Antibiotic Resistance Pattern and Extended Spectrum β-Lactamase Producing *Acinetobacter baumannii* Isolated From clinical samples

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Abstract
Background: The emergence of extended spectrum beta lactamase and carbapenemase production of *Acinetobacter baumannii* is a great concern and major cause of nosocomial infections due to its ability to production of extended spectrum beta lactamase and carbapenemase enzymes.
Objective: To assess Emergence of high prevalence of extended-spectrum beta-lactamase producing *Acinetobacter baumannii* among hospitalized patients.
Materials and Methods: A total of 50 clinical samples taken from admitted patients. Clinical specimens were collected aseptically and inoculated on blood agar and MacConkey agar media. Antimicrobial susceptibility test, and ESBL production were performed as CLSI guideline.
Results: Out of 50 clinical specimens 24% (12/50) were culture positive growth of *Acinetobacter baumannii* infection. Overall, 6/12 (50%) of isolates had have ESBL producing *Acinetobacter baumannii* infection. The overall multidrug resistance rate of the isolated bacteria was 88% (66/75). The majority of highest resistance rate was Amikacin in all 12 isolates (100%) and the lowest resistance was recorded toward Tigecycline only in 2 isolates (16.67%), respectively, while the more effective antibiotic against *Acinetobacter baumannii* was Colistin and the rate of resistance is 100%.
Conclusion: The incidence rates of ESBL, and antimicrobial resistant *Acinetobacter baumannii* infections are high. Therefore, treatment should be based on culture and antimicrobial test result and minimize the use of antibiotics empirically.
Keywords: *Acinetobacter baumannii*, antibiotic resistance, extended-spectrum β-lactamase genes.

Introduction

Acinetobacter baumannii is a typically short, almost round, rod-shaped (coccobacillus) Gram-negative bacterium. *A. baumannii* can cause infections in the blood, urinary tract, and lungs (pneumonia), or in wounds in other parts of the body. It can also “colonize” or live in a patient without causing infections or symptoms, especially in respiratory secretions (sputum) or open wounds (Lin and Lan, 2014).
It can be an opportunistic pathogen in humans, affecting people with compromised immune systems, and is becoming increasingly important as a hospital-derived (nosocomial) infection (Abdar et al., 2019). Bacteria of this genus lack flagella, whip-like structures many bacteria use for locomotion, but exhibit twitching or swarming motility. This may be due to the activity of type IV pili, pole-like structures that can be extended and retracted (Abd El-Baky et al., 2020).

Hospital-acquired infections are most commonly seen in critically ill patients; specific risk factors for developing an *A. baumannii* infection include prolonged hospital stays, immune suppression, advanced age, presence of comorbid diseases, major trauma or burns, previous antibiotic use, invasive procedures, and presence of indwelling catheters or mechanical ventilation (Wong et al., 2017). Due to the already poor prognosis of critically ill patients who acquire *A. baumannii* infections, it is difficult to attribute a definitive mortality rate (Freire et al., 2016); however crude morality rates have ranged from 23 to 68% (Morris et al., 2019); however, it is currently unknown as to whether host or bacterial factors are responsible for the difference in disease presentation between community and hospital infections (Morris et al., 2019).

The amount of colonization of *A. baumannii* is increasing in hospitalized patients, especially in patients who have been hospitalized for a long time or have received broad-spectrum antibiotics or anticancer drugs. Today, the spread of antibiotic resistance genes by creating multiple drug resistance (MDR) has become an important problem in the treatment of *A. baumannii* infection. Different previous studies have shown that *A. baumannii* is resistant to the majority of antibiotics including fluoroquinolones, cephalosporins, carbapenems, tetracycline, and aminoglycosides. Antimicrobial resistance of *A. baumannii* is mediated by acquired and inherent mechanisms, which include enzymatic changes, a mutation in the target genes, changes in the permeability of the outer membrane, and increased expression of the efflux pumps. Pumping mediation out of the bacteria, due to the mechanisms of the efflux, is one of the reasons for MDR (Moosavian et al., 2020).

The antimicrobial resistance in this nosocomial pathogen is mainly caused by β-lactamases inactivating enzymes, alteration of membrane porin channels, and mutations that change cellular functions. However, the most common mechanism of resistance is the production of hydrolytic enzymes of antimicrobial agents, including extended-spectrum β-lactamases (ESBLs) (Khoshnood et al., 2017). In recent years, the production of ESBL has become the main mechanism of resistance to β-lactam and other antibiotics in *A. baumannii*. ESBL enzymes confer resistance to penicillins, cephalosporins, monobactams and other antibiotic classes. Multidrug-resistant (MDR) patterns due to ESBL production in pathogenic bacteria are now becoming prevalent in hospitals worldwide, posing a public health challenge, including treatment failure, prolonged hospital stay and increased mortality rates (Jiang et al., 2020).

Recently, >300 different ESBL types have been described in Gram-negative bacteria. The bla_{TEM} and bla_{SHV} types have been recognized as the most prevalent ESBL genes conferring antibiotic resistance in pathogenic bacteria worldwide. Previous studies have revealed that the number of clinical isolates harboring the $bla_{\text{CTX-M}}$ gene type has also increased in the last few years. The $bla_{\text{CTX-M}}$ family

includes >130 β-lactamase variants classified into five distinct groups: *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-25}$ (Dirar et al., 2020).

The genetic diversity of ESBL-producing *A. baumannii* has progressively increased, posing challenges to hospital authorities due to their ability to confer antibiotic susceptibility and limit therapeutic options. The characterization of resistance genes encoding ESBL-producing *A. baumannii* is a powerful tool for developing evidence-based guidelines for combating antibiotic resistance in the clinical setting (Ibrahim et al., 2021, Abdar et al., 2019).

Regarding the growing importance of ESBLs in antibiotic resistance and its impact on treatment failure, this study was performed to evaluate the antimicrobial susceptibility patterns of clinical strains and determine the frequency of *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX}$ genes in *A. baumannii* strains isolated from several hospitals in Erbil city.

Materials and methods

Collection and identification of isolates

A total of 50 infectious samples were collected from sputum sources. Samples were immediately transferred to the laboratory in cooler with ice packs. Samples were inoculated on to blood agar (Merck, Germany) and MacConkey agar (Merck, Germany) and incubated aerobically at 37°C for 24 hrs., non-hemolytic, opaque and creamy colonies on blood agar and non-lactose fermenting colonies on MacConkey agar were further sub-cultured on MacConkey agar and incubated for another 24 hrs., at 37°C to obtained pure colonies. Primary identification of *A. baumannii* isolates was based on the colony morphology and Gram staining reaction. Standard biochemical tests such as citrate, oxidase, catalase, urease test, and indole production were used to identify the *A. baumannii* isolates with using VITEK 2 system.

Finally, identity of the isolates was confirmed via polymerase chain reaction (PCR) (Alpha PCRmax, UK) based on identifying 16S rRNA gene (Forward primer: CAC CTT CCG ATA CGG CTA CC and reverse primer: GTT GAC TGC CGG TGA CAA AC). The PCR program was as follows: 40 cycles of denaturation at 95 °C for 30 sec, annealing at 59°C for 45 sec, and extension at 72°C for 60 sec, with a final elongation step at 72°C for 10 min. The PCR products were detected by 1.2% agarose gel electrophoresis. The amplicon size of 16S rRNA gene was 372 bp (Hamasalih and Abdulrahman, 2020).

Antimicrobial susceptibility screening

According to the references of the Clinical and Laboratory Standards Institute (CLSI) (Humphries et al., 2021), antimicrobial sensitivity testing was carried out against the following antimicrobials using disk diffusion method; Amikacin AK 30 µg, Cefepime CFP 30 µg, Ceftazidime CAZ 30 µg, Ciprofloxacin CIP 5 µg, Colistin

CST 5 µg, Gentamicin G 10 µg, Imipenem IMP 30 µg, Levofloxacin LEV 5 µg, Meropenem MEM 10 µg, Netilmicin NET 30 µg, Piperacillin PIP 30 µg, Tigecycline TGC 30 µg, and Tobramycin TOB 10 µg (Bioanalyse, Turkey). A lawn of test *A. baumannii* was prepared by evenly spreading 100 µL inoculums ($1.5*10^8$ CFU/ml) according to 0.5 McFarland standard solution with the sterilized swab on top of the entire surface of Mueller Hinton Agar plate (Himedia, India). The disks were resolutely applied onto the agar plates surface within 15 minutes of inoculation (Bakr et al., 2021).

Genomic DNA extraction

According to the manufacturer's instructions, genomic DNA was extracted from pure cultures through the BETA BAYRN Genomic DNA Extraction Kit (BETA BAYRN, Germany); extract was eluted with an elution buffer of 50 µL. Before running PCR, extracts were stored at -20°C. The NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA) was used to evaluate DNA concentration and purity in which one µL of the genome DNA was used to define DNA concentration and purity.

Detection of ESBLs associated genes by PCR

The molecular analysis of ESBL-associated genes (bla_{SHV}, bla_{TEM}, and bla_{CTX}) in the most of ESBL producing *A. baumannii* strains was performed employing standard multiplex PCRs. The defined and the sequences of the primers used in this research was listed in Table 1 (Bakr et al., 2021).

Table 1. List of primers used for Multiplex PCR amplification.

Target gene	Primer	Sequence (5′-3′)	Amplicon size
bla_{TEM}	Forward	TCG CCG CAT ACA CTA TTC TCA GAA TGA	445-bp
	Reverse	ACG CTC ACC GGC TCC AGA TTT AT	
bla_{SHV}	Forward	ATG CGT TATATT CGC CTG TG	747-bp
	Reverse	TGC TTT GTT ATT CGG GCC AA	
bla_{CTX}	Forward	ATG TGC AGY ACC AGT AAR GTK ATG GC	593-bp
	Reverse	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	

The primer mixing for detecting ESBL-associated genes was made in an Eppendorf tube and 25 µL of the reaction medium were employed for the PCRs (7 µL Nuclease free water + 10 µL Master mix + 1 µL of each primer mix + 2 µL of genomic DNA template). The PCR cycling protocols were as shown below: initial denaturation for 10 minutes at 95°C, denaturation for 30 seconds at 95°C, annealing for 90 seconds at 60°C, elongation for 120 seconds at 72°C with 40 cycles, and final elongation for 10 minutes at 72°C. The PCR cycles were performed using an Alpha PCRmax

thermal cycler. Electrophoretically separated PCR amplicons with Red Safe dye on a 1% agarose gel and observed under UV light.

Data analysis

For statistical analysis, the GraphPad Prism (V. 9.3) software was used. The chi-square test and Fisher's exact test were used to assess the qualitative data. The discrepancy was regarded statistically significant for all analyses when the p-value was less than 0.05.

Results and discussion

Identification of *A. baumannii* isolates

In the Rozhawa Hospital in Erbil, Iraq, fifty samples were collected from sputum sources. Twelve isolates from these samples were identified as *A. baumannii* utilizing several biochemical tests and a VITEK 2 system with GN card. The research was conducted in the biotechnology laboratory of the biology department at Salahaddin University in Erbil, Iraq. *A. baumannii* isolates were identified by various tests that included: Gram-negative coccobacilli, oxidase negative, catalase positive, urease negative, citrate positive, and indole negative. In the present study, 12 non-duplicative *A. baumannii* isolates were collected from 4 (33.33%) females and 8 (66.67%) males with the mean age of 51.43±0.8 years. For additional validation of the identification of *A. baumannii* isolates, all twelve isolates were identified by PCR utilizing the specific 16S rRNA gene (Figure 1.)

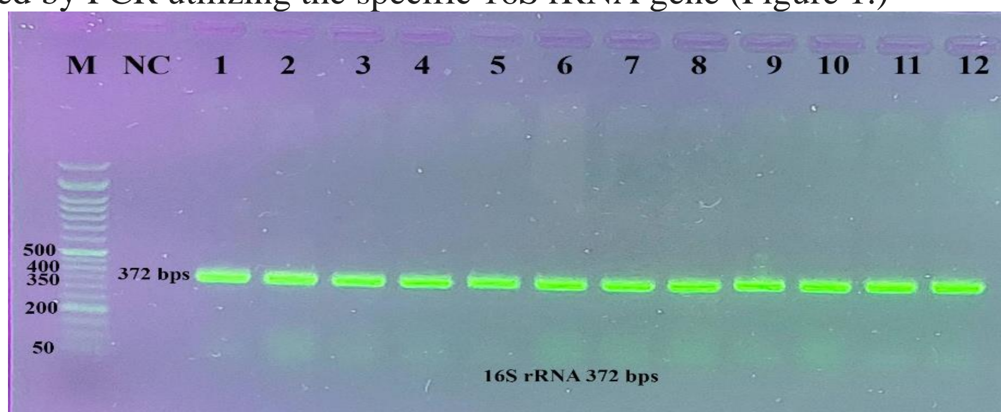

Figure 1. Agarose gel electrophoresis of 16S rRNA gene amplified. Lane M: DNA ladder 50bps, lane NC: negative control, lanes 1-12 positive amplicon for 16S rRNA gene at 372 bps.

Detection of ESBL–SHV, -CTX-M and -TEM genes

Of all 12 *A. baumannii* isolates, 83.33%, and 50% isolates were harboring HSV, and CTX-M genes, respectively. The TEM gene was found the most prevalent and present in the all studied isolates (Figure 2).

The results showed that most of the *A. baumannii* isolates were producing ESBLs (50%). Reports from Iran illustrate the high prevalence of drug resistance and multi drug resistance in *A. baumannii* especially against most effective antibiotics such as imipenem and meropenem (Feizabadi et al., 2008). Ting et al. (2013) investigated the drug resistance genes in 7 strains of imipenem-resistant *A. baumannii* including TEM, SHV, CTX-M, DHA, CIT, IMP, VIM, KPC, OXA-23. They detected TEM (100%) and OXA-23 (100%) genes among the isolates, but the other genes such as SHV, CTX-M, DHA, CIT, IMP, VIM, KPC could not be detected from 7 strains of imipenem-resistant *A. baumannii*. In the present study, consistent with Ting et al. (2013), just some of the genes have been detected including SHV (58%), TEM (20%) and VIM (30%). In another study by Shahcheraghi et al. (2011) in Tehran, Iran, they showed that the MBL encoding genes included bla VIM-2, bla SPM-1, bla IMP-2, bla GES-1, bla OXA-51, bla OXA-23 genes among 203 *A. baumannii* isolates. They reported that 6 isolates produce MBLs and 94 isolates produce OXA-type carbapenemase. Their finding suggests that in Tehran the prevalence of MBLs producing *A. baumannii* strains is lower than that of the present study from Hamadan City. They detect *bla*SPM-1, *bla*GES-1, *bla*OXA-51, *bla*OXA-23 genes among 6, 2, 94 and 84 isolates of the bacterium, respectively. The previous research by Rezaee et al. (2013) revealed genes coding for IMP, SPM-1, VIM, PER-1, VEB-1, TEM, SHV, GES-1, and CTX-M among 76 Acinetobacter spp. Also, they reported that 37% of isolates carried at least one of the *bla*PER-1 or *bla*TEM-1 genes and 13.15% of their studied isolates reported to harbor *bla*TEM-1 gene, which is similar to that of the present study (20%). Also, none of their studied *A. baumannii* isolates were harboring for *bla*VEB-1, *bla*SHV-, *bla*CTX-M-2 and *bla*GES-1.

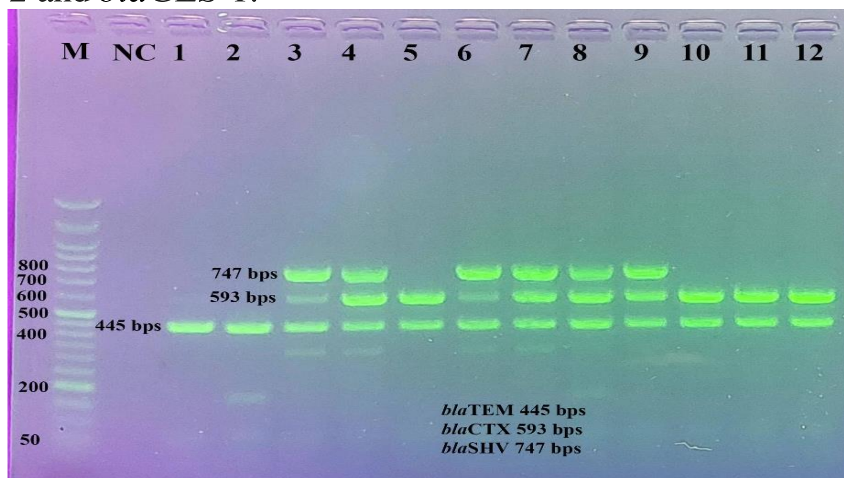

Figure 2. Agarose gel electrophoresis of multiplex PCR of ESBL genes (blaCTX, blaTEM, and blaSHV). Lane M: DNA ladder 100 bps, lane NC: negative control, lanes 1-12 positive for blaTEM gene with 445 bps, lanes 3-12 positive for blaCTX gene with 593 bps, lane 3,4, 6, 7, 8, and 9 positives for blaSHV gene with 747 bps.

Antimicrobial resistance

All isolates demonstrated complete resistance to amikacin. Colistin had the most significant efficiency towards *A. baumannii*, as all the isolates were sensitive to colistin. Sixty-two isolates (95.38%) were non-susceptible to ciprofloxacin and recorded the highest percentage of resistance after the amikacin antibiotic (Figure 3).

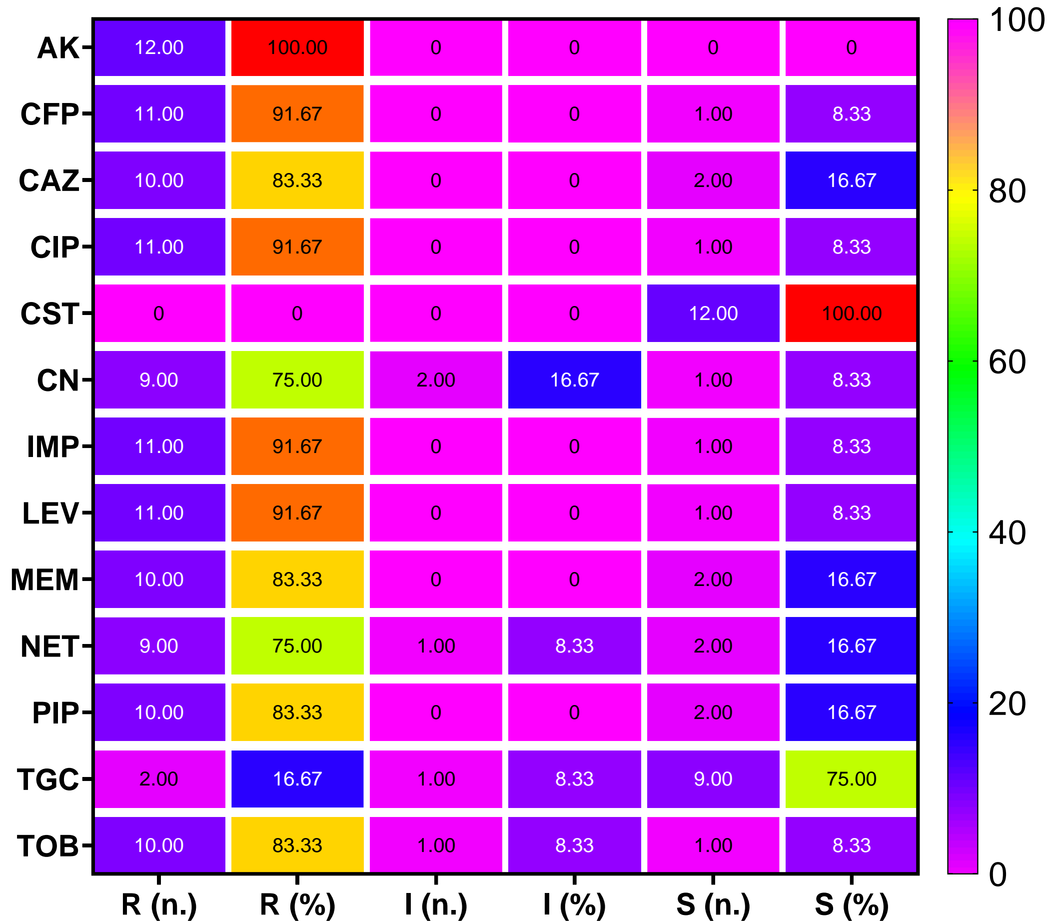

Figure 3. The heat-map clarifies the degrees of susceptibility (resistant, intermediate, and sensitive) of *A. baumannii* to different tested antimicrobial agents.

Acinetobacter baumannii is an opportunistic bacterium that could induce nosocomial illnesses due to antibiotic resistance (Jahangiri et al., 2019, Nowak et al., 2014). It has a significant capacity for colonization and dissemination among hospitalized patients due to its ability to adhere to a variety of surfaces and objects (Choi et al., 2010). This microorganism can tolerate a broad spectrum of current medications by gaining resistance determinants and up-regulating intrinsic resistance pathways (Zeighami et al., 2019). *A. baumannii* with multidrug resistance produces severe infections and significant mortality, particularly in immune-

compromised people (Howard et al., 2012, Poirel et al., 2011). In our analysis, all *A. baumannii* isolates were resistant to several antimicrobials. All were resistant to amikacin and sensitive to colistin which is consistent with findings from other Iranian studies (Vahdani et al., 2011, Saffari et al., 2017). Also, results of Zarifi et al. (2017) on the antibacterial susceptibility pattern revealed that in *A. baumannii* the high resistance was to all antibiotics except colistin, as resistance rates to imipenem, meropenem, ceftazidime, cefotaxime, cefuroxime, ceftriaxone, Cefepime, ertapenem and ampicillin/sulbactam were 97.9%, 98.1%, 96.4%, 97.9%, 99.3%, 97.9%, 97.9%,98.6% and97.1%, respectively. The most effective antibiotic against *A. baumannii* was colistin with susceptibility 97.9% followed by amikacin with sensitivity 27.1 % (Table 2).

Conclusions

In conclusion, resistance against 3rd generation cephalosporin is high which corresponds to the genotypic ESBLs production. The epidemiologic diversity of ESBLs encoding genes in A. baumannii may suggest that new ESBLs strains are constantly emerging. Forthcoming studies emphasis on study of other ESBL encoded genes. This study revealed both the need for more caution in antibiotic consumption and the alarming rate of resistance.

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