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Emergence of AmpC β-Lactamase– and Metallo-β-Lactamase–producing *Klebsiella pneumoniae* in Sebha, Libya

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ABSTRACT

The emergence of AmpC β-lactamase– and metallo-β-lactamase–producing Gram-negative bacteria has become a global concern. In the last 2 years, the resistance to broad-spectrum β-lactam antibiotics caused by *Klebsiella pneumoniae* has been noted among clinical isolates in Sebha Medical Center, Libya. During a period of 2 years, 22 nonrepetitive *K. pneumoniae* strains were obtained from neonates and identified in the microbiology unit. This study aimed to assess the antimicrobial resistance profile and phenotype of extended-spectrum β-lactamase (ESBL)– and AmpC β-lactamase–producing *K. pneumoniae* isolates. The antibiotic susceptibility of all isolates was evaluated according to the Clinical Laboratory Standards Institute guideline. Further screening using boric acid and ethylenediaminetetraacetate (EDTA) were performed to study metallo-β-lactamase (MBL and AmpC enzyme production. The study found that 100% of isolates were resistant to β-lactam group, and 91% were resistant to β-lactamase inhibitors. Moreover, 23% (5/24) of all isolates exhibited ESBL phenotype, and 59% (13/22) were AmpC enzyme producers. The resistance to other broad-spectrum antibiotics, for example, aminoglycosides and quinolones, was also observed in this study. This study reported the first case of carbapenem-resistant *K. pneumoniae* MBL in Sebha, in the south of Libya. This carbapenemase-producing strain exhibited remarkable resistance to cephalosporins, fluoroquinolones, and aminoglycosides. This study showed that AmpC and MBL screen tests were simple and could routinely be performed in the clinical laboratory using EDTA and boric acid.

Key words: AmpC enzyme, ESBL, *Klebsiella pneumoniae*, metallo-β-lactamase, neonates, opportunistic organism

INTRODUCTION

The emergence of antimicrobial resistance, especially to the β-lactam group, which is the most commonly used antibiotic to treat various infectious diseases, is particularly important because it limits the therapeutic options, thereby increasing the morbidity and mortality rates [1, 2]. In Enterobacteriaceae, antibiotic resistance is linked to different mechanisms, for example the production of a certain type of enzymes named as extended-spectrum β-lactamases (ESBLs). ESBLs are plasmid-mediated and efficiently hydrolyze penicillins, third-generation cephalosporins, and aztreonam, which are commonly used to treat infections [3, 4]. In addition, antibiotic resistance due to AmpC β-lactamases, 16S rRNA methylases, aminoglycoside-modifying enzymes, and carbapenemases has also been reported [5]. ESBL-producing bacteria remain susceptible to carbapenems, and the activity of these enzymes is inhibited by clavulanic acid [6, 7].

In *K. pneumoniae*, AmpC beta-lactamases are located on plasmids, while in other Enterobacteriaceae spp., these enzymes are either plasmid or chromosomally encoded [8, 9]. AmpC β-lactamases confer resistance to cephamycins (e.g., cefoxitin and cefotetan) and β-lactamase inhibitor combinations [6]. Moreover, *K. pneumoniae* has also been found to harbor carbapenemase enzyme (KPC), which confers resistance to carbapenems such as imipenem and meropenem [10]. The emergence of carbapenem-resistant Enterobacteriaceae spp. in general and *K. pneumoniae*, in particular, has become a major public health problem due to lack of effective antibiotics [11], increasing the morbidity

and mortality rate especially in critically ill patients [12, 13]. Carbapenemases are usually expressed by plasmids or transposons, which can easily be transmitted to other bacteria via horizontal gene transfer [14]. Resistance to other groups of antibiotics has also been reported in Gram-negative bacilli [15,16]. *K. pneumoniae* are among the most frequently isolated opportunistic pathogens and found to be highly resistant to multiple antibiotics such as aminoglycosides, fluoroquinolones, and sulfonamides [17,18].

After 2011, multidrug-resistant *K. pneumoniae strains* were isolated from Libyan refugees in European hospitals [19, 20, 21] and also from a Libyan patient transferred to a Tunisian hospital [22].

Other studies also documented the emergence of ESBL-, AmpC enzyme–, and carbapenemase-producing bacteria in Libya. They isolated ESBL- and AmpC-positive *E. coli* and *K. pneumoniae strains* from patients admitted to Libyan hospitals in Tripoli, Zleiten, and El Khoms, Libya [23, 24].

Although ESBLs and AmpC β-lactamases have been discovered years ago, little is known about the presence of these enzymes in Libya in general and Sebha in particular. Therefore, the early detection of multidrug-resistant pathogens may help to avoid their transmission in the hospital and inappropriate antibiotic therapy.

This study aimed to determine the frequency of ESBL-, AmpC-, and carbapenemase-producing *K. pneumoniae* among hospitalized patients in the Department of Microbiology, Sebha Medical Center, Sebha, Libya, the biggest hospital in the south of Libya.

MATERIALS AND METHODS

Clinical isolates

All the clinical isolates were taken from neonates between January 2015 and January 2017. A total of 22 nonduplicate clinical isolates of *K. pneumoniae* were recovered. All these isolates were grown on MacConkey agar and 5% sheep blood agar medium (Oxoid, England) and then aerobically incubated overnight at 37°C. They were identified using the API20E system (bioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed using the Kirby–Bauer disk diffusion method. All isolates were tested for their susceptibility to antibiotics according to the Clinical Laboratory Standards Institute (CLSI) guideline [25]. Fresh colony (or colonies) was suspended in sterile water and streaked on Mueller–Hinton (MHA) agar (Oxoid) using McFarland 0.5 as control and incubated at 37°C for 16–18 h. The diameter of the inhibition zone for each antibiotic test was interpreted according to the CLSI recommendations [25]. The following antibiotics were used in this test: penicillin G (5 µg), ampicillin (10 µg), amoxicillin (20 µg), augmentin (30 µg), erythromycin (30 mg), gentamicin (30 µg), ciprofloxacin (5 µg), ceftazidime (10 µg), ceftriaxone (30 µg), imipenem (10 µg), nalidixic acid (30 mg), tetracycline (30 µg), and chloramphenicol (30 µg). They were supplied by Oxoid.

Phenotypic detection of ESBLs using double disk synergy test

This study was performed according to the CLSI 2011 criteria [25]. The ESBL test was conducted when the organisms showed a zone of inhibition of <22 mm to ceftazidime or <23 mm to ceftriaxone. In this test, a suspension of a fresh colony was streaked on MHA (Oxoid). A combination of amoxicillin 20 µg and clavulanic acid 10 µg was placed centrally on the plate at 15-mm distance (center to center) from the disks containing ceftriaxone (30 µg) and ceftazidime (10 µg). The plates were incubated overnight at 37°C. An enhancement of the inhibition zone between the disks (ceftazidime/ceftriaxone) and clavulanic acid was considered as ESBL positive.

Screening for AmpC β-lactamase production using cefoxitin

The resistance to cefoxitin 30 µg was used for the screening of AmpC β-lactamase producers in *K. pneumoniae* isolates. Based on the CLSI 2011 criteria [25], all isolates showing an inhibition zone of <18 mm were considered as AmpC β-lactamase producers and subjected to a confirmatory test using boric acid and ethylenediaminetetraacetate (EDTA).

Phenotypic detection of AmpC β-lactamase production

The AmpC enzyme production by *K. pneumoniae* was phenotypically detected using boric acid as a β-lactamase inhibitor, as recommended by Kiener in

1978 [26]. Boric acid (20 µL) was dispensed onto commercially available cefoxitin disk. The disk was then left to dry at room temperature for 10 min. The agar plates (MHA with tested organism) were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around the cefoxitin disk with boric acid was compared with that without boric acid. The test was considered positive when the diameter of the growth-inhibitory zone around cefoxitin with boric acid was >5 mm larger than that without boric acid. Based on the procedure proposed by Black in 2005 with a slight modification [27], EDTA was added to the cefoxitin disk immersed in boric acid to increase the permeability of bacterial cells, thus promoting the release of β-lactamases into the environment. Then, the zone of inhibition around the cefoxitin disk with and without the EDTA was compared.

Phenotypic detection of carbapenemase production

The strain showing resistance to imipenem was further subjected to carbapenemase production assay. In this assay, imipenem and imipenem/EDTA disks were used. Further, 10 µL of EDTA (0.1M) was added to the imipenem disk and left to dry at room temperature for 10 min, as described by Yong 2002 [28]. The test organism suspension was adjusted to McFarland 0.5 standards and then inoculated on the Mueller–Hinton agar plate. Two imipenem disks, one with EDTA and the other without, were placed on the plate. The results of the two disks were compared after 16- to 18-h incubation at 37°C. An augmentation of the inhibition zone around imipenem with EDTA of at least 5 mm was considered as MBL positive.

RESULTS

A total of 22 bacterial isolates were analyzed in this study. Regarding the β-lactam group, all the isolates were found to be resistant to penicillin, ampicillin, amoxicillin, and cephalexin, while the resistant to augmentin and ceftriaxone was 91% (n = 21) and 82% (n = 18), respectively. The resistance to gentamicin and ciprofloxacin was 77% and 4.5%, respectively, while three isolates showed an intermediate response. The results also showed that 95% (n = 21) of the whole collection was resistant to erythromycin. Regarding the carbapenem group, only one isolate exhibited resistance to imipenem while the rest were sensitive. In addition, 4.5% were resistant to chloramphenicol while 14% were resistant to both tetracycline and nalidixic acid (Fig. 1). In this study, the majority of the strains were multidrug-resistant (data not shown). Out of 22 isolates, 18 were suspected to be ESBL producers and confirmed by double-disk synergy test (DDST). Only 5% were considered positive owing to the augmentation of the inhibition zone between ceftriaxone with clavulanic acid and ceftriaxone alone (Fig. 2A and 2B). Although the third-generation cephalosporins were used to detect ESBL production, the effect of ceftazidime and ceftriaxone as ESBL indicators was variable (Fig. 2A and 2B). The performance of ceftazidime in detecting ESBL was poor compared with ceftriaxone. In synergy with Augmentin, all ESBL-positive isolates showed enhancement of the inhibition zone with ceftriaxone but not with ceftazidime. Ceftriaxone, unfortunately, was not able to detect ESBL in 13 isolates. Indeed, the level of enzyme expression and the co-occurrence of other resistance mechanisms (AmpC enzymes, efflux, and altered porins) should be considered, which may result in different phenotypes among ESBL-positive isolates. The expression of AmpC enzymes was suspected in this study. Hence, strains (n =13) that could not be confirmed as ESBL positive were screened for AmpC β-lactamase enzyme production using the cefoxitin disk and confirmed using boric acid as an AmpC inhibitor. Boric acid alone could not confirm the expression of AmpC enzymes; however, when EDTA was added, the zone of inhibition around the cefoxitin disk with boric acid became obvious (Fig. 2D). The data showed that 12 out of 13 strains were AmpC producers and only one strain did not show any difference in the zone of inhibition around the cefoxitin disk with boric acid (inconclusive). Indeed, the inconclusive isolate needs further confirmation using a molecular technique to study its genotype.

In the present study, one strain was suspected to be a carbapenemase producer based on its resistance to imipenem. This imipenem-resistant strain was confirmed as MBL producer when the zone of inhibition around the imipenem disk increased >5 mm by adding EDTA compared with the imipenem disk alone (Fig. 2C). This imipenem-resistant isolate was resistant to all antibiotics used in this test.

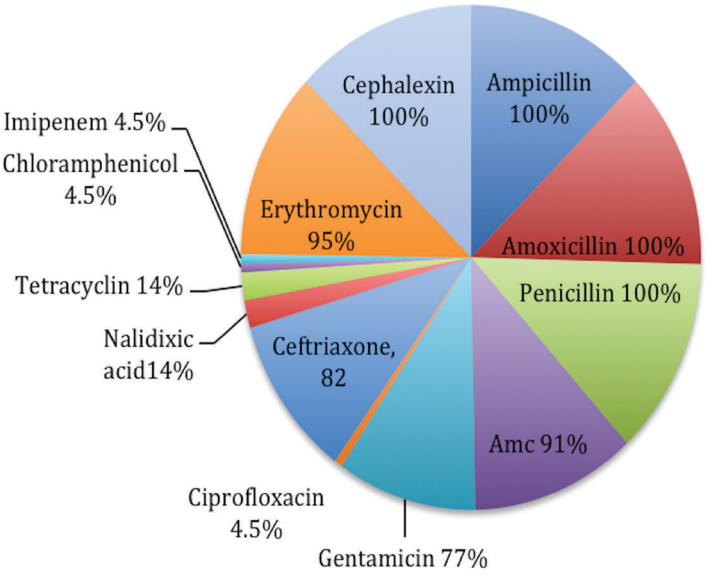

Figure 1: Antimicrobial resistance patterns of *K. pneumoniae* isolated from neonates at the Sebha Medical Center, Libya.

DISCUSSION

The *K. pneumoniae* infection is an increasing threat to health with increased morbidity and mortality rates due to the lack of effective antibiotics. The present study focused on the neonate department and highlighted the propagation of nosocomial pathogens during a period of 2 years (2015–2017). The infections by pathogenic organisms can be transmitted in neonates through breast milk [29, 30]. Moreover, in health-care settings, the health-care workers serve as a carrier for the transmission of microorganisms through their hands or contaminated gloves [31, 32, 33, 34]. The results showed that the rate of resistance to the β-lactam antibiotic group (penicillin, ampicillin, and amoxicillin) was very high, which might be attributed to the misuse, overuse, or improper use of this class of antibiotics in the hospital or in the community setting. This finding supported the results achieved by Abujnah and his group in 2015 [35]. They reported that 100% of the *K. pneumoniae* isolates were resistant to ampicillin. This finding also confirmed the previously reported results [36, 37, 38, 39]. Further, resistance to first- and third-generation cephalosporins was also high (100% and 82% resistance to cephalexin and ceftriaxone, respectively). This finding was expected because both of these drugs were extensively used in the hospital and community settings in the Sebha city. The results of several studies agreed with the findings of the present study [40, 41, 42].

Quinolones are not commonly the first choice for neonatal infections in the Sebha Medical Center, owing to their low resistance rate (4.5%) to ciprofloxacin. However, Apondi in 2016 [43] and Molina in 2015 [44] reported different results; they showed that the resistance rate to quinolones was 41.3% and 55.5%–60.6%, respectively. Abujnah in 2015 [35] found that 17% of *K. pneumoniae* strains were resistant to quinolones.

In the Sebha Medical Center, the aminoglycosides were used as the first line of treatment for infections caused by Gram-negative bacteria. Therefore, the resistance to gentamicin (77%) was high. A similar finding was reported by another study performed in Egypt [45]. Gentamicin resistance has also been reported as high in other Libyan hospitals [46]. The resistance of *K. pneumoniae* to gentamicin was 75%. Further, Apondi and his colleagues reported a similar result in 2016 [43]. They found that 83% of all isolates were resistant to gentamicin. On the contrary, González [47] showed a different result: 58.07% of all isolates were sensitive to gentamicin.

The carbapenem group has been shown to be effective against nosocomial infections caused by Gram-negative bacteria. It is considered the last reliable treatment for bacterial infections so far. In the Sebha Medical Center, the use of carbapenems has increased in the last few years because of the resistance of bacteria to other commonly used β-lactam antibiotics. The resistance to imipenem was very low in the

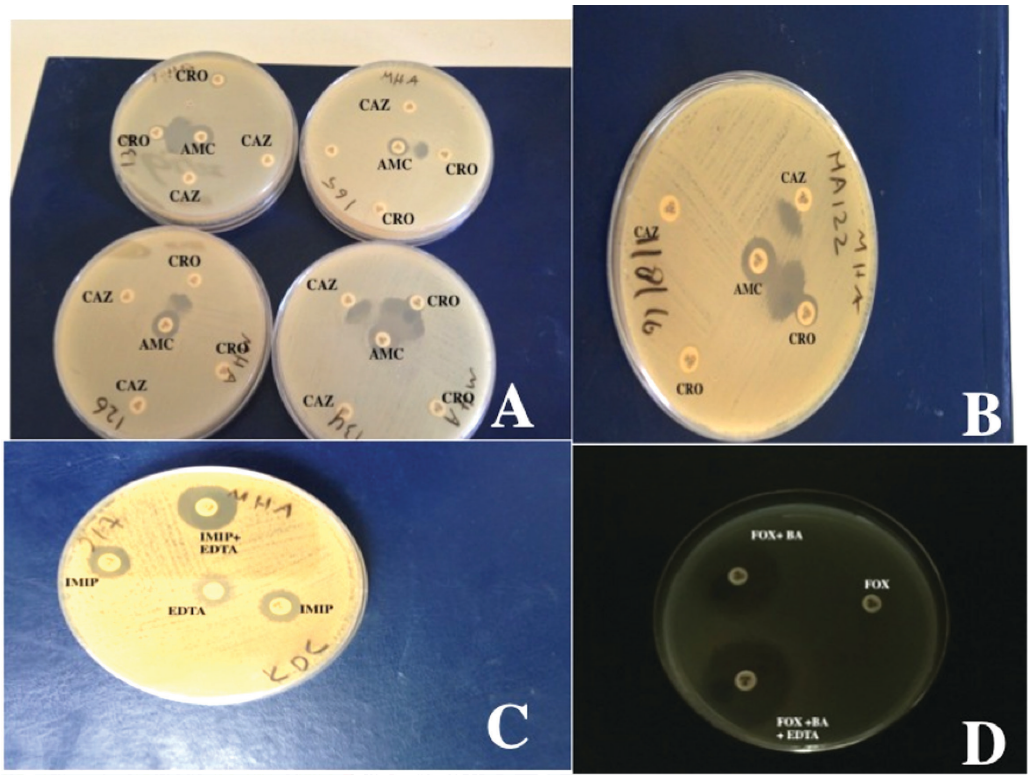

Figure 2: ESBL, AmpC lactamase, and carbapenemase enzyme detection in *K. pneumoniae* isolates from neonates. (A and B) Double-disk synergy test (DDST) (phenotypic detection of ESBLs); the augmentation of the zone of inhibition between clavulanic acid (AMC) and ceftriaxone (CRO) or ceftazidime (CAZ) disks. (C) An increase in the zone of inhibition around the imipenem disk with EDTA (IMIP/EDTA) compared with imipenem (IMIP) alone [phenotypic detection of carbapenemase enzyme (MBL)]. (D) Phenotypic detection of AmpC lactamase enzymes; an increase in the zone of inhibition around cefoxitin (Fox) with boric acid (BA) compared with cefoxitin (FOX) alone. The inhibition zone became more obvious after adding EDTA

present study compared with that in other studies [48, 49]. Indeed, imipenem-resistant *K. pneumoniae* isolate in this study showed extensive resistance to the most commonly used antibiotics.

In the present study, ESBL-, AmpC-, and carbapenemase-producing *K. pneumoniae* isolated from neonates admitted to the Sebha Medical Center, Libya, were characterized. The prevalence of ESBL and AmpC enzymes has increased worldwide [50, 51, 52]. However, the dissemination of ESBL and AmpC enzymes has also been reported in the neighboring country Tunisia [21, 38, 53, 54, 55]. In Saudi Arabia, Kader and his group (2005) also isolated ESBL-producing uropathogens from both inpatients and outpatients. They also reported a high resistance of these organisms to different antimicrobial agents [36]. Recently, the spread of ESBL and AmpC enzymes has been documented in Libya. In 2017, Zorgani and his group found that 54% of *K. pneumoniae* isolates were ESBL producers [23]. Further, in Zleiten and El Khoms, Libya, ESBL-producing *E. coli* was isolated from the fecal flora of pediatric age groups [24]. In the present study, ESBL enzymes were detected in 23% of all isolates and 59% were AmpC producers. Such coproduction of ESBL and AmpC enzymes was also reported by Zorgani in 2017. All AmpC enzyme producers were isolated from patients admitted to teaching hospitals in Tripoli, Libya [46]. Further, AmpC enzyme–producing *K. pneumoniae* isolates were also recovered from the pediatric department in Tripoli, Libya [23]. The prevalence of carbapenemase-producing *K. pneumoniae* has been recorded worldwide, including the Mediterranean region and North Africa [38, 21].

After Libyan conflict in 2011, *K. pneumoniae* isolates producing OXA-48 carbapenemase were recovered from patients transferred from Libya to Slovenia and Denmark [56, 20]. Moreover, OXA-48-producing *K. pneumoniae* isolates were isolated from patients admitted to Libyan hospitals [21]. This novel study

reported the emergence of AmpC enzyme– and carbapenemase-producing *K. pneumoniae* isolated from neonates admitted to the Sebha Medical Center, Libya.

The emergence of multidrug-resistant *K. pneumoniae* is alarming with a significant increase in the morbidity and mortality rates [57, 58]. In neonates with underdeveloped immunity, the prevalence of multidrug-resistant *K. pneumoniae* has been found as the main cause of neonatal infections especially in developing countries compared with developed countries [59, 60, 61, 62, 63, 64]. Further, the association between ESBL and AmpC enzymes produced by *K. pneumoniae* and multidrug resistance has been reported worldwide as the cause of mortality [65]. Although most of the isolates in this study showed resistance to multiple drugs, carbapenemase-producing *K. pneumoniae* exhibited resistance to all drugs. This finding was in agreement with other previously reported studies [60, 66]. Prolonged hospitalization and misuse and overuse of antibiotics were also considered risk factors, which increase the resistance rate among bacteria [63, 67]. However, this may be important especially in multidrug-resistant bacteria when the most common antibiotics are not effective, complicating the treatment of patients. In developing countries, the outspread of multidrug-resistant bacteria is considered as a clinical challenge because they are not usually detected using the routine susceptibility test and have a great potential to spread. The present study showed that the detection of ESBL, AmpC, and carbapenemase enzymes is not difficult and microbiologists can routinely do it to assess the outspread of multidrug-resistant bacteria, especially nosocomial pathogens. In developing countries where the molecular techniques are not always available, boric acid can help in detecting β-lactamase enzymes produced by nosocomial pathogens.

CONCLUSIONS

A periodic antibiogram study should be performed to limit the progress of multidrug resistance. This study highlighted the pattern of antibiotic susceptibility and prevalence of multidrug resistance among Gram-negative pathogens most likely acquired during patient stay in the hospital. Healthcare workers should routinely be screened for the presence of multidrug-resistant bacteria, and their education regarding hand hygiene and convenient aseptic techniques should be encouraged. Further, appropriate and effective good policies and drugs should always be considered to avoid the spread of multidrug-resistant bacteria especially among immunosuppressed patients.

DECLARATIONS

Availability of supporting data

All data generated or analyzed during this study are included in this manuscript; some supplementary files will be available from the corresponding author on request.

Competing interests

The authors declare no competing interests.

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