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PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF FIVE ANTIMICROBIAL RESISTANCE GENES ASSOCIATED WITH *Klebsiella pneumoniae* ISOLATED FROM BURN INFECTION PATIENTS

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ABSTRACT

The current study was carried out for the phenotypic and genotypic characterization of five antimicrobial resistance-associated genes in *Klebsiella pneumoniae* isolated from burn infection patients. Total one hundred three (103) bacterial samples (strains) were isolated from the 103 burn infection patients admitted at Middle Euphrates Burns Center of AL-Kufa City Iraq. Out of total isolated bacterial samples (103), there were 31 isolates (30%) identified as *Pseudomonas sp.*, 23 isolates (22.3%) as *K. pneumoniae*, 22 isolates as *Staphylococcus sps.* (21.4%), 11 isolates as *E. coli* (10.6%), 8 isolates as *Acinetobacter sps.* (7.8%), 5 isolates as *Enterobacter sps.* (4.9%), while the lowest prevalence (3 isolates) was reported for the *Proteus spp.* (3%). The antimicrobial sensitivity test indicated that all isolated *K. pneumoniae* have resistant (100%) against standard antibiotic Amoxicillin. While Imipenem is the only antibiotic that can inhibit the growth of all 23 isolates. Further, according to the phenotypic detection method, there were 14 isolates (61%) capable of production of extended spectrum beta lactamase (ESBL). Genotypic method to detect the presence of five antibiotic resistance genes by polymerase chain reaction proved that 13 isolates (56.5%) were *Tem* gene, 18 isolates (78.2%) were positive for *Shv* gene, 8 isolates (34.7%) were positive *Ctxm* gene, three isolates (13%) were positive for *Oxa* gene and 10 isolates (43.7%) positive for *AmpC* gene. Results of the study can be concluded that *K. pneumoniae* is the second causative agent that causes burn infection and has higher antibiotics resistance. Extended spectrum beta lactamase of *K. pneumoniae* was higher prevalence in burn infection and harbored many beta lactamase genes.

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1 Introduction

Hospital-acquired infections, such as burns, skin infections, and eventually reproductive system infections, urinary tract infections, blood, gastrointestinal soft tissue infections, and respiratory tract infections, are the leading causes of hospitalization (Rup et al., 2021). Burn injuries are of public health importance in both developed and developing countries. It is associated with several complications such as functional disability, severe scarring, and contractures of the affected body part (Jeschke et al., 2020). Burn injuries affect the skin to a large extent (Aljanaby et al., 2018; Hansen et al., 2019; Neog et al., 2021).

Infection is the most common complication of burn patients after admission, leading to higher morbidity and mortality (Navon-Venezia et al., 2017). The presence of multidrug-resistant pathogenic bacteria increases the impact of hospital-acquired infections (El-Mokhtar et al., 2021). Among the most common bacteria associated with burn infection, *K. pneumoniae* is one of the most widespread bacteria and the second pathogen after *Pseudomonas* (Sharahi et al., 2020). This might be due to the antibiotic resistance in bacteria, these resistance bacteria have antibiotics resistance plasmid and have high resistance against various antibiotics such as ampicillin and carbenicillin (Yap et al., 2013). Multidrug-resistant bacteria and extended spectrum beta lactamase with high prevalence distributions in both hospitals and the general population growth in *K. pneumoniae* and other Gram-Negative bacteria (Legese et al., 2017). Almost 390 distinct forms of ESBLs, including *bla* genes such as *tem* and *ctxm*, have been worldwide recognized (Barguigua et al., 2011). Furthermore, multidrug resistance and ESBL develop in *K. pneumoniae* when antimicrobials such as beta-lactams, cephalosporins, aminoglycosides, and others are used, resulting in a loss of care in

hospitals and ambulatory patients (Tang et al., 2017). The purpose of this study was to evaluate the incidence of *K. pneumoniae* as a causal agent in burn infections in hospitalized patients and also establish their antibiotic resistance patterns and associated gene using phenotypic and genotypic approaches.

2 Materials and Methods

2.1 Study design and numbers of burn swabs

This is a cross-sectional descriptive study carried out at the laboratory of Kufa University, Faculty of science, department of microbiology, Iraq during the period between August to December 2020. A total of 103 burn swabs (male and female) were collected from hospitalized patients at Middle Euphrates Burn Center in AL-Kufa City.

2.2 Ethical considerations

Before starting the study, the written consent and ethical approval were taken from all the selected 103 patients. Every swab sample was taken by an experienced physician or a healthcare team member from the hospital, and the collected swab samples were taken to the microbiology laboratory, University of Kufa, Iraq for further processing (Figure 1).

2.3 Culturing of Burns swabs and bacterial identification

The collected swab samples were stroked on the blood agar and brain heart infusion broth for 24 hours at 37°C for suitable bacterial growth. Bacterial isolation has been determined by blood hemolysis on blood agar, gram stain, oxidase test, catalase test, imvic test, motility test, coagulase test, and growth on MacConkey agar (MacFaddin, 2000; Aljanaby, 2013; Aljanaby, 2018).

Figure1 Burn swab samples collected from the patients admitted to the Middle Euphrates Burn Center of AL-Kufa City.

2.4 Antibacterial susceptibility test

Isolated bacterial strains were subject to antibiotic sensitivity or resistance tests, for this eleven different antibiotic discs were utilized: the majority of them being Oxoid™ discs made in the United States. Resistance to antibiotic assays was evaluated using disc diffusion and the Kirby-Bauer method on Mueller Hinton agar (Oxoid™, USA, PO5007A) (Bauer et al., 1966). For estimation of antibiotic sensitivity or resistance, the used antibacterial discs have Penicillin G 50 µg (CT0004B), Cefotetan 30µg (CT0268B), Gentamicin Sulfate 15 µg (CT0300B), Ceftazidime Sulfate 15 µg (CT0407B), Levofloxacin Sulfate 15 µg (CT0311B), Ciprofloxacin Sulfate 5 µg (CT0436B), Gentamicin Sulfate 15 µg (CT0317B), Ceftazidime Sulfate 15 µg (CT0432B), Levofloxacin Sulfate 15 µg (CT0326B), Gentamicin Sulphate 15 µg (CT0311B), CeftazidimeSulphate 15 µg (CT0056B) antibacterial solution was used in the above-mentioned concentration. To find out the diameters of inhibition zones, one unassisted eye measurement is sufficient. Subsequently, clinical and laboratory standards of CLSI guidelines 2020 were used to find out the exact diameter of inhibition zones. A bacterial isolate that was resistant to at least three different antibiotic classes is considered an MDR strain, If any bacterial isolate was resistant to all antimicrobial classes except two or three were considered as extensive drug resistance(XDR) (Leber, 2020).

2.5 Phenotypic detection method

Double disc synergy test (MDDST) as described by Kaur et al. (2013) was used to distinguish *E. coli* expressing extended spectrum beta lactamase (as described above) that phenotypically express EBSBA from regular *E. coli* (as described above). Every step in streaking and plating bacterial isolates (turbidity was adjusted to a 1 McFarland tube and 0.5 Himedia India) was carried out using a sterile swab (Himedia, India) on Mueller Hinton agar (Oxoid™, USA). Then, an AMC antibiotic disc (30µg) was inserted in the middle of the

agar plate, and the other aforementioned medications, such as CRO, CTX, and CAZ antibiotics were dispersed around the AMC disc (15 mm from center to center). Every plate was incubated at 37 °C in a microaerobic atmosphere for 24 hours, and then each plate was plunged into an incubator at 37°C temperatures for the next 24 hours. An increase in the inhibitory zone around the AMC disc was considered a good outcome for the extended spectrum beta-lactamase (Majeed & Aljanaby, 2019).

2.6 Extraction of total DNA

From the isolated bacterial strain DNA extraction was carried out as per the method described by Aljanaby & Alhasnawi (2017a). For this, aged water with five distinctively clean bacterial colonies was added to 500 microliters of distilled water, and the resulting solution was heated on a water bath for 30 minutes. This was followed by centrifugation @ 7500 rpm for 20 minutes and the supernatant was collected.

2.7 PCR and thermal cycling conditions

Five different primer sequences for the selected five genes which were employed in the present investigation and thermal cycling conditions are presented in tables 1 and 2. The PCR product samples were examined using gel electrophoresis with 2% agarose, to produce five µl aliquots. The electrophoresis was done using an electric current of 80 volts for 90 minutes and molecular size DNA nucleic acid marker (1000bp ladder, 50 to 1500 bp). Ethidium bromide was used as a staining agent (2 mg/ml), and observation was taken under ultraviolet light.

2.8 Statistical analysis

The percentages have been used in this study to compare all data and antibacterial resistance (Aljanaby, 2018; Tong et al., 2021).

Table1 Primers used in this study

Gene	Oligo Sequence (3'→5')	Product Size (bp)	Reference
<i>SHV</i>	F- GGCCGCGTAGGCATGATAGA R- CCCGGCGATTTGCTGATTTC	714	Ensor et al. (2009)
<i>TEM</i>	F- CAGCGGTAAGATCCTTGAGA R- ACTCCCCGTCGTGTAGATAA	643	
<i>CTX-M</i>	F-AACCGTCACGCTGTGTTAG R-TTGAGGCGTGGTGAAGTAAG	766	
<i>OXA</i>	F-ATATCTCACTGTTGCATCTCC R- AAACCCTTCAAACCATCC	618	Tuwaij et al. (2020)
<i>AMPC</i>	F-ATCAAACTGGCAGCCG R-GAGCCCGTTTATGCACCCA	550	Paterson et al. (2003)

Table 2 Programs of PCR thermo cycling conditions

Gene	Initial Denaturation (°C/ Time)		Cycling condition (C° / Time)			Number of Cycles	Final extension (°C/ Time)	Reference
	Denaturation	Denaturation	Annealing	Extension				
<i>TEM</i>	95 °C/5 min	94 °C/30 sec	52 °C/45 sec	72 °C/45sec	30		72 °C/7min	Ensor et al. (2009)
<i>SHV</i>	95 °C/5 min	94 °C/30 sec	55 °C/60 sec	72 °C/45sec	30		72 °C/7min	
<i>CTX-M</i>	94 °C/5 min	94 °C/45 sec	57 °C/45 sec	72 °C/45sec	30		72 °C/7min	
<i>AMPC</i>	94 °C/5 min	94 °C/45 sec	60 °C/45 sec	72 °C/60sec	35		72 °C/5min	Paterson et al. (2003)
<i>OXA</i>	94 °C/5 min	94 °C/60sec	55 °C/60 sec	72 °C/60sec	30		72 °C/10min	Tuwaij et al. (2020)

Figure 2 The number and percentage of total bacterial isolates obtained from the patients suffering from the burn infection.

Figure 3 Single metallic green colonies of *K. pneumoniae* onto chrome agar at 37°C for 24h.

3 Results

3.1 Total bacterial isolates

Figure 2 showed the numbers and percentages of bacterial isolates isolated from 103 patients (53 male and 50 female) with burn infections. The results of the current study revealed that among the 103 respondents, there were 31 (18 male and 13 female) patients were suffering from the infection of *Pseudomonas spp.*, 23 (12 male and 11 female) were from *K. pneumoniae* followed by 22 (11 male and 11 female) from *Staphylococcus spp.*, and 11 (4 male and 7 female) from *E. coli* (Figure 3). While there were 8 patients (3 male and 5 female) who were suffering from the *Acinetobacter spp.* infection, 5 (3 male and 2 female) were from *Enterobacter spp.*, and the lowest prevalence was reported for *Proteus spp.* with 3 isolates (2 male and 1 female).

3.2 Screening of antibiotics resistance

The results presented in Table 3 revealed that all 23 *K. pneumoniae* isolates had complete resistance (100%) against standard antibiotic Amoxicillin and Ceftriaxone while these isolates had high

resistance (91.3%) to Amoxicillin + Clavulanic acid combination. On the other hand, the results of this study demonstrated that *K. pneumoniae* also had resistance to Cefotaxime (73.9%), Ceftazidime (43.4%), Tetracycline (69.5%), Tobramycin (60.9%), and Ciprofloxacin (47.8%). While, there was low resistance against Levofloxacin (17.1%), Gentamicin (13%), and Amikacin (4.3%). Among the tested antibiotics, Imipenem was the only antibiotic that inhibited the growth of all 23 isolates.

3.3 Type of resistance

Results given in figure 4 suggested that 87% of *K. pneumoniae* isolates have multidrug resistance (MDR), while the remaining 13% have XDR, and no isolates have PDR characteristics (bacterial isolate resistance to all subclasses in all classes of antibiotics).

3.4 Extended-spectrum beta-lactamase (ESBL) phenotypic test

This test was carried out according to Double Disc Synergy Test (DDST). The results proved that out of 23 isolates, 14 isolates (61%) were capable of production of ESBL (Figure 5).

Table 3 Antibiotics sensitivity of the 23 *Klebsiella pneumoniae* isolates.

Antibiotics	Sensitive N (100%)	Intimidated N (100%)	Resistance N (100%)
Amoxicillin (25 µg)	0 (0%)	0 (0%)	23 (100%)
Amoxiclav (30 µg)	2 (8.7%)	0 (0%)	21 (91.3%)
Cefotaxime (30 µg)	5 (21.7%)	1 (4.4%)	17 (73.9%)
Ceftazidime (30 µg)	8 (34.8%)	5 (21.8%)	10 (43.4%)
Ceftriaxone (30 µg)	0 (0%)	0 (0%)	23 (100%)
Levofloxacin (30 µg)	13 (56.8%)	6 (26.1%)	4 (17.1%)
Imipenem (10 µg)	20 (87%)	3 (13%)	0 (0%)
Tetracycline (30 µg)	7 (30.5%)	0 (0%)	16 (69.5%)
Ciprofloxacin (5 µg)	7 (30.8%)	5 (21.7%)	11 (47.8%)
Gentamicin (15 µg)	19 (82.7%)	1 (4.3%)	3 (13%)
Amikacin (30 µg)	22 (95.7%)	0 (0%)	1 (4.3%)
Tobramycin (10 µg)	5 (21.7%)	4 (17.4%)	14 (60.9%)

Figure 4 Type of resistance (Numbers and percentages) in 23 isolates of *K.pneumoniae* Figure 5 Phenotypic confirmatory test of ESBL for *K. pneumoniae* by Double Disc Synergy Test.

3.5 Molecular detection of antibiotics resistance associated genes

All the antibiotic resistance-linked gene findings were given in figure 6. Of the 23 isolates, there were 13 (56.5 %) isolates that had *tem* gene (Figure 7), 18 isolates were found positive for the *shv* gene (Figure 8), 8 (34.7 %) isolates were harboring the *ctxm* gene (Figure 9), three (13 %) were positive for *oxa* gene (Figure 10) while 10 isolates (43.7%) were positive for the *AmpC* gene (Figure 11).

Figure 6 Prevalence of antibiotic resistance genes in *K. pneumoniae*

Figure 7 The prevalence of *Tem* gene in *K. pneumoniae* amplified by PCR and separated by agarose gel electrophoresis

Figure 8 The prevalence of *Shv* gene in *K. pneumoniae* amplified by PCR and separated by agarose gel electrophoresis

Figure 9 The prevalence of *Ctxm* gene in *K. pneumoniae* amplified by PCR and separated by agarose gel electrophoresis

Figure 10 The prevalence of *Oxa* gene in *K. pneumoniae* amplified by PCR and separated by agarose gel electrophoresis

Figure 11 The prevalence of *AmpC* gene in *K. pneumoniae* amplified by PCR and separated by agarose gel electrophoresis

4 Discussion

Antibiotics are one of the most widely used therapies for the treatment of many kinds of bacterial infections. Antibiotic resistance is a significant clinical issue in the treatment of bacterial infections globally; in the current study, all isolates were evaluated for antibiotic resistance as per the CLSI-recommended antimicrobial drugs (2020). Frequent uses of antibiotics without sensitivity testing are the primary factor that significantly

contributes to the development of multidrug resistance, which resulted in the selection and spread of antibiotic resistance in the bacteria under clinical care (Hernando-Amado et al., 2019). The fast development of antibiotic-resistant pathogens disables the effectiveness of the treatments which is developing a dangerous issue for public health (Borges et al., 2016).

In the current study, the results presented in Table 3 revealed that all 23 *K. pneumoniae* strains have complete resistance against

standard antibiotic Amoxicillin and Ceftriaxone, while these strains high resistant (91.3%) against Amoxicillin + Clavulanic acid, and no resistance was reported against Imipenem. These results are in agreement with the findings of Kandela (2011) those who reported the most striking results that *K. pneumoniae* isolates were resistant to Cefotaxime (80 %), and highly resistant to Cephalosporin, and moderately resistant to Ceftazidime. Similarly, Lari et al. (2013) reported *K. pneumoniae* antibiotic resistance against various antibiotics such as Cefotaxime (91%), Tobramycin (86%), Tetracycline (80%), Gentamicin (72%), Amikacin (71%), Amoxicillin/ clavulanic acid (71%), Chloramphenicol (63%) and Imipenem (54%) in Iran.

In other studies, Mariya & Hatkar (2015) have reported moderate to higher *K. pneumoniae* antibiotic resistance to Amikacin, Salih et al. (2018) against Nalidixic acid, and Aljanaby & Alhasnawi (2017b) against Amoxicillin and Amoxicillin with Clavulanic acid combination. Results of the current study are contradictory to the findings of Al Sanjee et al. (2020), those who reported lower *K. pneumoniae* resistance against the most commonly used antibiotic drugs such as Amoxicillin (20%), Cefotaxime (0%), Ciprofloxacin (70%), and Gentamycin (40%), this variation might be due to experimental conditions or absence of the antibiotic resistance plasmid or gene.

There are two types of antibiotic resistance i.e. acquired and natural resistance (Landecker, 2016). Natural resistance refers to the capability of an organism to avoid antibiotic effects by having inherited properties. This kind of resistance was reported in various kinds of gram negative bacteria such as *E. coli* which has natural resistance against Penicillin G due to the absence of specific antibiotic receptors on its surface (Lewies et al., 2019). On the other hand, there is an acquired resistance that is determined as quality alterations in the genetic materials (mutation) of the organism which helps microorganisms to defend themselves against antibiotics (Bengtsson-Palme et al., 2018). Further, horizontal gene transfer is the way in that bacteria acquired their resistant genes from a donor bacterium (Soucy et al., 2015).

Many *K. pneumoniae* strains have antibiotic resistance due to the presence of *tem* and *shv* plasmid-encoded class A enzymes (Miryala et al., 2020). Mutations in these *tem* and *shv* genes have resulted in extended spectrum beta lactamase enzymes, which have been well characterized in the Enterobacteriaceae family, especially in *K. pneumoniae* (Aljanaby & Alhasnawi, 2017a). The results of the current study revealed the presence of 56.5% *tem* (Figure7) and 78.2% *shv* gene from the genetic combination of *K. pneumoniae* which helpful in the establishment of antibiotic resistance of this bacteria (Figure 9). In contrast to this, Ahmed et al. (2013) reported only 10% of the *shv* gene from the *K. pneumoniae* isolates. Other studies by Izadí et al. (2014) indicated that 43% of *K. pneumoniae* bacterial isolates were

ESBL's positive outpatient and have *tem* (87%), *shv* (69.64%) gene while 48.21% isolates have the presence of both *tem* and *shv* gene. The results of Aljanaby & Alhasani (2015) showed that the prevalence of *shv* and *tem* gene was 91.66% and 100% respectively.

Further, the results of the current study reported that 8 (34.7%) isolates were positive for the *ctxm* gene (Figure 9). It has been reported that there has been a clonal expansion of *ctxm* gene rating *K. pneumoniae* isolates in Saudi Arabia, Egypt, Iran, Oman, Lebanon, the Gulf Council, Iraq, Kuwait, and Turkey (Zowawi et al., 2014; Yezli et al., 2015). Additionally, in conjunction with *ctxm* and virulence genes creating multidrug-resistant *K. pneumoniae*, the therapy combinations in this treatment regimen might further exacerbate infections and lower the probability of successful therapy (Derakhshan et al., 2016).

The molecular detection of antibiotics resistance genes revealed that 3 (13%) isolates were positive for the *oxa* gene (Figure10). This result is contradictory to the findings of Sugumar et al. (2014), and Amirkamali et al. (2017), those who reported the presence of 46 and 37.3% *oxa* gene respectively. Further, results presented in figure11 suggested that 10 isolates (43.7%) were positive for the *AmpC* gene. This result is similar to Gupta et al. (2012) who reported that 32% of isolates have the *AmpC* gene. Class D lactamases are also called oxa-cillinases or oxa-lactamases because they can hydrolyze oxalic acid more efficiently than benzylpenicillin. These enzymes were first discovered in the late 1960s and early 1970s and showed hydrolytic activity against penicillin and oxacillin (King et al., 2016).

Conclusions

K. pneumoniae is a second causative agent that causes burn infection and had a high ability to resistant antibiotics. Further, extended spectrum beta lactamase *K. pneumoniae* was high prevalence in burn infection and harbored many beta lactamase genes.

Conflict of Interest

This study does not have any conflict of interest

Source of Funding

There was no fund in this study, fund by authors themselves

Data Availability

Data presented in this study are available on fair request to the corresponding author.

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