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PHENOTYPIC AND MOLECULAR DETECTION OF *ACINETOBACTER BAUMANNII* ISOLATED FROM PATIENTS IN DUHOK CITY-IRAQ

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ABSTRACT:

Acinetobacter baumannii (*A. baumannii*) is an opportunistic pathogen and one of the most importnat Multidrug resistant microorganisms responsible for a vast array of nosocomial infections. 41 (6.8 %) isolates of A. baumannii were obtained from a total of 603 clinical samples of burn and sputum during the period from March 2018 to February 2019. Twenty two isolates (3.6%) of *A. baummanni* were recovered from burn infections and nineteen isolates (3.2%) from sputum specimens. Identification and characterization of these isolates was accomplished by the aid of selective media (CHROM agar Acinetobacter) and was finally confirmed by VITEK 2 identification system test. Molecular identification utilizing genus and species-specific primers to detect the 16S rRNA and $bla_{\text{OXA-51}}$ was also applied. The Antibiotic resistance testing was done by the Kirby-Bauer disc diffusion method, 24.4% of the isolates were Multi-Drug Resistance (MDR), while 65.9% were extensive drug resistance (XDR). All isolates were absolutely resistant (100 %) to most of the antibiotics in use: cefixime, cefotaxime, ceftriaxone, ceftazidime, piperacillin-tazobactam, amoxicillin-tazobactam, while the resistant profile for the other antibiotics can be represented as follows 98 %, 95 %, 90 %, and 83 % For both ciprofloxacin and norfloxacin, gentamycin and meropenem, amikacin, and azithromycin and imipenem, respectively. On the other hand, levofloxacin has a moderate effect on the isolates shown by 51% resistant isolates followed by doxycycline for which 39% of the isolates were resistant. Colistin was the only antimicrobial agent that has an intense effect as the majority of the isolates were sensitive. All the selected isolates of *A. baumannii* were successfuly produced band corresponded to the intended genes. Accurate and early detection of such bacteria is essential for stimulates effective treatment specifically in intensive care units. Molecular techniques have been successfully applied with high specificity using 16S rRNA and blaOXA-51-like gene as a simple and reliable method to differentiate *A. baumannii* strains.

KEYWORDS: *Acinetobacter Baumannii, Blaoxa-51, Polymerase Chain Reaction, 16s Rrna.*

1. INTRODUCTION

Acinetobacter baumannii is one of the most important pathogens responsible for hospital-acquired infections, particularly in intensive care units (ICUs) (Banerjee *et al.,*2018) . This bacterium can be easily isolated from water, soil, and health care environments (Ecker *et al.,* 2006). *A. baumannii* as a nosocomial opportunistic pathogen is resistant to a wide range of antibiotics and responsible for multiple infections, including bacteremia, pneumonia, meningitis, urinary tract infections, and surgical wounds (Ghajavand *et al.,* 2015). *Acinetobacter* infections have been historically associated with military and injured soldiers in combat due to direct environmental contamination of wounds (Ecker *et al.,* 2006). In a report described an increasing number of *A. baumannii* bloodstream infections in patients at military medical facilities that were treating service members injured throughout Gulf War and in Afghanistan during Operation Enduring Freedom. This has the potential to become a serious problem in military and veterans' hospitals, where soldiers returning from active duty worldwide are treated in the same environment as other patients (Scott *et al*., 2004). In the last 20 years, outbreaks caused by carbapenem-resistant *A. baumannii* isolates have become a major worldwide concern (Visca *et al.,* 2011) and infections caused by such pathogen are associated with increased morbidity and mortality (Henig *et al.,* 2015). Due to the high prevalence rate of this infection as well as various patterns of antibiotic resistance in different geographical areas, an investigation on prevalence rate and antibiotic sensitivity pattern in different part of the world is essential (Ghajavand *et al.,* 2015). The ability to gain multiple virulence factors, including resistance determinants such as serum resistance, motility, efflux pumps and iron acquisition mechanisms, in addition to its ability to develop multidrug resistance and to survive in hospital environments for prolonged periods has helped it to emerge as a successful opportunistic nosocomial pathogen (AL-Kadmy et al., 2018). During the past decade, incidence of nosocomial outbreaks by *A. baumannii* has been described mostly in burn, surgical and intensive care units (Jafari and Karbasizade, 2014). Most surveillance studies report high mortality rates among patients with *A. baumannii* bacteremia and may be associated with considerable morbidity and mortality reach to more than 58% (Falagas and Rafailidis, 2007; Chuang *et al.,* 2011). Since most outbreaks may a raise from a limited number of clonal lines with specific characteristics, it's essential to develop techniques for rapid identification and characterization of this pathogen and take measures to control the spreading of it among patients. Molecular techniques proved its effectiveness for identification of pathogenic bacteria at species level, detection the virulence genes, and study the genetic relationship of the micooroganisms (Sevillano and Gallego, 2011). The management and prevention spreading of *A. baumannii* in the health care

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settings require to identify potential reservoirs of this organism, the modes of transmission and investigate *A. baumannii* clones cause the outbreaks from epidemiologically unrelated strains, a comparison of isolates at the subspecies level is required by application of molecular typing methods (Sadeghi *et al.,* 2016). This study involved the isolation and identification of *A. baumannii* from sputum specimens, burns and wounds infections of hospitalized patients in Duhok hospitals using conventional and molecular methods, in addition, the investigation of the susceptibility of *A. baumannii* isolates to different antimicrobial agents.

2. MATERIAL AND METHODS

2.1 Sample collection:

A total of 603 clinical burns and sputum samples were collected during the period from March 2018 to February 2019 at the following hospitals; 226 sputum specimens were collected from patients attending Azadi teaching hospital as well as 111 sputum samples from Duhok Emergency hospital; Moreover, 266 burn infections samples were obtained from patients admitted to Duhok cosmetic and burn hospital. Regarding to the gender, 351 samples have been obtained from female, while 252 were collected from male Table (1).

Table (1): The sample collection from different hospitals from male and female pateints

Hospital	Female	Male
Duhok Azadi Teaching Hospital (ICU)	120	106
Emergency Hospital (ICU)	61	50
Duhok Cosmetic and Burn hospital (Burned wound)	170	96
	351	252
Total	603	

2.2 Identification of *A. baumannii*:

The preliminary conventional phenotypic tests including; growth on MacConkey agar, catalase and oxidase tests, and other standard recommended tests were applied (Forbes *et al*., 2007; and Golanbar *et al.,* 2011). For definitive phenotypic identification of these isolates, they were cultured on CHROMagar Acinetobacter (CHROMagar, France), MDR *A. baumannii* have been detected after adding MDR supplement (CR102) used for this purpose, in addition to VITEK 2 identification system test using ID-GNB cards, according to the manufacturer's instructions.

2.3 Antibiotic susceptibility test:

Seventeen antibiotic disks (Bioanalyse/ Turkey) have been used in this study. Antibiotic sensitivity testing was accomplished by the Kirby-Bauer disc diffusion method. All plates were incubated at 37°C for 18 h. After incubation, the diameter of the inhibition zone was measured to the CLSI reference for *Acinetobacter spp* (CLSI., 2016).

2.4 Molecular methods:

The isolates were identified to the genus level using genus-specific PCR assay by 16SrRNA primer as previously described (Ghaima *et al.,* 2016). In brief, a 150 -bp fragment of the 16SrRNA was amplified using pair of primers F (5′-CAGCTCGTGTCGTGAGATGT-3′) and R (5′-CGTAAGGGCCATGATGACTT-3′). The amplification reaction volume was 25 µl, and the cycling conditions were as follows: an initial denaturation at 95°C for 3minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Species-specific PCR assay with *OXA-51* gene using F primer 5′-TAATGCTTTGATCGGCCTTG -3 and R ′5 -TGGATTGCACTTCATCTTGG -3′ was also applied to confirm the identity of the isolates with expected amplified product 353bp (Ghaima *et al.,* 2016). The amplification reaction volume was 25 µl, and the cycling conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 40 seconds, 52°C for 45 seconds, 72°C for 40 seconds and a final extension at 72°C for 6 minutes. The amplified products were resolved through electrophoresis and analyzed on 1.5% agarose gel-stained ethidium bromide.

2.5 Biostatic analysis:

Statistical Package for Social Sciences (SPSS) version 22.0 and Microsoft Office Excel were used to analyze the results of the current study. Chi-square test was used and $P < 0.05$ was considered to be statistically significant (Sokal and Rohlf, 2009).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Strain identification: In this study, 603 clinical samples were collected from different hospitals in Duhok City, Forty one (6.8%) of A. baumannii isolates were obtained in total, 19 (3.2%) were isolated from the sputum samples of inpatients admitted at the intensive care unit and 22 (3.64%) from burn infection (Table 2). Twenty three accounting 63.4% obtained from females while 15 strains (36.5%) were collected from males.

Table 2: Distribution of A. baumannii isolates according to the source and location of isolation

Location		Source	No. of samples	No Percentage of isolates in each hospital	No. of positive isolates %
Duhok Azadi Teaching Hospital	ICU	Sputum	226	13(5.8%)	13 (2.1 %)
Emergency Hospital		Sputum	111	6 (5.4%)	6 (1 %)
			337	19(5.6%)	19 (3.2%)
Duhok Cosmetic and Burn hospital	Burn units	Burned wound	266	22(8.3%)	22 (3.6%)
Total			603		41 (6.8%)

Fifty four suspected isolates as *A. baumannii* with late lactose fermenter on MacConkey agar were negative to oxidase test and positive to catalase test appeared on CHROMagar as bright red colonies after 24 hr., incubation at 37ºC (Figure 1-A), this medium is also specific for MDR *A. baumannii* as it contains MDR Acinetobacter screening supplements.

Although this Chromogenic medium is essential for detection of *Acinetobacter* and MDR *Acinetobacter* sp., other bacterial species like *Stenotrophomonas maltophilia*, *Pseudomonas aueroginosa* were grown and appeared with similar colonies but smaller size than *Acinetobacter*, by which they can be distinguished from each other (Figure 1-B). Indeed, further confirmatory tests were required. In this regard, VITEK 2

Identification System test using ID-GNB cards was utilized according to the manufacturer's instructions. Out of 54 suspected isolates, only 41 isolates were confirmed as *Acinetobacter* while other 13 isolates stood for other bacteria, 3 isolates were identified as *A. ursingii*, 3 as *Stenotrophomonas maltophilia* and 7 samples were identified as *Pseudomonas aueroginosa*. Thus, the sensitivity of CHROMagar result was 75.9%. While VITEK 2 Identification System test showed 100% accuracy after comparison with molecular methods.

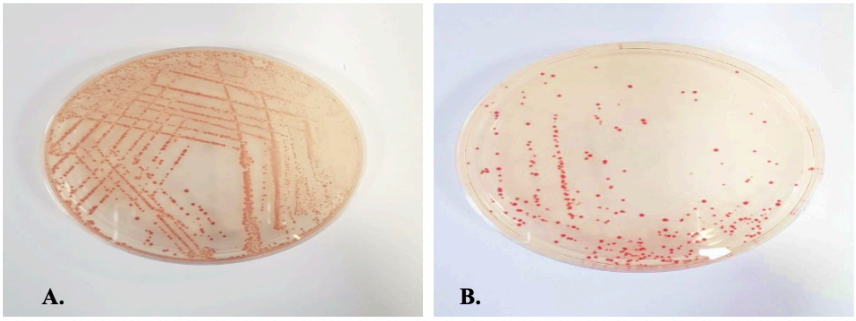

Figure 1-A: Colonies of *Acinetobacter baumannii,* B. colonies of *Stenotrophomonas maltophilia* after 24 hrs. of incubation at 37ºC on CHROMagar Acinetobacter.

3.1.2 Antibiotic Susceptibility Test: The results of antimicrobial susceptibility test showed that the forty one *A. baumannii* isolates varied in their sensitivity toward antibiotics used in this study. Ten categories of antimicrobial agents have been used in this study as shown in Table (2). It is worth mentioning, only 10 (24.4%) isolates were appeared to be resistant to a minimum of 3 classes of selected antibiotics, in other words, MDR *A. baumannii*. While 27 isolates accounting 65.9% were susceptible to two or fewer of antimicrobial categories, considered as XDR *A. baumannii* strains. None of these isolates showed its resistancy against all classes of antimicrobial agents. Colistin was the only antimicrobial agent that has potent effects on all 41 isolates with 100% sensitivity rate. In other hand, all isolates were resistant (100%) to cefixime, cefotaxime, ceftriaxone, ceftazidime, piperacilin-tazobactam, amoxicillin-tazobactam, with high resistance rate to ciprofloxacin, norfloxacin and trimethoprim-Sulfamethoxazole with resistant rate 98% , 98% and 95%, Respectively. Gentamycin, meropenem and amikacin showed low efficacy of these isolates with resistant rate 93%, 93% and 90%, orderly. Azythromycin and imipenem have high resistance rate 83%. Levofloxacin has a moderate effect on these isolates 51%, followed by doxycyclin 39%.

Table 2: Antimicrobial Susceptibility test Results of 41 *Acinetobacter baumannii* isolates

No	Name	Code	No. of susceptible isolates of *A. baumannii*	Sensitivity %	No. of resistant isolates of *A. baumannii*	Resistancy %
	Amino glycoside					
1	Amikacin	AK	4	10%	37	90%
2	Gentamycin	CN	3	7%	38	93%
	Cephalosporins					
3	Cefixime	CFM	0	0%	41	100%
4	Cefotaxime	CTX	0	0%	41	100%
5	Ceftriaxone	CRO	0	0%	41	100%
6	Ceftazidime	CAZ	0	0%	41	100%
	Quinolones					
7	Ciprofloxacin	CIP	1	2%	40	98%
8	Levofloxacin	LIV	20	49%	21	51%
9	Norfloxacin	NOR	1	2%	40	98%
	Tetracycline					
10	Doxycyclin	DO	25	61%	16	39%
	Penicillin					
11	Piperacilin-Tazobactam	PRL	0	0%	41	100%
	Trimethoprim					
12	Trimethoprim-Sulfamethoxazole	SXT	2	5%	39	95%
	Macrolides					
13	Azythromycin	AZM	7	17%	34	83%
	lactam/lactamase Inhibitor					

14	Amoxicillin-Tazobactam	AMC	0	0%	41	100%
	Carbapenems					
15	Imipenem	IPM	9	22%	32	88%
16	Meropenem	MEM	3	7%	38	93%
	Polymyxin					
17	Colistin	CT	41	100%	0	0%

3.1.3 Detection of *A. baumannii* at molecular level: All isolates were identified as *Acinetobacter* spp. at molecular level using 16SrRNA region, 41 isolates successfully produced amplicon with 150-bp corresponding to the target region (Figure 2). Moreover, these isolates were subjected to molecular confirmation as *Acinetobacter baumannii* using primer for detection bla_{OXA-51} gene with 353-bp amplicon size. The results of PCR showed that this gene exists in all 41 isolates which had been identified as *A. baumannii* (Figure 3).

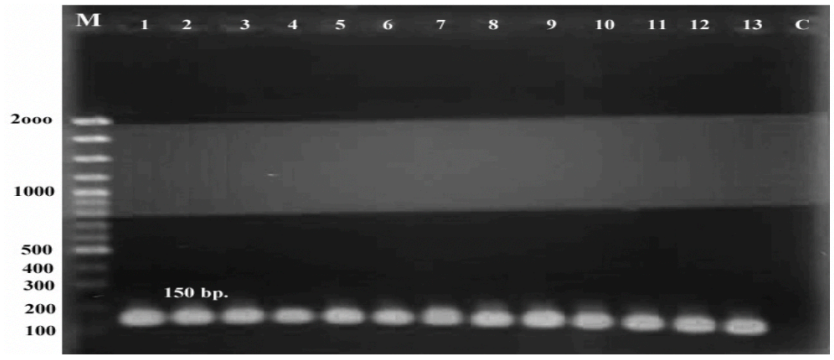

Figure 2. The PCR results of 16S rRNA gene of *A. baumannii* isolates. Lane (M): Molecular marker (2000bp ladder), Lane (1-13): show positive results with positive bands of 150-bp, lane (C): Negative control. Electrophoresis was performed on 1.5% agarose gel run with 5V/CM.

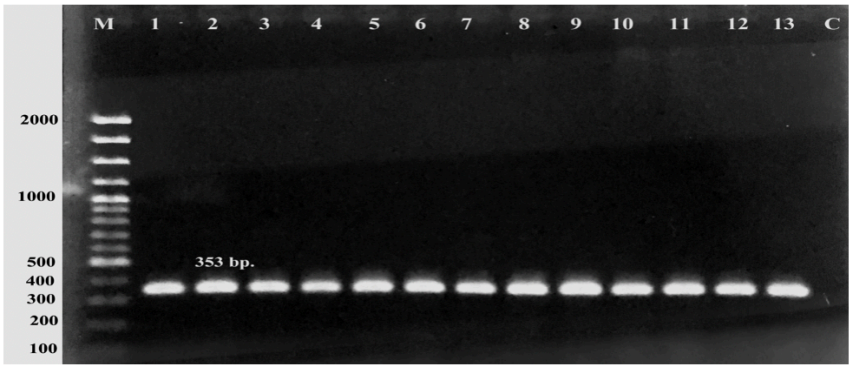

Figure 3. The PCR results of OXA-51 gene of A. baumannii isolates. Lane (M): Molecular marker (2000bp ladder), Lane (1-13): show positive results with positive bands of 353-bp OXA-51 gene of A. baumannii, lane (14): Negative control. Electrophoresis was performed on 1.5% agarose gel ran with 5V/CM.

4. CONCLUSION

The emergence of MDR A. baumannii strains at health care institutions in our region as well as globally require more surveillance concern on the molecular characterization and the epidemiology of A. baumannii strains enrolled in our region.

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