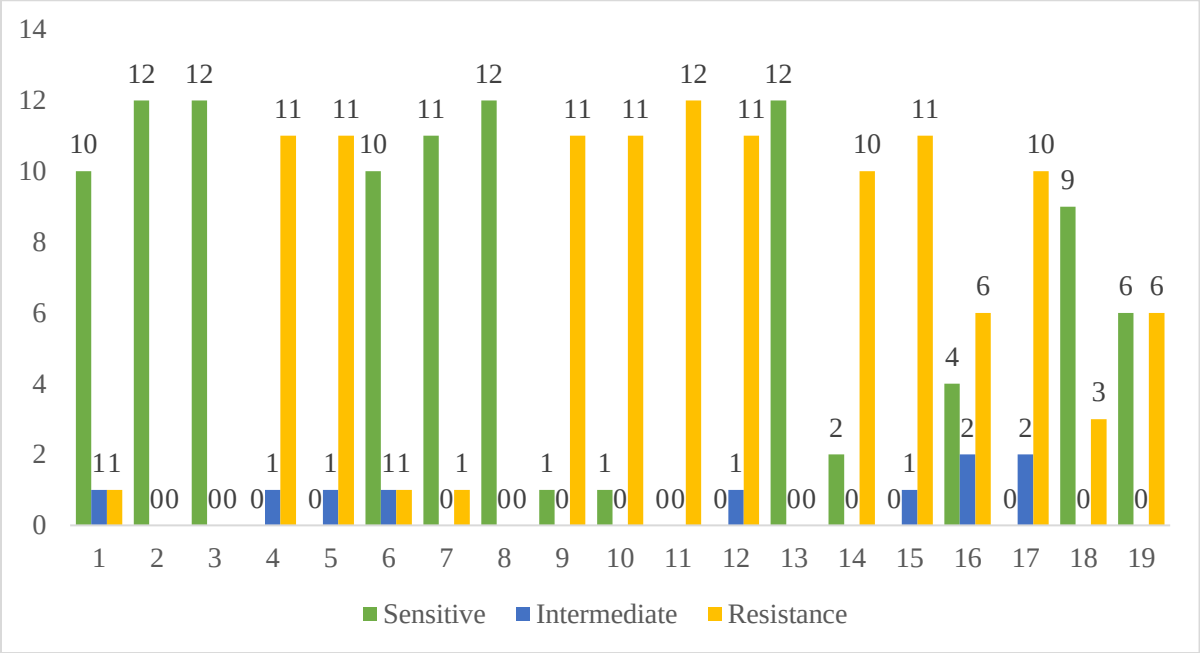

Figure 2: Antibiotic sensitivity against nineteen isolates of *P. aeruginosa*.

P. aeruginosa isolated appeared high resistance to antibiotics showing in table 3 and figure 3.

Table 3: Antibiotics sensitivity categories of P. aeruginosa isolated from ear infection patients.

Categories	Antibiotics	No. of isolates Sensitive	No. of isolates Intermediate	No. of isolates Resistant
Aminoglycosides	Amikacin	12 63.15	0	7 36.85
	Gentamicin	8 42.11	2 10.52	9 47.37
	Tobramycin	9 47.37	0	10 52.63
Beta-lactam/beta-lactamase inhibitor	piperacillin / tazobactam	9 47.37	0	10 52.63
	Ticarcillin/Clavulanic acid	7 36.85	0	13 63.16%
	Imipenem	10 52.63	1 5.26	8 42.11

Carbapenems	Meropenem	10 52.63	0	9 47.37
Cephalosporin's	Cefepime	8 42.11	1 5,26	10 52.63
	Ceftazidime	9 47.37	6 31.58	4 21,05
Fluoroquinolones	Ciprofloxacin	8 42.11	0	11 57.89
Penicillin's	Ticarcillin	5 26.31	0	14 73.69
	Piperacillin	6 31.57	0	13 68.43
P value =		0.011	0.000	0.000

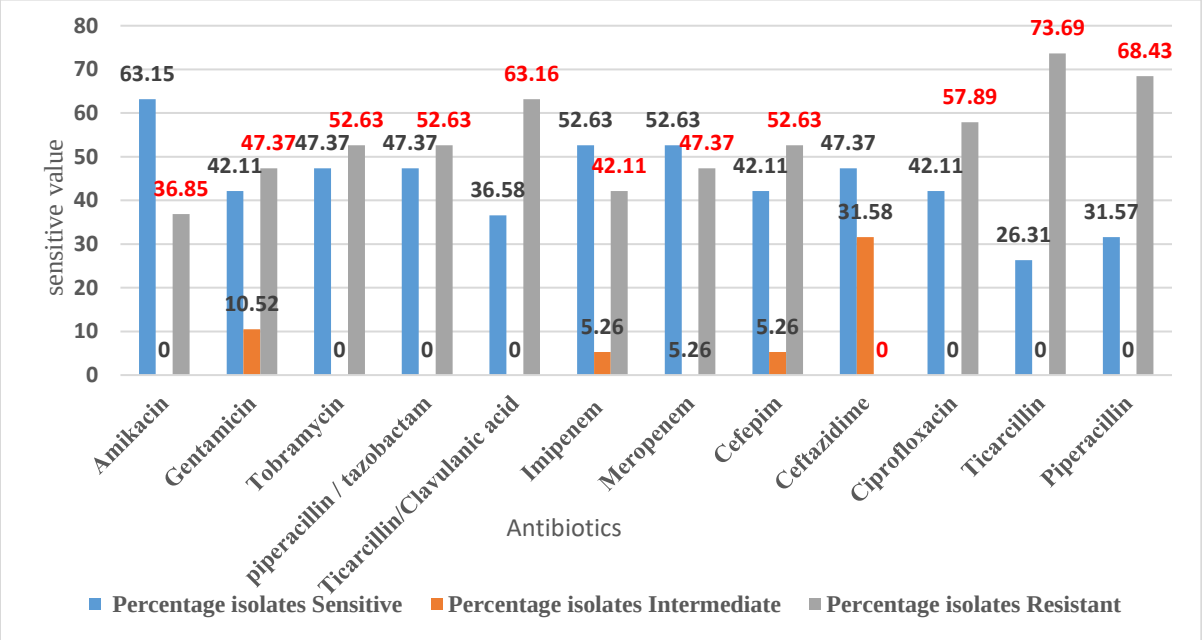

Figure 3: Percentage of antibiotics sensitivity of *P. aeruginosa* isolated from ear infection patients.

Multi-drug Resistance of Isolates:

The sensitivity of results regarding P. aeruginosa isolates showed high drug resistance, with high significant value P<0.000. Table 4 shows categories and types of the antibiotics resistance of P. aeruginosa isolates, as figure 4 shows the antibiotics resistance types of P. aeruginosa isolates.

Table 4: Shows categories and antibiotics resistance types of *P. aeruginosa* isolates.

Resistance type	No. resistance isolates	Categories of antibiotics
MDR	2 (10 %)	≥ 1 agent in ≥ 3 categories
XDR	8(42%)	≥ 1 agent in all but ≤ 2 categories
PDR	1(5%)	resistant to all antimicrobial agents
P value=	0.000	

*** MDR= Multidrug resistance. *XDR= Extensive drug resistance. * PDR = Pandrug resistance.**

The problem of antibiotics resistance is worldwide. Showed *P. aeruginosa* isolates from the current study were multiple antibiotics resistant. *P. aeruginosa* is one of the most important bacterial pathogen seriously contributing the problem of hospital infections, it's a serious issue that requires urgent attention.

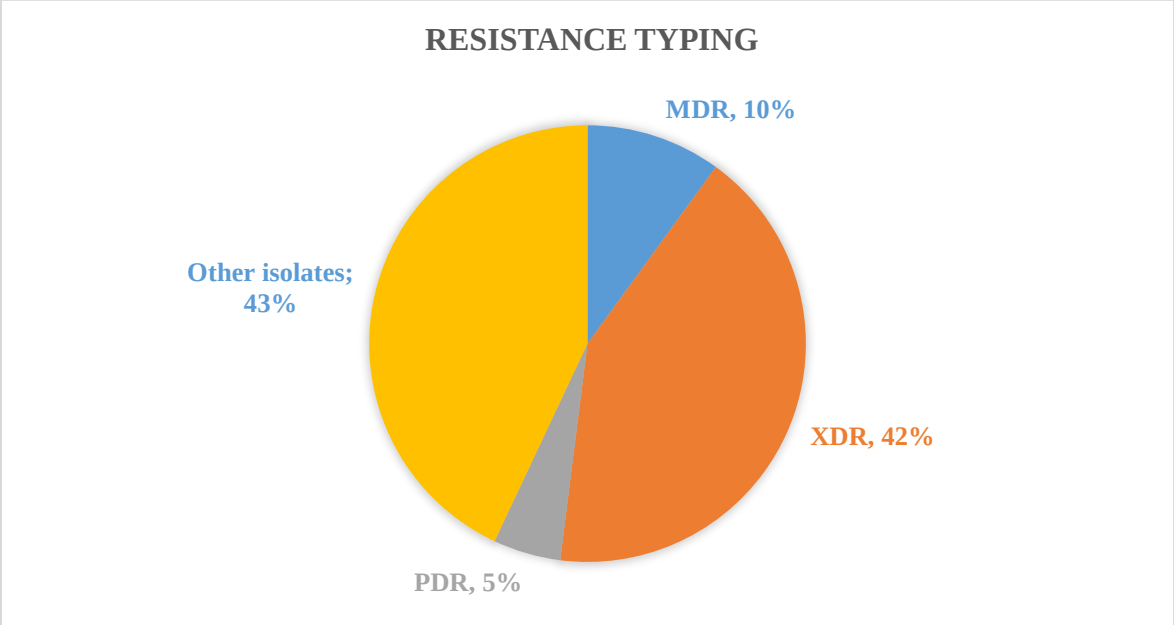

Figure 4: Shows the antibiotics resistance types of *P. aeruginosa* isolates.

Concluding from present study and previous studies that the reason resistance of *P. aeruginosa* to antibiotics due to them possession of many different mechanisms to resistance, included the ability to change the permeability of membrane, pumps Efflux, produces beta-lactamase enzymes, Biofilm forming and Plasmid-R which carries genes for resistance to various antibiotics(Bassetti *et al*., 2018).

Evaluation of virulence factors:

Virulence factors study *P. aeruginosa* isolates, which include form biofilm, produce protease enzyme and (MBLs). Bacteria form biofilm become more resistance to changeable environment condition such as pH, antibiotics, disinfectants and phagocytosis(Singh *et al*., 2017). Results showed 89% of isolates were ability to biofilm form, the biofilm producer's isolates exhibited three different categories: 11.76% of isolates were produced weak -biofilm, were 41.17% are intermediate biofilm, while 47.05% of isolates were a strong biofilm(Jalil *et al*., 2017). The study agreed with Abdulhaq (2020) who found out 94% of isolates can biofilm form, and another study done by Abd El-Galil (2013) who found out that 84% isolates were producers of biofilm.

Results showed 79% of isolates were ability to protease enzyme production which appearance on skimmed milk agar as transparent edge, results agreed with Al-Shwaikh *et al.*, (2015) which found 81% of *p.aeruginosa* were able protease production, and the study agreed with Hameed (2017) who found 75% of *P.aeruginosa* isolates are produce to protease enzyme. There is a relationship between production of protease enzyme by *P. aeruginosa* isolates and resistance antibiotics, as the increased the percentage of protease production by bacteria, the increased the number of antibiotics that the bacteria resisted(Fernández *et al*., 2012).

Results showed the ability of *P. aeruginosa* isolates to production the metallo beta-lactamase enzymes (MBLs), using method the IMP-EDTA combined disk test, were 9 (47.36%) out of 19 isolates can produced these enzymes, (figure 5) Shows virulence factors production by *P. aeruginosa* isolates. Metallo-β-lactamases are a diverse set of enzymes that catalyze the hydrolysis of a broad range of β-lactam drugs including carbapenems. The dissemination of the genes encoding these enzymes among *P. aeruginosa* isolates has made them an important cause of antibiotics resistance.

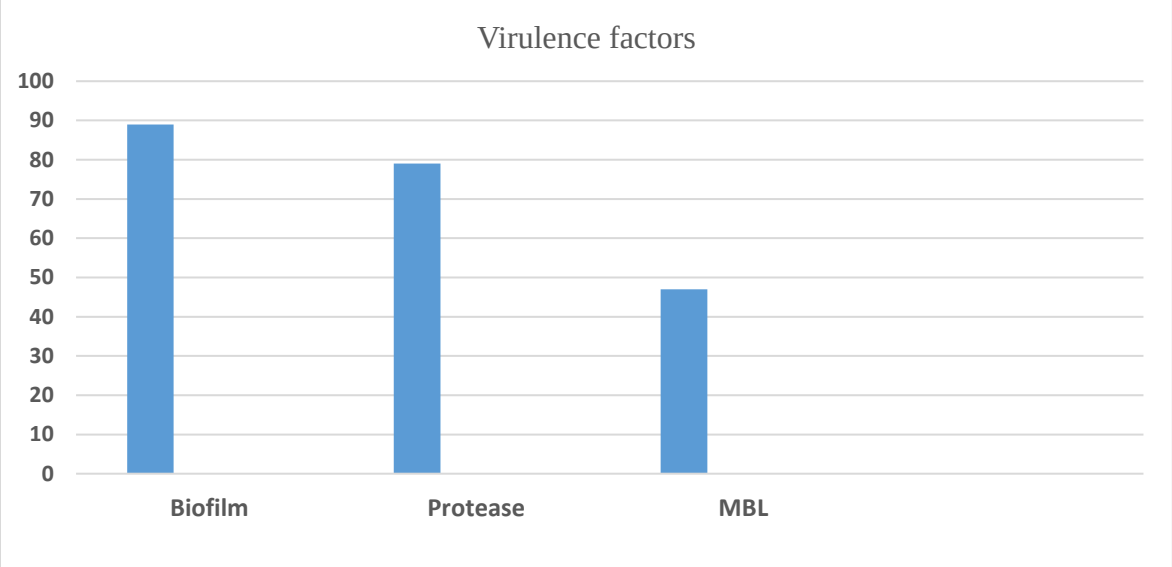

Figure 5: shows percentage to virulence factors production by *P. aeruginosa* isolates.

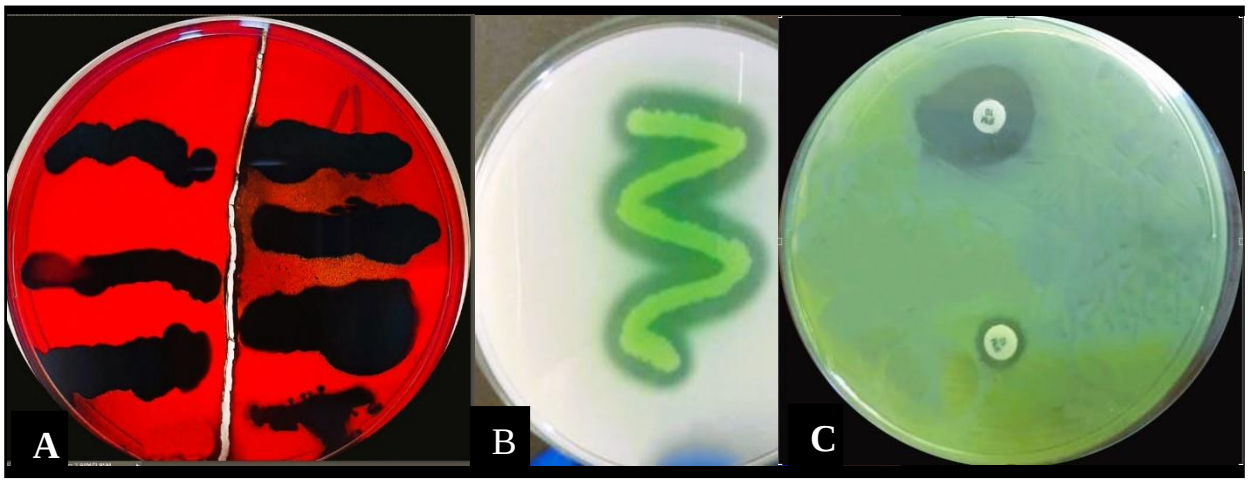

Figure 6: Shows virulence factors produce by *P. aeruginosa* isolates: A; Congo Red Agar medium shown biofilm formation, black colonies color indicates strong biofilm. B; Skim milk agar shown protease production, transparent edge indicates protease enzyme. C; MBLs production, inhibition zone around the Imipenem disc with EDTA indicates positive test.

Molecular genetic identification:

In DNA extraction, the results showed demonstrated DNA bands from all isolates, in which the agarose gel electrophoresis analysis revealed one band in each well corresponding to the isolates genomic DNA.

After that Exoenzyme genes detection (Y, T, U, S) using PCR technique, that P. aeruginosa isolates possess these genes with different ratio. After completing the PCR program for the four genes of P. aeruginosa, the gel electrophoresis gave a band corresponding to Exoenzyme band size (exoY: 309bp, exoT: 405 bp, exoU: 406 bp, exoS: 410 bp). Results showing 17(89.47%) out of 19 clinical isolates were carrying exoY gene, 15 (78.94%) out of 19 isolates were contain exoT gene, as well as showing 13 (68.42%) from 19 isolates contain exoU gene, and 11(57.89%) out of 19 P. aeruginosa isolates were contain exoS gene, which corresponding with DNA Ladder.

To Sequence analysis to exoenzyme genes (Y, T, U, and S), 13 PCR products were sent to Macrogen Company to confirm the presence of exoenzyme genes in P. aeruginosa isolates, which included four genes (exoY, exoT, exoU, and exoS), which varied in their presence among P. aeruginosa isolates. After identification using Vitek 2 system, P. aeruginosa were identified by comparing the exoenzyme genes region sequence with sequences located in the public database at the Gene Bank. The exoenzyme genes region sequences of all isolates were homology to the located sequences in the NCBI site. The percentage of identity was 100% as global similarity, it was found that they belonged to the P. aeruginosa Consequently, there was no requirement to enter the same sequences into the database at the Gene Bank.

Phylogenetic Tree:

Phylogenetic tree comprised of 13 genes belonging to *P. aeruginosa*, based on the Exoenzyme (*exoY, exoT, exoU* and *exoS*) region sequences of DNA constructed from UPGMA MAFFT, (figure) show that.

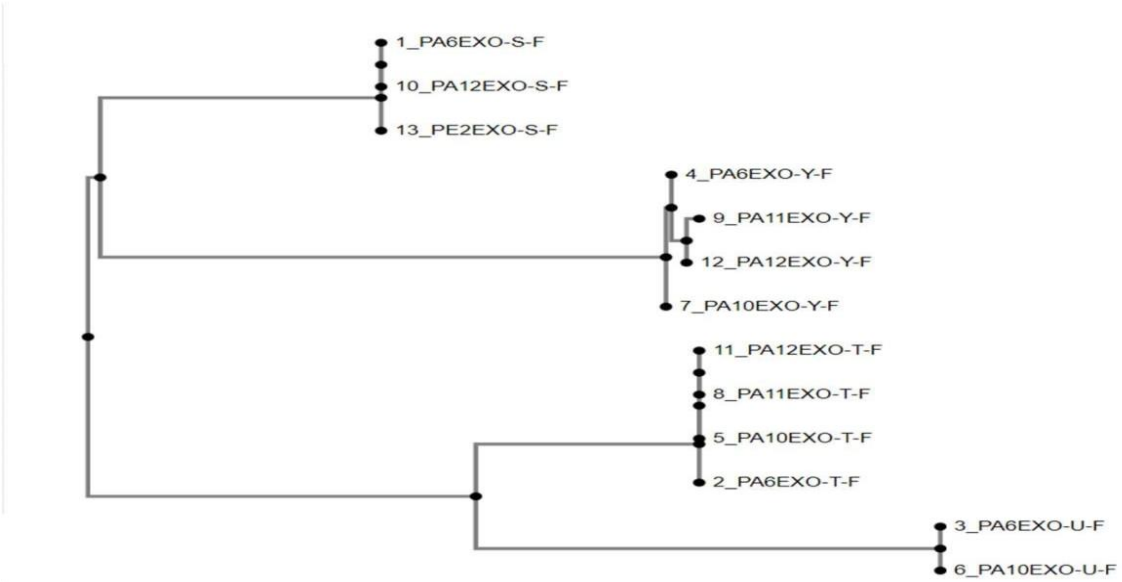

Figure 7: Rooted Neighbor Joining phylogenetic tree showing phylogenetic relationship of virulence genes (*exoS*= 3, *exoY* =4, *exoT*= 4, *exoU*= 2) that is constructed from UPGMA MAFFT.

Conclusion:

The results of the study indicate the *P. aeruginosa* is one of the most microorganism that cause to ear infection, most cases appeared in individuals over 50 years of age. Can rely on Vitek2 system in identification of bacterial species and also the determination of the MIC value of antibiotics against bacterium isolates, with high precision. The study showed that there is a correlation between the production of virulence factors and the resistance to antibiotics, isolates showed high drug resistance, with high significant value P<0.000. the results showed bacterium carried exoenzyme genes in varying proportions. It is worth noting necessity further studies to determine the causes of multiple resistances to antibiotics and detect the genes responsible for resistance and conduct more studies on other bacterial species that cause ear infections.

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