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Research Article

Molecular Screening of Ambler class C and extended-spectrum β-lactamases in multi-drug resistant Pseudomonas aeruginosa and selected species of Enterobacteriaceae

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ABSTRACT

Background and objective: As a major clinical concern worldwide, the occurrence of extended spectrum β-lactamases (ESBLs) has been increasingly reported in Pseudomonas aeruginosa. The goal of the study for the detection the occurrence of β-lactamase bla-OXA specifically, the OXA II gene and III in study isolates and its role in the resistance pattern.

Patients and Methods: One-hundred of clinical specimens were obtained from different clinical sites. Antibiotic susceptibility testing was performed for the study isolates of Pseudomonas aeruginosa according to the recommendations laid down by CLSI. ESBLs confirmatory test had been done by double-disc synergy test. Bacterial DNA extraction was achieved using automated DNA extraction unit, SaMag, Italy. RT-PCR was used for amplification of β-lactamase (blaOXA) genes and detected using agarose gel electrophoresis.

Results: Out of 100 clinical specimens, 75 (75%) were culture-positive. Of these, 35 (46.7%) isolates were diagnosed as P. aeruginosa, 23 (30.7%) E. coli isolates and 17 (22.6%) K. pneumonia. Based on the confirmatory test for the extended-spectrum β-lactamase production, 40 (53.33%) isolates were considered to produce ESBLs, including 22 (55%) P. aeruginosa, 8 (20%) K. pneumoniae, and 10 (25%) E. coli isolates. RT-PCR was then used to detect the presence of the OXAII and III genes in the K. pneumoniae, E. coli, and P. aeruginosa clinical isolates. Five (14.28%) isolates of P. aeruginosa were producing OXAII while three (8.57 %) of them produced OXAIII. Two(8.69%) isolates from E. coli for OXAII and III gene and 2(11.76%) isolates from K. pneumonia were positive for the presence of OXAII while Five (29.4%) of them produced OXAIII.

Conclusion: PCR is so helpful for the identification of specific β-lactamase genes. Also, the study suggested that a significant presence of blaOXA-II and blaOXA-III genes among study isolates were detected and the highlighting for the need for suitable infection control strategies to effectively treat patients and prevent the further distribution of these resistant organisms.

Keywords: ESBL; carbapenemase; oxacillinase; multidrug resistance

INTRODUCTION

P. aeruginosa is a pathogen that is typically involved in extreme nosocomial infections such as pneumonia, inflammation of the urinary tract, and sepsis. In several classes of antimicrobial agents, including βlactams, aminoglycosides, and fluoroquinolones, P. aeruginosa is also highly resistant.

Several mechanisms can cause the development of β-lactam resistance in this opportunistic pathogen: (1) genetic mutations leading to stable overexpression of AmpC, a chromosome mediated cephalosporinase; (2) acquisitions of transferable genetic markers for a variety of β -lactamases; (3) overproduction of efflux pumps; and (4) decreased permeability. [1]

An increasing number of extended-spectrum β-lactamases (ESBLs) of Ambler class A, carbapenemases (Metallo-β-lactamases) of class B, and extended-spectrum oxacillinases (OXAs) of class D have been reported in clinical strains of P. aeruginosa [2-4,5]. ESBLs are a rapidly increasing group of β-lactamases that hydrolyze aztreonam and broad-spectrum cephalosporins. The expression of ESBL also confers tolerance to penicillin and narrow-spectrum cephalosporin. [1]

ESBLs are blocked by inhibitors of β-lactamase such as clavulanic acid. Gram-negative bacilli

have been found in over 200 different ESBLs. Thirty-two ESBLs, which can be classified into two molecular classes A and D and further divided into eight subgroups, have been identified in P. aeruginosa: TEM-, SHV-, CTX-M-, PER-, VEB-, GES-, BEL-, and OXA-type β-lactamases. [6]

On the other hand, AmpC β -lactamases have been identified in Enterobacteriaceae and P. aeruginosa and are class C cephalosporinases. Class C molecules contribute to the intrinsic resistance to penicillins and cephalosporins of Enterobacteriaceae and P. aeruginosa, as well as the combination of β -lactam and β -lactamase inhibitors [6]. Resistance to ceftazidime in P. aeruginosa is primarily due to overexpression of its naturally occurring cephalosporinase-type AmpC. [7]

It is realized that resistance of Enterobacteriaceae to various β-lactamases of Class A, B, and C has been reported in Taiwan [8-14]. ESBLs (focusing on OXA types) have only been recorded in one study. P. aeruginosa in Taiwan [15]. In 1294 P. aeruginosa isolates, including OXA-14 (0.2 %), OXA17 (2.8 %), and OXA10 (0.6 %), three ESBLs belonging to the OXA-types were identified.

In Taiwan, surveillance of the different types of ESBLs found in P. aeruginosa has not been recorded. The Clinical Laboratory Standards Institute (CLSI) has proposed the double-disc synergy test (DDST) as a phenotypic test for ESBL in Escherichia coli and Klebsiella pneumonia [16].

However, due to the sensitivity of a phenotypic test, only a limited subset of studies have documented the application of DDST to P. aeruginosa [17]. Phenotypic ESBL detection can obscure chromosomal Amp C cephalosporinase in P. aeruginosa although AmpC is not affected by β-lactamase inhibitors (e.g. clavulanic acid)[18]. Only with the addition of cloxacillin, ESBL detection in P. aeruginosa is obscured by the addition of cloxacillin.

The ESBL group of enzymes is found widely and causes a severe infection on human health leading to various diseases. These enzymes stimulate hydrolysis of the β-lactam ring and thereby, inhibit these antibiotics. Thus, the therapeutic choices available for combatting resistant bacteria remain limited.[19-20,21]

PATIENTS AND METHODS

Study patients

A total of 100 clinical specimens were obtained from different clinical sites, including burns, wounds, otitis media, and urinary tract infection, during the duration from January 2018 to August 2018. These specimens were collected from specialized units in AL-Ramadi Teaching Hospital. In addition to biochemical and confirmatory tests, study isolates were bacteriologically diagnosed in accordance with conventional, macroscopic, and microscopic tests. The isolates were preserved in brain heart infusion broth containing 20 % glycerol. P. aeruginosa ATCC 27853 and E. coli, ATCC 25922 were used as international quality control.

Antimicrobial susceptibility tests

The susceptibilities of the study isolate to ceftriaxone (30 µg), ceftazidime (30 µg), imipenem (10 µg), cefoxitin (30 µg), aztreonam (10 µg), and cefepime (30 µg) were determined using the Standardized Kirby Bauer method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) [22].

Preliminary Phenotypic Detection of ESBLs

The Kirby-Bauer disc diffusion technique was used to conduct primary ESBL screening. Briefly, this method includes the preparation of a suspension of the target bacteria at a standardized density. The bacterial suspension is used to swab the surface of Mueller- Hinton agar plates, after which antibiotic-impregnated discs were aseptically placed on the agar surface, and the bacteria were allowed to grow for 18 h [22]. The antibiotic discs were placed on the surface of the medium evenly with sterile forceps and then incubated at 37°C for overnight incubation. Then, the inhibition zones were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) [22].

Confirmatory detection of ESBLs

In the confirmatory test for ESBLs, all isolates which reveal resistance to third-generation cephalosporins (ceftazidime and ceftriaxone) were submitted to the double-disc synergy test. In this test, amoxiclav (amoxicillin+ clavulanic acid, 30/10 µg) disc was placed in the center of the inoculated plate, and ceftazidime (30 µg) and cefotaxime (30 µg) disks alone are set at 10 mm distance from the amoxiclav disc. The plates were overnight incubated at 37°C for 16–18 h. The isolates are interpreted as ESBL if there clear synergy of the edge of the inhibition zone of the antibiotic disc toward the amoxiclav disc[19,22].

The Extraction of DNA

The SaMag bacterial DNA extraction kit is used for the automated extraction of genomic DNA from the study isolates (SaMag, Cepheid, Italy) using the SaMag-12 automated nucleic acid extraction system. The extraction process included lysis, binding, washing, and elution steps. Initially,

by taking five colonies of bacteria growth on the MacConkey agar, a bacterial suspension was carried out and applied to 2 ml brain heart infusion broth and incubated at 37 °C for 24 hours, One ml of bacterial suspension was transferred to 1.5 ml microcentrifuge tube at 5000 g for 5 minutes, then discarding supernatant and applied 220 µl of BL2 buffer to the pellet and mixed for 5-10 s by vortexing. Thereafter, 200 µl of suspension was taken into the sample tube and 10 µl of positive control was applied to all tubes of the unit. Finally, these containing study samples tubes were inserted into the automated DNA extraction unit (Samag-12) [19]

The molecular technique for the detection of ESBLs encoding genes

The genomic DNA samples were used as templates for specific polymerase chain reaction (PCR) amplification and detection of the OXA gene. Specifically, the OXA-2F forward (5'-CCAAAGGCACGATAGTTGT-3′) and OXA-2B reverse (5′- GCGTCCGAGTTGACTGCCGG-3′) and OXA-1A forward(5'-AGCCGTTAAAATTAAGCCC-3′) and OXA-1B reverse (5′-CTTGATTGAAGGGTTGGGCG-3′) and primers were used in conjunction with Maxime PCR Pre-Mix kits (Intron Biotechnologies, Seongnam, Korea). The optimum conditions for detection of the OXA group I, II gene including initial denaturation at 95 °C for three min followed by 30 cycles each, of denaturation (95 °C for 45 s), annealing (50 °C for 45 s), and amplification (72 °C for 45 s); a final extension step (72 °C for 7 min) was achieved.

Agarose gel electrophoresis was performed to determine DNA amplicon sizes after extraction. Standard DNA ladders were used to determine the size of the DNA bands 11. The electrophoresis proceeded at 5 V/cm2, in TBE buffer, for 1.5 h. When electrophoresis was completed, each gel was read using ultraviolet light (336 nm).

Statistical analysis

The data were analyzed using Excel (Microsoft, Redmond, WA, USA), Minitab (ver. 17, Minitab, State College, PA USA), and SPSS (ver. 24, IBM, Armonk, NY, USA). The Chi-square test was used to investigate the association between antibiotic susceptibility (sensitivity, resistance, intermediate), ESBL production, and OXAII and OXAIII gene prevalence among the bacterial isolates; P-values < 0.05 were considered statistically significant

RESULTS

A total of one hundred clinical specimens were collected during this study. Among these specimens, seventy-five (75%) were culture-positive for Gram-negative bacteria, while fourteen (14%) represent for Gram-positive bacteria. Eleven samples (11%) were showing no growth under aerobic conditions (see the result table 1).

These isolates were screened using the Kirby-Bauer disc diffusion method and showing that 30 (85.7%) P. aeruginosa isolates were resistant to ceftriaxone, 22 (62.8%) to ceftazidime, 9 (25.7%) to aztreonam, 16 (45.7%) to cefepime, 30 (85.7%) to cefoxitin and 5 (14.2%) to imipenem. The K. pneumonia isolates were resistant to ceftriaxone 15 (88.2%), ceftazidime 14 (82.3%), aztreonam 15 (88.2%), cefepime 9 (52.9 %), cefoxitin 13(76.4%), and 3(17.6%) to imipenem.

The E. coli isolates were demonstrating resistance to ceftriaxone 21 (91.3%), ceftazidime 19 (82.6%), aztreonam 18(78.2%), cefoxitin 20 (86.9%), cefepime 8 (34.7%) and imipenem 2 (8.7%).

Based on the confirmatory test for the extended-spectrum β-lactamase production using the double-disk synergy test, 40 (53.33%) isolates were considered to produce ESBLs, including 22 (55%) P. aeruginosa, 8 (20%) K. pneumoniae, and 10 (25%) E. coli isolates.

PCR was then used to detect the presence of the OXAII and III genes in the K. pneumoniae, E. coli, and P. aeruginosa clinical isolates. Five (14.28%) isolates of P. aeruginosa were producing OXAII while three (8.57 %) of them produced OXAIII. Two(8.69%) isolates from E. coli for OXAII and III gene and 2(11.76%) isolates from K. pneumonia were positive for the presence of OXAII while Five(29.4%) of them produced OXAIII.

Table 1. The study Gram-negative study isolates versus site of specimen collection

Bacteria	Burn wounds n (%)	Non-burn wounds n (%)	Urinary tract n (%)	Otitis media (%)	No. of isolates n (%)
P. aeruginosa	24(68.57%)	-	-	11(31.43%)	35(46.7%)
E. coli	11(47.83)%	-	12(52.17%)	-	23(30.7%)
K. pneumoniae	8(68.57%)	4(23.5%)	-	5(29.4%)	17(22.6%)

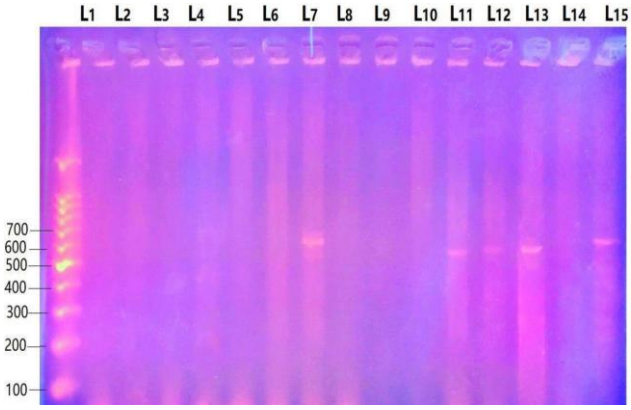

Fig.1: Distribution of OXAII gene in study isolates. Agarose gel electrophoresis (2%) with Novel Juice dye agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. Bands with OXAII gene (700bp) obtained from P. aeruginosa isolates, which showed that positive results are represented by (L7, L11, L12, L13, and L15) while L1, L2, L3, L4, L5, L6, L8, L9, L10, and L14 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker.

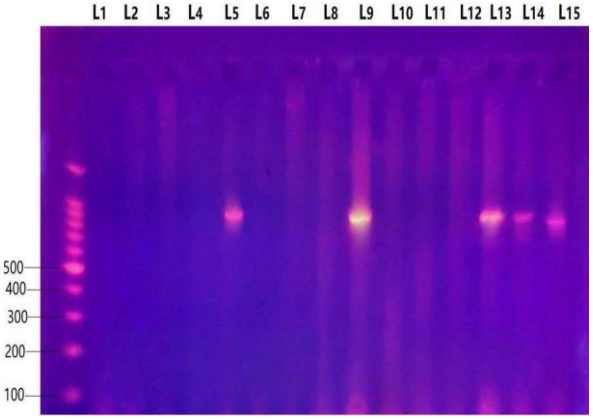

Fig.2: Distribution of OXAIII gene in study isolates. Agarose gel electrophoresis (2%) with Novel Juice dye agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. Bands with OXAIII gene (908bp) obtained from P. aeruginosa isolates, which showed that positive results are represented by (L5, L9, L13, L14, and L15) while L1, L2, L3, L4, L6, L7, L8, L10, L11, and L12 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker.

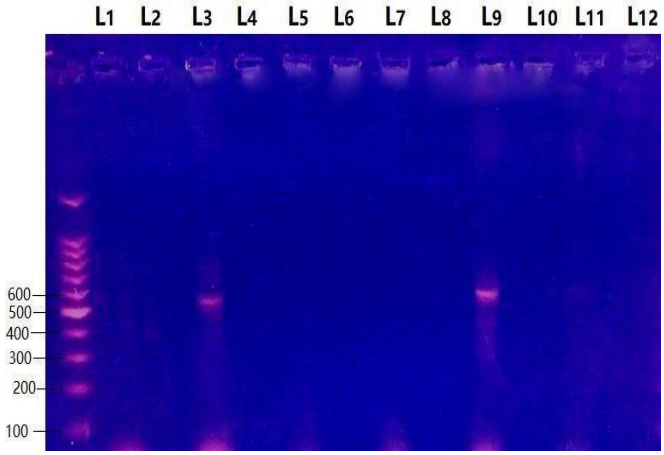

Fig.3: Distribution of OXAII gene in study isolates. Agarose gel electrophoresis (2%) with Novel Juice dye agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. Bands with OXAII gene (700bp) obtained from E. coli isolates, which showed that positive results are represented by (L3 and L9, while L1, L2, L4, L5, L6, L7, L8, L10, L11, L12, L13, L14, and L15 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker.

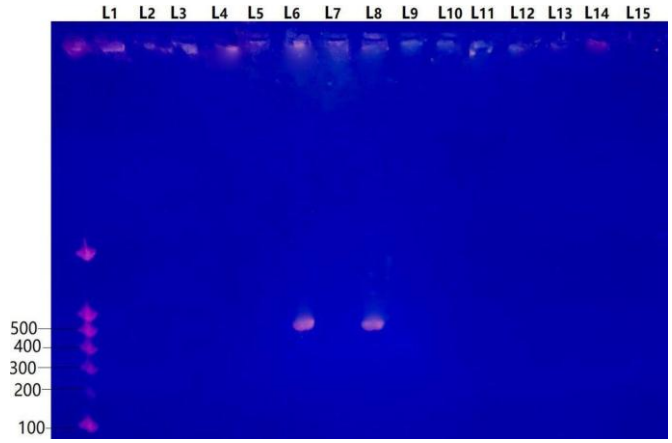

Fig.4: Distribution of OXAIII gene in study isolates. Agarose gel electrophoresis (2%) with Novel Juice dye agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. Bands with OXAIII gene (908bp) obtained from E. coli isolates, which showed that positive results are represented by (L6and L8, while L1, L2, L3, L4, L5, L7, L9, L10, L11, L12, L13, L14, and L15 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker.

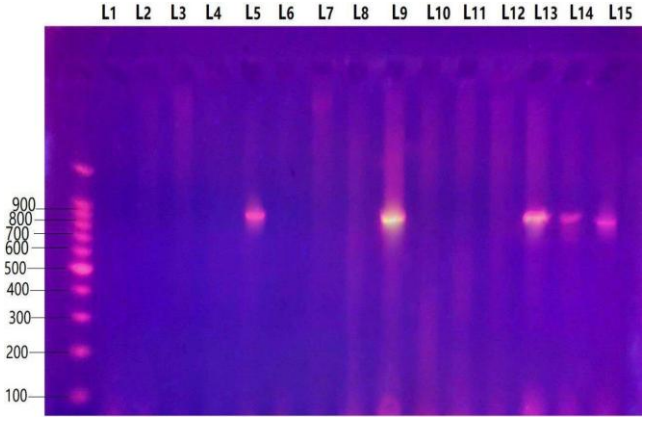

Fig.5: Distribution of OXAIII gene in study isolates. Agarose gel electrophoresis (2%) with Novel Juice dye agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. Bands with OXAIII gene (908bp) obtained from K. pneumoniae isolates, which showed that positive results are represented by (L5, L9, L13, L14, and L5, while L1, L2, L3, L4, L6, L7, L8, L10, L11, and L12 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker.

DISCUSSION

P. aeruginosa and Enterobacteriaceae have been classified as major pathogens. Since they are rapidly evolving resistance to antibacterial agents and by restricting therapeutic options, they pose a threat. The pathogenic agents are involved in several nosocomial infections, such as urinary tract infections (UTI) [23], infections of the bloodstream [24], pneumonia [25,26]. Infections of the lower respiratory tract [27] and wounds [28]. Mostly the majority of enzymes are acquired from clinically important oxacillinases which genes are embedded on gram-negative pathogen plasmids (P. aeruginosa, Acinetobacters, and Enterobacteriaceae) present in integeron or transposons and were widely reported Either the chromosomal genes or the transferable genes that are found on the plasmids or the transposons encode the different β-lactamases. These enzymes have been found commonly in Klebsiella and E. coli. However, all members of Enterobacteriaceae and other gram-negative bacilli have been reported to generate these enzymes[32].

The study isolates of Pseudomonas aeruginosa, E. coli, and K. pneumoniae reported that the highest rates of sensitivity to the antimicrobial agents. The inappropriate infection control in terms of inappropriate treatment of infections antibiotic use is likely to be an important predisposing factor in the appearance in hospital settings of resistant bacteria. These data demonstrate the need to create both surveillance of local and national antimicrobial resistance system to monitor the potential appearance of resistance

inside control the potential occurrence of resistance inside our system with healthcare. Additionally, the findings of the current research show that ESBL-producing genotypes are mainly derived from Intensive Care unit patients. This may represent a higher level of clinical as patients admitted to these wards are likely to experience an impact. Further, chronic predisposing factors and the broad range of antibiotics and therapeutic manipulations are revealed through in the invasive device use. It should therefore be observed that the development of nosocomial pathogens varied significantly based on various regions and countries, economies, hospitals, Also, in the common hospitals in different places. Factors for patients as well as age, the severity of infection, immune system, and duration of the presence of these microorganisms are also influenced by hospitalizations. Also, factors correlated with hospitals, such as efficiency, broad-spectrum antibiotics, and the diagnosis and use of therapeutic methods identified also should be taken into consideration Account, though information on all these variables is limited.

P. aeruginosa is a rising healthcare global concern and is referred to as a multidrug-resistant organism. In the present research, CAZ had a high rate of resistance of In agreement with the findings obtained by Woodford et al,**33** approximately 44.91% (53/118),. (2008) and Li et al. (2015)33, who found 47.00 and 51.60 % respectively in CAZ resistance rates. Studies in the United States, Europe, and the Mediterranean region, however, (Sader et al., 2014)34, and Turkish (Vahaboglu et al., 1997)35 showed lower rates of resistance to CAZs (16.10-24.00 and 28.00 %, respectively). A higher rate of CAZ resistance has been shown in other studies, such as in Iran (100.0%) (Mirsalehian et al., 2010)36. Among the 35(46.7%) isolates of P. aeruginosa researched in This study identified 22 (55%) as ESBLs producer, which was identical to the study results, while in other study conducted in China (Li et al., 2015) 33 (37.90 %). This number was less than that, however, Documented by Vaez et al. (2015) 37 and (2010) by Mirsalehian et al 36 (51.80 and 87.05 %, respectively).[31]

In this study, 19 (25.34%) of 75 isolates were PCR-positive for b-lactamase genes. Although Shih-Ping Lin, et al .,2012 38 revealed that35 (61.4%) of 57 P. aeruginosa isolates were PCR-positive for genes of β-lactamase. [1] Due to the limited number of surveillance studies finding clinical strains producing OXA-1 β-lactamases and the difficulty of finding OXA-1 β -lactamases, the prevalence of blaOXA-1 mediated resistance in India is not known. This mechanism of resistance has been reliably identified by clinical microbiology laboratories. In the present analysis, the phenotypic frequency of blaOXA-1 among K. pneumoniae was shown in Indian medical research. 39 % of centers, with 20.3 % of them generally identified genotype [33].

Aghazadeh M and et al (2016)[30]. The results of the PCR indicated that 56% of isolates bore the Genes of OXA Category I. For OXA Group II and OXA Group III, 26 and 19 percent of isolates were PCR-positive, respectively. The highest rate of resistance to most antibiotics was found in group I OXA isolates relative to group II or III genes. These findings showed that cephalosporin, anti-pseudomonas penicillin, and Carbapenems were highly immune. β-lactamases, enzymes that open the β-lactam ring and inactivate antibiotics. Different genes found on chromosomes or plasmids encode these enzymes. In the Ambler classification, these enzymes, which are often called extended-spectrum β-lactamases (ESBL), are divided into four categories, from A to D. Various class D ESBLs of Ambler, such as OXA-type ESBLs, were established and most commonly found in P. aeruginosa [34]. The frequency of OXA-1, OXA-group I, II, and III genes registered in France was 26 %, 5 %, 4 %, 4 %, and lower compared to France, respectively. [30] while the results of our study showed The OXA group II gene was expressed by only Five (14.28%) isolates of P. aeruginosa while three (8.57 %) of them produced OXAIII. Additionally, in Korea. The frequency of genes identified for OXA-group I, II, III, and OXA-1 was 34%, 6%, 16%, and 11%, respectively. Some variables can affect the diversity of antibiotic susceptibilities, such as diversity of antibiotic use and geographical difference [35].

Two(8.69%) isolates from E. coli for OXAII and III gene and 2(11.76%) isolates from K. pneumonia were positive for the presence of OXAII while Five(29.4%) of them produced OXAIII. Just two isolates of Pseudomonas aeruginosa expressed the OXA group I gene, Shih-Ping Lin et al, 2011[1] reported. In this analysis, there was no detection of CTX-M, PER, BEL, OXA group II and OXA group III genes.

Most OXA enzymes are located in the bacterial genome, but many of the genes of oxacillinase are part of class 1 integron gene cassettes[36] that are usually associated with plasmids or transposons that promote the transmission of OXA genes among bacteria. The major reason behind the spread of the OXA gene is the spread of a single transferrable form of Incl/M. 62 kb [37] conjugative plasmids in that resistance is

transferred from one strain to others. The OXA enzyme has been used in recent years. The activity has increased significantly with carbapenemase. Furthermore, In certain clinically important species, these enzymes are widely distributed, Acinetobacter baumannii[38], Pseudomonas aeruginosa, K., for instance. Pneumoniae and With E. coli respectively[39,40]. The type-β-lactamases of OXA, however, Oxacillinase has been found mainly in P. aeruginosa. The bacterial isolate containing higher OXA variants exhibits high levels of Resistance level not just to β-lactams but cephalosporins [28]. Antibiotic therapy needs to be based on laboratory confirmation of susceptibility which will help prevent the occurrence and spread of antibiotic-resistant strains.

The study recommended that PCR is so helpful for the identification of specific β-lactamase genes. Also, the study suggested that a significant presence of blaOXA-II and blaOXA-III genes among study isolates were detected and the highlighting for the need for suitable infection control strategies to effectively treat patients and prevent the further distribution of these resistant organisms.

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