

Comparative Study of Some Antibiotic Resistance, Extended Spectrum β -Lactamases (ESBLs), Metallo β -Lactamases (MBLs) Produced in *Pseudomonas aeruginosa* Isolated from Clinical and Food Samples

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Abstract: A total number of 33 isolates of *Pseudomonas aeruginosa* were collected from different clinical samples, such as: burn, wound and urine from patients attending Al-Yarmouk teaching hospital and some private clinical laboratories in Baghdad city through the period from October to December 2016. On the other hand, 21 isolates of *P. aeruginosa* were collected from 38 different food samples; such as: vegetables and fruits, from different local markets in Baghdad city during the period from November to December 2016. All isolates were identified by using different bacteriological and biochemical assays and confirmed by Vitek-2 identification system. The antimicrobial susceptibility test for clinical and food isolates towards 17 antimicrobial agents disks was performed, the results showed that all clinical isolates were sensitive to Tobramycin but resistant to Cefepime, cloxacillin and Azthromycin, while all food isolates were sensitive to Ciprofloxacin but resistant to Cefepime. The resistant of *P. aeruginosa* clinical isolates towards Cefotaxime, Cefazidime and Imipenem was 75.8 %, 51.5 % and 75.8 % respectively while the food isolates of *P. aeruginosa* showed 52.4%, 95.2 % and 28.6% resistant for same antimicrobial agents respectively. Double disk synergy method is used to detect of *P. aeruginosa* producing Extended spectrum β -lactamases (ESBLs), 51.5 % of clinical isolates and 38 % of food isolates had the ability to produce ESBLs enzymes. Production of Metallo β -lactamases (MBLs) was performed by Imipenem – EDTA combined disk method. The results reveal that 51.5 % of clinical isolates and 47.6 % of food isolates were MBLs producers.

Keywords: *Pseudomonas aeruginosa*, Extended spectrum β -lactamases (ESBLs), Metallo β -lactamases (MBLs), resistance to antimicrobial agents

1. Introduction

Pseudomonas aeruginosa is Gram-negative bacteria, rod shape, about 1_5 μ m length and 0.5_1 μ m width, non spore forming, motile by single polar flagellum, capable of both aerobic and anaerobic growth [1, 2].

P. aeruginosa found in moist environment: sewage, soil, water, lake, streams, river, fresh water, fresh fruits and vegetables [2]. These bacteria are an opportunistic pathogen and a major cause of nosocomial infections in immunocompromised patients: malignansis, cystic fibrosis, burns, etc. [3].

P. aeruginosa, generally, is environmentally acquired and spread person - to - person rare. These bacteria can be transmitted through respiratory care equipment, irrigating solutions, catheters, infusions, cosmetics, dilute, antiseptics, and cleaning liquids and even through toilet soaps [4].

Food plants may be contaminated by *P. aeruginosa* through insufficiently-treated water and fertilizer or may be compromised by the used of biocides during cultivation. Contamination can occur in the field during harvesting, processing, distribution and even at use [5]. Oral ingestion of *P. aeruginosa* in water and food dose not necessary pose a risk of infection in healthy people, but the risk in immunocompromised patients and babies. *P. aeruginosa* has

significant importance in food microbiology not only as a food borne pathogen, but as one of the major spoilers of food and also as a marker of the hygiene qualities of food and water for human conception, *P. aeruginosa* also acts a plant pathogen causing systemic infection in plant leading to plant death especially in legumes [6].

1.1 Antimicrobial agents resistant in *P. aeruginosa*

The resistance capability of *P.aeruginosa* toward a range of antimicrobial agents is possible by several mechanisms : low permeability of outer membrane, efflux pump, B-lactamase production, aminoglycoside-modifying enzymes and biofilm formation [1, 4]. It has become increasingly clear the resistant development in *P. aeruginosa* is multifactorial with mutation in genes encoding porins, efflux pump, penicillin binding protein and chromosomal B-lactamase, all contributing to resistance to B-lactam, carbapenems, aminoglycoside and fluoroquinolones [7].

1.2 Beta Lactamase in *P. aeruginosa*

Production of B-lactamase enzymes are the most widespread and effective mechanism through which bacteria can become resistant to B-lactam drug, by hydrolyzing B-lactam ring (8). B-lactamase are classified into three major groups [9, 10]:

1) Class A: ESBL

hydrolyze penicillin, not extended cephalosporins, first, second, third and fourth generation (cefotaxim, ceftazidin, ceftriaxon), aztreonam but not cephomycin. These enzymes encoded by different gene located either on chromosome or plasmid, ESBL inhibited by clavulanic acid and sulbactam, these enzyme undergoing continue mutation causing the progression of more than 300 new different ESBL [3, 11].

2) Class B (carbapenemase): These enzymes hydrolyze a variety of B-lactam agents such as penicillin, cephalosporins and carbapenems (imipenem and meropenem), the genes responsible for the production of MBL are carried on transferable plasmid but can also be part of the chromosome. **These enzyme can be divided into:**

- a) **Serin B-lactamase:** presence of serine in their active site, inhibited by tazobactam and clavulanate.
- b) **Metallo-Beta lactamase:** having at least one zinc atom at their active site, inhibited by EDTA or thiol-based compound as chelator for Zn^{+2} [12, 13].

3) Class C (Amp C) (Cephalosporinase): These enzymes are chromosomal or plasmid mediated enzyme, confer high level of resistance to many B-lactam antibiotics, third generation cephalosporin, aztreonam and cephomycins (cefoxitin, cefotaten) [10].

The other mechanisms for drug resistance to the B-lactam group of antibiotics are loss of outer membrane proteins; alter target site and over expression of the up-regulating efflux system [11, 13].

Production of these enzymes make the treatment of infections caused by *P. aeruginosa* very difficult, because they become resistant to many antimicrobial agents that used as a drug of choice, so the detection of these enzyme needed to be done prior to treatment to avoid therapeutic failures and nosocomial outbreaks. [8, 14].

So the aim of this study is comparing between clinical and food isolates of *P. aeruginosa* susceptibility towards antimicrobial agents and their ability to produce ESBLs and MBLs enzymes.

2. Materials and Approach

2.1 Collection of samples

Isolates of *P. aeruginosa* (33 isolates) are recovered from clinical samples: burns, wounds, and urine from patients attended Al-Yarmouk teaching hospital and some private clinical laboratories in Baghdad city through the period October to December 2016.

P. aeruginosa were isolated from a total of 38 samples of vegetables and fruits samples; including: cucumber, zucchini, apple, tomatoes, Lemon, parsley, mint, strawberries, green pepper, pear, carrot, Rocca, leek, Cress and Spanish that collected from different local markets in Baghdad city through the period from November to

December 2016. By using sterile equipments and media, the samples were transferred to the lab for isolation and identification of *P. aeruginosa*. The samples were cut by sterile knife on sterile trays to small pieces, 5 gm of these pieces were weight and placed into sterile plastic container contains 45 ml of sterile D.W [15, 16].

2.2 Isolation and Identification of *P. aeruginosa* isolates

All isolates are identified by using bacteriological and biochemical tests and Gram stain to detect their response to stain, shape and arrangement, then the identification is confirmed by Vitek-2 identification system that indicated the identification of tested isolates as *P. aeruginosa* by using [9, 11].

2.3 Antimicrobial activity

2.3.1 Antimicrobial susceptibility test

Agar disc diffusion method of Kirby-Bauer modified base on CLSI [4, 17] was followed to perform the susceptibility test of 17 different antimicrobial agents toward the clinical and food *P. aeruginosa* isolates.

The results were interpreted according to Clinical Laboratories Standards Institute (CLSI, 2005). The isolate was interpreted as susceptible, intermediate or resistant to particular antimicrobial agents by comparison with standard inhibition zones.

2.3.2 Detection of Extended Spectrum β -Lactamases (ESBLs)

ESBLs enzymes production in *P. aeruginosa* was detected by double disk synergy test (DDST) [11, 8, & 18].

Mueller Hinton agar was inoculated with standardized inoculum (corresponding to 0.5 McFarland tube). Using sterile cotton swab and left for 10 min. to dry, then AMC disk (20 μ g Amoxicillin and 10 μ g Clavulanic acid) was placed in the center of the plate and test disk of 3rd generation cephalosporins (Ceftazidime- CAZ 30 μ g, Cefotaxime -CTX 30 μ g, and Aztreonam -ATM 30 μ g) disks were placed at 20 mm distance (center to center) from AMC disk, the plate was incubated overnight at 37°C.

Enhancement of the inhibition zone of any one of three disks towards AMC suggested the presence of ESBLs.

2.3.3 Detection of Metallo β -Lactamases (MBLs)

Imipenem - EDTA combined disk test was performed to identify metallo - β -Lactamases (MBLs) *P. aeruginosa* producing isolates [8, 13].

The test organism (Standardized to corresponding to 0.5 McFarland tube) was inoculated on Mueller Hinton agar by sterile cotton swab, and left to dry for 15 min, after that EDTA solution (0.5 M) that was prepared by dissolving 18.61 gm of Na-EDTA in 100 ml D.W and adjusting its pH to 8.0 by using NaOH. The mixture was sterilized by autoclave.

Two Imipenem (10 µg) disks were placed on the surface of cultured Mueller Hinton agar plate at the distance of 30 mm. EDTA solution (10 µl) was added to one of them, then the plate incubated at 37°C for 18-24 hrs. The inhibition zone of Imipenem and Imipenem –EDTA disks were compared after 18-24 hrs. if the increase of inhibition zone with Imipenem – EDTA disk was > 7mm than the inhibition zone of Imipenem disk alone it is considered MBLs positive.

3. Results and Discussion

3.1 Isolation and Identification

P. aeruginosa accounts for 10% of all hospital acquired infections; it is the second most frequently recovered pathogen from intensive care unit patients [2].

A total of 33 isolates of *P. aeruginosa* were isolated from clinical samples: burn (n=12 (36.4%)), wounds (n=12 (36.4%)) and urine (n=9 (27.3%)) from patient attending to Al- Yarmouk teaching hospital and some private clinical laboratories in Baghdad city through the period from October to December 2016.

The prevalence of *P. aeruginosa* isolated from wounds (37.5%) was differ from [11] (19%) in bangaladish; [9] (20%) in Egypt; [3] (2.6%) in Iran and [13] (9.3%) in Baghdad respectively, While the prevalence of *P. aeruginosa* isolates in burns were (37.5%) in this study which is differs from another local study [13] it was (29%), and finally the prevalence of *P. aeruginosa* isolated from urine sample was (25%). This result was relatively lower than [3] (33.6%) but it is higher than [11] (12%) and [13] (6.8%).

The fresh vegetables and fruits are carried strains of *P. aeruginosa* considered to be a major vehicle for disseminated of these bacteria [15].

A total of 33 vegetables and fruits samples were collected from different local markets in Baghdad city through the period from November to December 2016. Out of total 33 vegetables and fruits samples, *P. aeruginosa* was isolated from 21 (55.3 %) samples.

The contamination of vegetables and fruits by *P. aeruginosa* may occur during harvesting, handling, processing and transit, vegetables and fruits may come in contact with soil, insects and water which they are represented important sources of contamination in field including run off from nearby animal pastures and irrigation from contaminated sources [15,16], also the presence of *P. aeruginosa* in animal faeces (which is used as fertilizer) is suggested a possible source of contamination [19]. The presence of high number of viable bacteria may leads to increase spoilage as well as the possibility of produce- associated out break [5]. All isolates from clinical and food samples are cultured on different bacteriological media; such as: Nutrient agar, MacConkey agar and cetrimide agar for identification by morphological properties of colonies. For further identification, microscopic identification is done by using

Gram stain. *P. aeruginosa* appeared Gram negative small bacilli arranged as single bacilli or in pairs; finally, identification of all isolates were confirmed by Vitek- 2 identification system [4].

3.2 Susceptibility of *P. aeruginosa* isolates from clinical and food samples towards antimicrobial agents

Antimicrobial susceptibility was performed by CLSI method [20] for 17 different antimicrobial agents that listed in (table 1). It was investigated by using Kirby-Bauer method (figure 1). The results of clinical and food samples were showed in (figure 2) respectively.



Figure 1: Susceptibility of *P. aeruginosa* isolates towards antimicrobial agents by using Kirby-Bauer method

Table 1: Antimicrobial agents used in susceptibility test

Antibiotics	Code	Concentration	Group
Ceftazidime	CAZ	10	Third generation of cephalosporins
Cefotaxime	CTX	10	Third generation of cephalosporins
Cefixime	CFM	5	Third generation of cephalosporins
Cefepim	FEP	30	Fourth generation of cephalosporine
Piperacillin	PRL	30	Penicillin
Cloxacillin	COX	5	Penicillin
Ciprofloxacin	CIP	10	Second generation of fluoroquinolones
Amikacin	AK	30	Aminoglycoside
Tobramycin	TOB	30	Aminoglycoside
Neomycin	N	30	Aminoglycoside
Aztreonam	AT	30	Monobactam
Vancomycin	VA	30	Glycopeptides
Imipenem	IPM	10	Carbpenems
Meropenem	MEM	10	Carbpenems
Azthromycin	AZM	15	Macrolids
Erythromycin	E	15	Macrolids
Colistin	CT	10	Polymixin

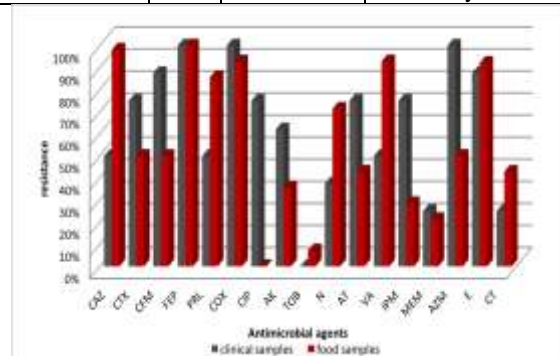


Figure 2: Susceptibility of *P. aeruginosa* isolates from clinical and food samples to antimicrobial agents

Among the 17 antimicrobial agents, maximum sensitivity of clinical isolates was found with Tobramycin 100% sensitive, while 100% of the isolates resistant to Cefepime, Cloxacillin and Azthromycin (figure 2).

The results show 51.5 % of clinical isolates were resistant to Ceftazidime, which was approach to [11] in Bangaladish, (54.5%) while they differed from [8], [9], [3], [10], [4] and [21]. They found that the resistant to Ceftazidime was (9.5%), (86%), (14.7%), (60.34%), (64%) and (19.5%) in Nigeria, Egypt, Iran, India, Saudi Arabia respectively, but this result was less than local study by [13] and other study by [19] they reported that the resistant to Ceftazidime was 100%.

For Cefotaxime, (75.8 %) of clinical isolates were resistant, which is approach to [9] in Eygpt (72%), but it is higher than [3] in Iran that reported that (22.4%) of isolates were resistant to Cefotaxime; while the resistant of clinical isolates to Ceftazidime are 51.5%.

The resistant of Imipenem towards clinical isolates in our study was (75.8 %) which is agree with [8] in Nigeria (28.4%) that is sensitive to Imipenem, but this result disagree with [11], [9], [3] and [4] who they mentioned that the results were (93.3%), (100%), (17.2%) and (82.5%) respectively sensitive to Imipenem.

The diversity in susceptibility of clinical samples to the same antimicrobial agents varies with time due to increase the usage of these agents, and to the geographical area.

High resistant to some antimicrobial agents due to intensive using of them in clinical practice so appropriate therapeutic protocols and regular monitoring system must be followed to prevent the wide spread of resistant determinant [17].

Pattern of antimicrobial susceptibility revealed that Ciprofloxacin was most effective against food isolates of *P. aeruginosa*, while 100% of the isolates are resistant to Cefepime (Figure 3), these results agree with [5], [14] and [19] in Jamaica, Egypt and Nigeria who reported that all isolates were sensitive to Ciprofloxacin.

This study reveals that 52.4% of food samples are resistant to Cefotaxime and 95.2%, are resistant to Ceftazidime which is higher than [5] and [19] who found that 79% and 45% of their tested isolates were resistance to Ceftazidime. 28.6 % of food samples were resistant to Imipenem which differs from [5] and [19] who they found that 100% of their isolates were sensitive to Imipenem.

Clinical isolates of *P. aeruginosa* are highly resistant to antimicrobial agents when compared to food samples, this may due to the constant exposure to the antimicrobial agent in hospitals environment.

3.3 Multi- Drug Resistant of *P. aeruginosa* isolates to antimicrobial agents

Multi drug resistant (MDR) is defined as resistance of bacteria towards three or more antimicrobial agents [7].

Multidrug resistance to nosocomial infections pathogens has been increasing around the world. Occurrence of MDR in *P. aeruginosa* is growing which leads to limiting the therapeutic options [3, 4].

The results of antimicrobial susceptibility towards *P. aeruginosa* from clinical and food samples in this study show that they all are considered as MDR isolates (Table 2 & 3).

Table 2: Multi-drug resistant of clinical *P. aeruginosa* isolates

Groups	No. of resistant antimicrobial	No. of resistant isolates	Percentage of resistance
1	1-6	5	15.2 %
2	7-11	12	36.4 %
3	12-17	16	48.5 %

Table 3: Multi-drug resistant of food *P. aeruginosa* isolates

Groups	No. of resistant antimicrobial	No. of resistant isolates	Percentage of resistance
1	1-6	2	9.5 %
2	7-11	14	66.7 %
3	12-17	5	23.8 %

In clinical samples; similar results are observed in local study by [13] but in other studies; such as [3] in Iran and [9] in Egypt, who reported that only (16.37%) and (56%) of the clinical isolates showed MDR phenomena respectively; while the results of food isolates are matching with [4] & [5] who mentioned that their isolates of *P. aeruginosa* from food samples were MDR.

P. aeruginosa is intensively resistant to significant number of antimicrobial agents, and they can easily have acquired resistant to new antimicrobial agents by mutational changes or acquisition of genetic material [7], and the bacterial have several resistant mechanisms; such as: efflux pumps, outer membrane which provides an effective intrinsic barrier in cell wall, biofilm formation and various classes β -lactamases production [22].

3.4 Production of EXBL in *P. aeruginosa* isolates from clinical & food samples

Double disk synergy method was used to detect *P. aeruginosa* producing Extended spectrum β - lactamases (ESBLs) enzymes, the increasing of inhibition zone against any one of three disks towards Amoxicillin - clavulanic acid is suggested the presence of ESBLs producing isolates [13] (Figure 3 & 4).

The detection of isolates that produce these enzymes is important to avoid therapeutic failure and nosocomial outbreak [8].

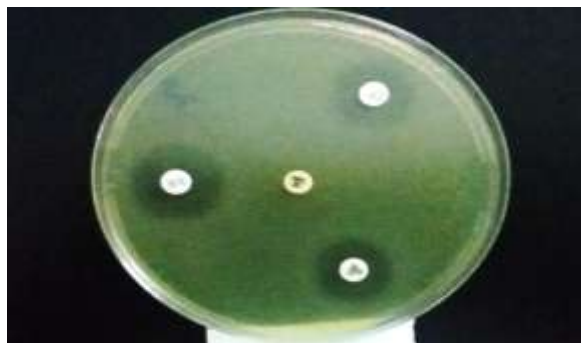


Figure 3: Non ESBL producing *P. aeruginosa*



Figure 4: ESBL producing *P. aeruginosa*

The results showed four clinical isolates (51.5 %) were ESBLs producers, which is the same results reported by [9] in Egypt (51%), also the results of this study are approach to [21] who reported that (59%) of clinical isolates in Saudi Arabia were ESBLs, but these results are differed from two local studies in Baghdad by [23] in Kirkuk and [24], who noticed that their isolates were ESBLs (72.2%), (100%) respectively; and it is also differed from other studies by [8], [11] and [3], who found that ESBLs producing isolates were (16.7%), (39.2%) and (6.89%) of their all total clinical isolates respectively.

In food samples, (38.1 %) were ESBLs producers. This finding is very higher that findings of [18] and [19] in Nigeria, they found that the production of *P. aeruginosa* producing ESBLs isolates in food samples of their studies was (7.14%) and (18%) respectively.

Production of ESBLs allows *P. aeruginosa* bacteria to be tolerated against penicillin, cephemycin, carbapenes and extended spectrum cephalosporins; such as: cefotaxime, ceftriaxone and cftazidime, which they are represented the drug of choice for *P. aeruginosa* infections treatment [3].

ESBLs are frequently plasmid encoded, plasmids are carried elements encoding resistant to other drug class like aminoglycoside, these plasmids are mobile genetic elements and can also facilitate the dispersal of genes or genetic material that encoded for resistance of antimicrobial agents among bacterial population and also can serve as vehicle for other resistance mechanisms. Incidence of these genes in plants for human consumption is dangerous because of the risk transmission of resistance strains to human food, and may people consumption food without washing or cooking [19].

3.4 Production of MBLs in *P. aeruginosa* isolates from clinical & food samples:

Metallo- β -lactamases enzymes producing *P. aeruginosa* isolates were detected by Imipenem –EDTA combined disk method (Figure 5 & 6).

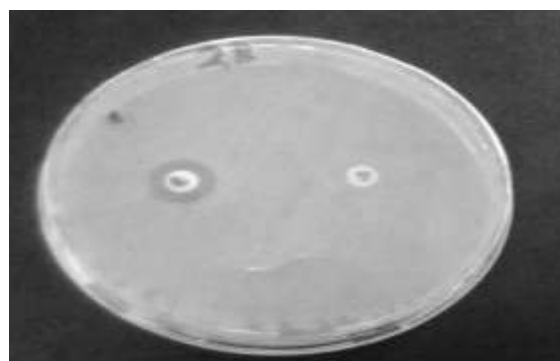


Figure 5: Non MBL producing *P. aeruginosa*



Figure 6: MBL producing *P. aeruginosa*

This study showed that (51.5 %) of clinical isolates produced MBLs enzymes by increasing inhibition zone more than 7 mm in EDTA- Imipenem combined disk as it compared with Imipenem disk alone [8, 13]. these results are similar to a local study in Baghdad city by [23] who found that (50%) of clinical *P. aeruginosa* isolates were MBLs producers, but it is higher than other local study by [13] who reported that (5.33%) of the isolates produced MBLs enzymes; on the other hand, this result is less than what [8] reported in his study (70%).

There is no study has detected *P. aeruginosa* MBLs Producing isolates from food samples, so there is no comparison with this result that showed one isolate (47.6 %) of *P. aeruginosa* from food samples was MBLs producers.

MBLs producers isolate leads to serious therapeutic failure because they carry multidrug resistant gene.

Decrease the susceptibility to Imipenem is a great concern for treating infections caused by *P. aeruginosa* because only available treatment except Imipenem is Polymixin B which it is potentially toxic [13]. Another fact can be detected from this study is the high production of ESBLs & MBLs in clinical isolates than food isolates, (figure 7), that may be refers to continuous exposure of clinical infection to antimicrobial agents and this make the bacteria modify their mechanisms to tolerate these agents.

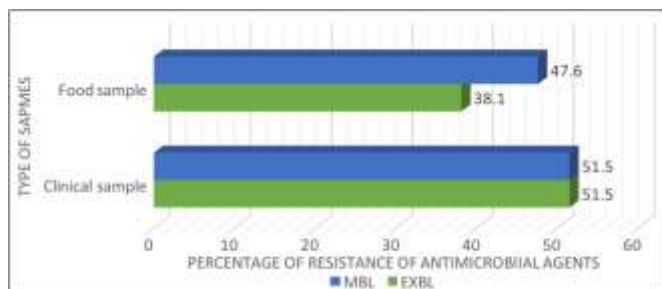


Figure 7: Percentage of ESBLs & MBLs Production in *P. aeruginosa* in clinical & food samples

4. Conclusions

Finally, the conclusions of this study is referred to highly resistant of *P. aeruginosa* isolates from food samples towards antimicrobial agents in addition to their ability to produce ESBLs & MBLs, which makes these isolates carrying virulence factors that transfer to human by contaminated, unwashed food; such as: fruits & vegetables leading to serious infection could not be treated by common drug of choice as the same as clinical isolates.

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