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Co-expression of Extended Spectrum β-lactamase (ESBL) and AmpC β-lactamase among *Pseudomonas aeruginosa* clinical isolates in sebha medical center

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Abstract The aim of this study was to assess the resistance rate and pattern in *Pseudomonas aeruginosa* isolates recovered from patients admitted to Sebha medical center, Libya. *P. aeruginosa* is a known opportunistic pathogen which has become of great concern due to its high resistance to a wide range of antibiotics. This study was performed to evaluate the frequency of the Extended spectrum β-lactamase (ESBL) and AmpC β-lactamase enzymes in *P. aeruginosa* clinical isolates. Thirty-one non-repetitive clinical samples of *P. aeruginosa* were studied for their antibiotic sensitivity, ESBL and AmpC β-lactamase production. The phenotypic screening test for antibiotic showed 100% resistance to Penicillin, Ampicillin, Amoxicillin and β-lactamase inhibitor. The majority of the isolates were resistant to third generation cephalosporins (Ceftriaxone and Cefotaxime). In this study 29% of all isolates were resistant to gentamicin and 19% were resistant to ciprofloxacin. While all isolates were sensitive to Imipenem, 97% were resistant to Nalidixic acid and Fucidic acid. The resistance to Tetracyclins and Chloramphenicol was 94% and 90% respectively. All isolates exhibited ESBL phenotype but only 48% (15/31) confirmed as Ampc β-lactamase enzymes producers using Boric acid and EDTA.
Key wards: AmpC β-lactamase enzymes, ESBL, MDR, and *Pseudomonas* spp.

انتاج انزيمات البيتا لاكتاميز ذات الطيف الممتد بواسطة بكتريا الزائفة الزنجارية المنتشرة خلال العزلات السريرية في مركز سبها الطبي

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الملخص كان الهدف من هذه الدراسة هو تقييم معدل المقاومة والنمط في عزلات الزائفة الزنجارية المعزولة من المرضى الذي تم إدخالهم مركز سبها الطبي، ليبيا. بكتريا الزائفة الزنجارية هي إنتهازية ممرضة الأمر الذي جعلها مثيرة للقلق الشديد بسبب مقاومتها العالية لمجموعة كبيرة من المضادات الحيوية. تم إجراء هذه الدراسة لتقييم مدى إنتشار إنزيمات الطيف الممتد البيتا لاكتاميز ES-lactamase [ESBL] و Amp C-lactamase في العزلات الإكلينيكية لبكتريا الزائفة الزنجارية. تمت دراسة واحد وثلاثين عينة سريرية غير متكررة من الزائفة الزنجارية حساسيتها للمضادات الحيوية وانتاجها لانزيمات اللاكتاميز الممتدة الطيف lactamase–I AmpC و ESBL. أظهر اختبار الفحص المظهري للمضادات الحيوية مقاومة 100٪ للبنسلين، الأمبيسلين، أموكسيسيلين، ومثبط لانزيم β-lactamase. فكانت غالبية العزلات مقاومة للجيل الثالث من السيفالوسبورينات (سيفترياكسون وسيفوتاكسيم). وقد كانت في هذه الدراسة 29٪ من جميع العزلات مقاومة للجنتاميسين و 19٪ مقاومة للسيبروفلوكساسين. بينما كانت جميع العزلات حساسة لايمبينيم ، كان 97٪ مقاومين لحمض Nalidixic وحمض Fucidic. بلغت مقاومة التتراسيكلين و كلورامفينكول 94٪ و 90٪ على التوالي. أظهرت جميع العزلات النمط الظاهري لـ ESBL ولكن 48 ٪ فقط (31/15) أكدت أنها منتجة لإنزيمات Ampc-lactamase باستخدام حمض البوريك و EDTA.

الكلمات المفتاحية: إنزيمات إنزيم lactamase–I AmpC ، الطيف الممتد بيتا لاكتاماز (ESBL)، والأدوية المتعددة المقاومة، الزائفة الزنجارية

Introduction

Pseudomonas aeruginosa has become one of the most frequently isolated multidrug resistant nosocomial pathogen and it is associated with significant morbidity and mortality[1]. *P. aeruginosa* is intrinsically resistance to the most

available antibiotic, but acquired resistance through horizontal transmission of mobile genetic elements has also been reported[2]. Extended spectrum β-lactamase (ESBL) and AmpC β-lactamase roduction considered as the main mechanism of β-lactams resistance in enterobacteriaceae and other gram-negative bacilli. In addition, AmpC β- lactamases confers resistance to all β-lactams except fourth-generation cephalosporins and almost exists with multidrug resistance (MDR) [3][4]In gram-negative bacilli, AmpC β- lactamase enzymes are chromosomally encoded, though some are plasmid mediated as in enterobacteriaceae [5][6] and mutation in *ampD* is associated with overexpression of AmpC β-lactamase[7][8]The overexpression of AmpC genes may give a false negative ESBL test [9]which make the treatment even difficult. The efflux system over production and reduced permeability has also been reported to increase the resistance of *P. aeruginosa* to β-lactam antibiotics [10]Further, the resistance of *P. aeruginosa* to carbapenems has also been documented[11][12] [13]

The multidrug resistant *pseudomonas* strains pose serious clinical challenge to the public health, because the limited therapeutic options. However, the emergence of MDR *P. aeruginosa* strains are usually associated with prolonged stay in the hospital [14] and it is prevalent among intensive care unit patients more than other hospital patients.

Up to date, particularly in Sebha little information known regarding the prevalence of ESBL and and AmpC β-lactamase produced by *Pseudomonas*. This study will be the first report regarding the prevalence of extended spectrum β-lactamases among *P. aeruginosa* clinical isolates in the southern region of Libya. Indeed with increase of the frequency of Multidrug resistant pathogens, the monitoring of ESBL/Ampc production has become essential for surveillance and to provide effective treatment. The detection of ESBL activity in the presence of AmpC enzymes has become problem especially in the developing countries where the molecular techniques are not always available. Therefore, simple laboratory methods, for instance, clavulanic acid to detect ESBL and boronic acid to detect AmpC enzyme phenotypes are recommended and easy to perform in the routine laboratory work. The aim of this study was to characterize the ESBL and AmpC β-lactamase expressed by *P. aeruginosa* clinical isolates recovered from different sources in Sebha medical center, Libya.

Material and methods

1- Samples collection

All clinical samples were collected during the routine investigation and processed at Microbiology unit at laboratory department in Sebha medical center, Libya. The samples were collected from different patients and different sources (wound, abscess, ear, urine, oropharyngeal and rectal swabs) in a period from January 2015 to January 2017. The majority of the samples were collected from inpatients (23 clinical samples and 2 from incubator and sink), while 6 isolates from outpatient department (details are available in Table (1). A total of 31 non-duplicate clinical isolates were recovered on MacConkey's agar and 5 % sheep blood agar medium (Oxoid, UK) then incubated overnight at 37° C. All isolates were stained by gram stain and confirmed as *Pausudomonas* by using oxidase reagent. All strains were then confirmed as *P. aeuroginosa* by growing them on Kings medium B base. The designation numbers (MA) were given for all isolates and stored at -70 °C for further study.

Table 1: Distribution of *P. aeruginosa* isolates by departments used in this study

MA	Source	Department
MA9	Wound	Male Surgical ward
MA28	Urine	Out Patient department
MA54	Urine	Pediatric
MA57	Wound	Female Surgical ward
MA62	Skin Abscess	Neonate
MA71	Chest wall abscess	Female Surgical ward
MA89	Abscess	Male Surgical ward
MA95	Urine	Out Patient department
MA96	Abscess	Male Surgical ward
MA100	Otitis media	Out Patient department
MA110	Wound	Male Surgical ward
MA117	Leg abscess	Male Surgical ward
MA129	Oropharyngeal swab	Neonate
MA131	Oropharyngeal swab	Neonate
MA135	Oropharyngeal swab	Neonate
MA154	Wound	Male Surgical ward
MA166	Oropharyngeal swab	Neonate
MA169	Rectal swab	Neonate
MA189	Rectal swab	Neonate
MA170	Rectal swab	Neonate
MA200	Incubator	Neonate
MA202	Postoperative wound infection	Intensive care unit
MA211	Postoperative wound infection	Obstetric department
MA213	Postoperative wound infection	Obstetric department
MA219	Otitis media	Out Patient department
MA222	Urine	Out Patient department
MA229	Oropharyngeal swab	Neonate
MA232	Oropharyngeal swab	Neonate
MA241	Wound	Male Surgical ward
MA254	Stool	Out Patient department
MA267	Sink	Neonate

2- Determination of antibiotic susceptibility

According to Clinical Laboratory Standard Institute CLSI [15], The antimicrobial susceptibility test was performed for all isolates using Kirby Bauer disc diffusion method. Fresh colony was suspended in sterile water and the turbidity was adjusted to McFarland 0.5 standard and then streaked on Mueller-Hinton (MHA) agar (Oxoid, England). Plates were then incubated at 37 ° C for 16–18 h. Penicillin G (5µg), Ampicillin (10µg), Amoxicillin (20 µg), Augmentin (30 µg), Gentamicin (30µg), Ciprofloxacin (5µg),

Cefotaxime (30 µg), Ceftriaxone (30 µg), Imipenem (10 µg), Nalidixic acid (30 mg), Tetracycline (30 µg) and Chloramphenicol (30 µg) (Oxoid, UK) were applied. The diameter of inhibition zones was interpreted as recommended by CLSI [15]

3- Double disc synergy test for ESBLs phenotype detection

This experiment was done according to CLSI 2011 recommendations. Detection of the ESBL was performed on Mueller-Hinton agar plates (MHA) (Oxoid UK). (Amoxicillin 20 µg + clavulanic acid 10 µg) were kept at distance of 15 mm (center to center) to discs containing ceftriaxone (30 µg) and Cefotaxime (30µg) and incubated over night at 37 °C. The test was considered as positive when the zone size around the antibiotic disc increased towards the Amoxicillin + clavulanic acid disc.

4- Screen for AmpC β-lactamases production

The resistance to Cefoxitin was used as indicator for AmpC β-lactamases production by *P. aeruginosa* isolates. The phenotypic detection of AmpC enzymes production was carried out using boric acid as β-lactamases inhibitor[16]. In this test, cefoxitin disc was immerged in 20 µl of Boric acid and then left to dry at room temperature for 10 minutes. The plates were then incubated at 37°C for overnight. The diameter of the growth-inhibitory zone around Cefoxitin disc with boric acid was compared with that without boric acid. The results considered as positive when the diameter was >5 mm larger than that without boric acid. To enhance the release of β-lactamases 10 µl of EDTA (0.1 M) was added to Cefoxitin and boric acid [17]Then the zones of inhibition around the Cefoxitin discs with and without EDTA were compared.

Results

Our data showed 100% resistance to all β-lactam drugs used in this study (Penicillin, Ampicillin, Amoxicillin) as well as to β-lactamases inhibitors (clavulanate). Further, the resistance to third generation cephalosporines was 94% and 100% resistance to Ceftriaxone and Cefotaxime respectively. Our study also showed that resistance to Gentamicin was 29%. Moreover, the resistance rate was in both tetracycline and chloramphenicol 94% and 90% respectively. All isolates were resistant to Cefoxitin (100%), while 97% were resistant for both Nalidixic and Fucidic acids. Interestingly, our study did not detect any resistance to Imipenem and it was the most effective drug as it showed the maximum sensitivity rate of 100%. Ciprofloxacin was the second most effective antibiotic with resistance rate is 19% (see Fig. 1).

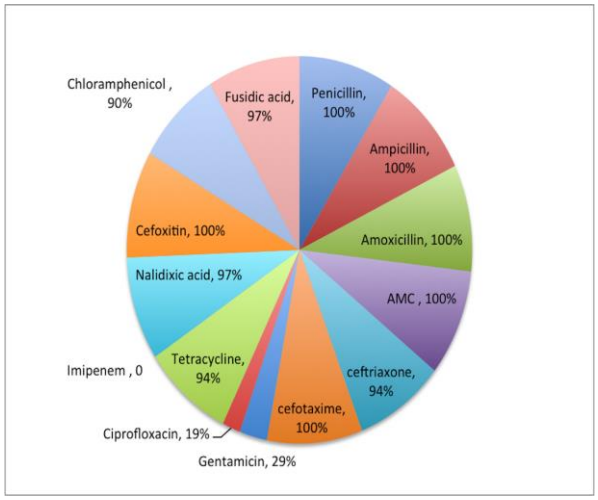

Figure1: Antimicrobial resistance patterns of *P. aeruginosa* isolates collected from patients attended Sebha medical center, Libya.

Our data also showed that some strains exhibited resistance to different groups of antibiotics. These strains were mainly isolated from neonate department 39% followed by surgical departments 29% and outpatient department 19% (Fig. 2).

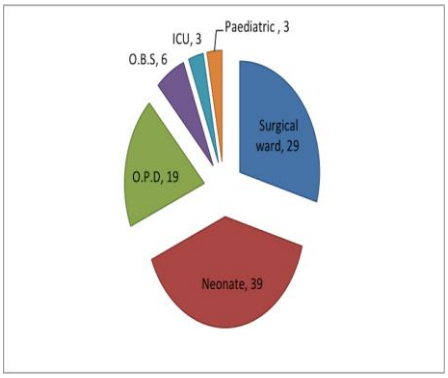

Figure 2: Distribution of Multidrug resistant (MDR) *P. aeruginosa* strains among different departments at Sebha medical center, Libya.

In the double disc synergy test for ESBL detection, the test did not show any augmentation of the zone toward the Cefotaxime or Ceftriaxone, so the isolates were suspected to be ampC β-lactamase co-producers. To confirm AmpC β-lactamase co-production by *P. aeuroginosa* isolates, all strains were tested for their susceptibility to cefoxitin. The isolates that exhibited reduced zone around Cefoxitin were suspected to be AmpC β- lactamase producers and confirmed by adding boric acid to Cefoxitin disc. A ≥5 mm increase in the zone diameter of Cefoxitin in combination with boric acid was considered positive for AmpC production compared to that with Cefoxitin alone. When EDTA was added, the zone around Cefoxitin with boric acid has increased and became more obvious (Fig. 3).

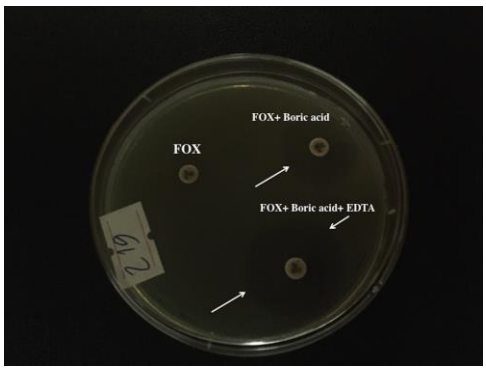

Figure3: Phenotypic detection **of** ESBL and AmpC lactamase enzymes production by in *P. aeruginosa clinical* isolates. It show An augmentation of the inhibition zone around the Cefoxitin (FOX) with boric acid and more with EDTA compared to cefoxitin (FOX) alone.

Despite most of the isolates were resistant to third generation cephalosporins, none of them were confirmed as ESBL producer by double disc synergy test. On the other hand, only 15 were confirmed as Ampc producers by adding boric acid and EDTA although all exhibited Cefoxitin resistant.

Discussion

P. aeruginosa has become one of the most frequently isolated nosocomial pathogen in the hospital and its resistance to almost any of the available antibiotic has increased the mortality and morbidity[18], [4][19].

P. aeruginosa pathogen can survive in moist environment (tapes, sinks) and has ability to form biofilm which increases the virulence of the organism [20][21] [22][23] In health care settings, Ventilators have been reported as a source of *P. aeruginosa,* but health care workers can also be a reservoir for this organism [24][25][26].

In this study 31 clinical isolates were collected from different departments in Sebha medical center, Libya. Considering the distribution of *P. aeruginosa* isolates, the majority were isolated from Neonate (39%), followed by surgical wards (29%) and then from outpatient department (19%). This could be due to low immunity in neonate, prolonged hospitalization and other risk factors including systemic diseases such as Diabetes and using of immunosuppressive therapy [27] Regarding neonate department, the majority of isolates were obtained from patients but two were from incubator and sink. This confirms findings from other studies that reported this organism as highly adapted organism and it can survive in the moist environment for example sink and faucets on the water [25][26]

In the present study, all isolates were obtained from hospitalized patients and they were highly resistant to β-lactams antibiotics such as Ampicillin, Amoxicillin and penicillin. This finding was similar to that obtained from other studies [28][29] Moreover, our data showed that the resistance of *P. aeruginosa* isolates to cephalosporins was high and this result is consistent with the one reported by [29]. Although several studies have confirmed such high resistance to ceftriaxone[30] [31][32] yet the current study showed a higher value of resistance. Such resistance to cephalosporins could be attributed to the indiscriminate use of this antibiotic as broad-spectrum empirical therapy in the last years. Other researchers have also reported a high resistance to ceftriaxone with similar result to the present study [33] [34]Interesting findings show that, our results differed from those reported by Ibukun et al, who found that 79.4% of *P. aeruginoas* isolates were highly sensitive to ceftazidime. The data in the present study showed that all *P. aeruginoas* isolates were resistant to β-lactamase inhibitors (e.g. clavulanic acid) (100%), and this has been confirmed by other studies[35][36]

Gentamicin was found to be the best antibiotic of choice to treat *P. aeruginosa* on 2005 in Sebha, South Libya, and our present study showed that this antibiotic can still be used to treat this organism where the resistance rate is 29%. Other studies have also reported a similar result with low resistance to aminoglycosides [36]

P. aeruginosa isolates in our study were found to be highly susceptible to Imipenem followed by Ciprofloxacin and this could be due to restricted use of these antibiotics in this hospital. This result is in agreement with [37]where they reported that*P.aeruginosa* isolates were 100% sensitive to Imipenem which shows promising effect in the treatment, while other studies showed different results with varying degree of susceptibility to imipenem ([38], [39]

Also high resistance rates were observed for other antibiotics such as chloramphenicol, Tetracycline, Nalidixic acid and fucidic acid. This suggests that these antibiotics cannot be included in the treatment strategy for *P. aeruginosa* infections. Such high resistance to chloramphenicol has also been reported[40][41]

According to [42]the isolates in our study considered as multidrug resistant (MDR) since they exhibited resistance to three or more antibiotics. In addition, the percentage of MDR among all isolates was somewhat high and all of them showed a resistance to more than three antibiotics. The MDR *P. aeruginosa* isolates were mainly disseminated among inpatient and in particular neonates than outpatient and this is likely to be related to increased antibiotic use. This finding is in agreement with other researchers who have recorded a similar result [43]

Despite the fact that all isolates were resistant to third generation of Cephalosporin, none of them was confirmed as ESBL producer by confirmatory test recommended by CLSI, as they were 100% resistance to β-lactamase inhibitors (inconclusive data). However, the ESBL detection may be masked by co-production of Ampc enzymes, and these isolates when tested by β-lactamase inhibitors (e.g. calvulanic acid), they are enhanced to over produce Ampc enzymes and gave negative results. The resistance to β-lactams can also be due to other mechanisms rather than β-lactamases production (e.g. loss of proteins on outer membrane and efflux pumps [44]

While all isolates were resistant to Cefoxitin, only 15 were found as AmpC enzymes producers by using boric acid and EDTA. In *Pseudomonas* spp. the resistance to Cefoxitin, however, may be mediated by other mechanisms for instance loss of outer membrane protein or altered target sites [45]. Other studies have also reported a similar result where they found that the resistance to Cefoxitin can be exhibited by both Ampc production and loss of porin expression via mutation in the porin gene [45][46] For this reason, further investigations regarding ESBL and Ampc enzymes production are needed and molecular typing with plasmid profile of the multidrug resistant *P. aeruginosa* isolates, could provide that.

Conclusion

Emergence of ESBL, AmpC-type β-lactamases and MDR among *P. aeruginosa* strains is a serious problem which is complicated by significant health problem resulting in increasing morbidity, mortality and high health care cost. Luckily, all isolates of *P. aeruginosa* were fully sensitive to Imipenem. Regular anti-microbial susceptibility monitoring is therefore vital and essential to control the misuse of antibiotics.

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Conflict of interest

We have no conflict of interest

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