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Clonal Diversity and High Prevalence of Oxa-23 among Carbapenem Resistant *Acinetobacter baumannii* Isolates in Egypt

Running title: Clonal Diversity among Carbapenem Resistant *Acinetobacter baumannii* Isolates

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Abstract

Background and Aim: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is becoming a global threat especially to hospitalized patients. We aimed to address the magnitude of CRAB causing healthcare-associated infections in patients admitted to a tertiary healthcare hospital in Egypt and to study their genetic and epidemiologic diversity. **Materials and Methods:** Twenty-six CRAB isolates representing 48% of all *Acinetobacter baumannii (A. baumannii*) isolated in the study period were identified by microbiological culture methods and verified by presence of *bla-oxa*$_{51}$. Antimicrobial susceptibility was tested by disc diffusion and MIC was determined by VITEK2 compact system. Phenotypic expression of metallo-β-lactamases (MBLs) was determined by MBL IP/IPI E-test. Carbapenemase encoding genes were identified by PCR and clonal relatedness was studied by pulsed field gel electrophoresis (PFGE) using *Apa1* and PulseNet protocol**. Results:** All *A.*

baumannii isolates were multi-drug resistant (MDR). Colistin and minocycline showed the highest sensitivities of 100% and 61.1%respectively. MBLs were phenotypically detected in 20/26 (76.9%) of the isolates while $bla_{\text{OXA-23-like}}$ was the main carbapenem resistance gene recorded in 61.5% followed by $bla_{\text{NDM-1-like}}$ (26.9%) and $bla_{\text{GES-like}}$ (7.7%). PFGE typing showed high diversity as most of the isolates were < 80% similar. **Conclusion:** Carbapenem resistance among *A. baumannii* isolates is increasing dramatically in our geographic region. $Bla_{OXA\text{-}23\text{-}like}$is the most common gene in CRAB isolates in our hospital setting. In addition, $bla_{NDM\text{-}1\text{-}like}$ and $bla_{GES\text{-}like}$ harboring *A. baumannii* isolates are exhibiting a considerable spread in hospital environment in Egypt. The clonal diversity of our CRAB isolates suggests that it could be due to horizontal dissemination of mobile genetic elements rather than propagation of a certain clone.

Key Words: *A. baumannii,* carbapenem resistance, GES, NDM, OXA-23, PFGE.

1. Introduction

Multidrug-resistant *Acinetobacter baumannii* (MDR-AB) is globally responsible for nosocomial outbreaks particularly in immune compromised patients in intensive care units (ICUs) **(Uwingabiye et al., 2017).** Carbapenems have been commonly used as the treatment of choice for MDR-AB infections **(Park et al., 2016),** however, *A. baumannii* resistant to carbapenems have been increasingly reported worldwide limiting drastically the range of therapeutic alternatives **(Lob et al., 2016)**.

Patients with carbapenem-resistant *A. baumannii* (CRAB) infections have higher mortality rates, longer duration of hospitalization, and higher health-care costs **(Park et al., 2013).**

The World Health Organization (WHO) has included CRAB among the critical list of bacteria that pose the greatest threat to human health, and prioritizing research and development efforts for new antimicrobial treatments **(WHO, 2017).**

Resistance to carbapenems among *A. baumannii* isolates is predominantly attributed to beta (β) lactamases; a diverse group of enzymes; belonging to the class D- OXA type carbapenemases, in addition to the emerging bla_{NDM} gene belonging to class B metallo-β-lactamase (MBL) and some members of class A **(Uwingabiye et al., 2017).** Class B-MBLs are prevalent in East Asia, Western Europe and African countries. It confers resistance to all β-lactams except aztreonam **(Aghamiri et al., 2016).**

Throughout the years, many typing approaches from phenotypic to molecular methods for *A. baumannii* epidemiological studies have been proposed. Currently, the use of phenotypic typing methods have declined considerably and been progressively replaced by molecular methods (**Rafei et al., 2014).** Pulsed-field gel electrophoresis (PFGE) is one of the molecular typing methods and is becoming a prototype for understanding the fundamental mechanisms of *Acinetobacter* infections in hospital settings to investigate the clonal relationship among bacterial strains and its geographical spread **(Aljindan et al., 2018).** Several studies from Egypt have pointed to the high prevalence of carbapenem resistance among *Acinetobacter* clinical isolates particularly in ICUs **(Mohamed and Raafat, 2011; Nasr and Attalah, 2012; Fouad et al., 2013; Shalaby et al., 2016; Alkasaby and Zaki, 2017).**

This work aimed to address the magnitude of *A. baumannii* infections in our tertiary care hospital, to study the resistance profile of CRAB clinical isolates and to study their genetic and epidemiologic diversity by investigating their carbapenemase resistant genes and the clonal relatedness of the clinical isolates.

2. Material and Methods

2.1. Bacterial identification and antimicrobial susceptibility Twenty-six CRAB isolates were recovered from 724 Gram negative clinical samples received and processed in Microbiology laboratory of Theodor Bilharz Research Institute (TBRI) from hospitalized patients during the study period from January 2016 to December 2016.Isolates were identified preliminary by conventional culture and API 20NE (BioMérieux, France) and confirmed by using VITEK2 Compact System (BioMérieux, France) according to manufacturer guidelines.

Antimicrobial susceptibility testing was initially performed by the disc diffusion method **(CLSI, 2018).** MICs of the tested antibiotics were obtained by using VITEK2 Compact system (BioMérieux, France). Susceptibility testing was performed for the following antibiotics; ticarcillin (TIC), piperacillin (PIP), piperacillin/tazobactam (PIP/TZP), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IPM), meropenem (MEM), amikacin (AK), gentamicin (CN), tobramycin (TOB), ciprofloxacin (CIP), pefloxacin (PEF) minocycline (MIN), colistin (CST), trimethoprim/sulfamethoxazole (SXT). Results were interpreted according to Clinical Laboratory Standards Institute breakpoints (CLSI) **(CLSI, 2018).**

Isolates that showed resistance to imipenem and meropenem with MIC values ≥8 µg/ml **(CLSI, 2018)** were considered carbapenem resistant and used for subsequent investigation.

2.2. MBL screening

Isolates were screened for MBL presence using the MBL IP/IPI E-test (BioMérieux, France) as per the manufacturer's instructions.

2.3. PCR amplification of carbapenemase-encoding genes

DNA was extracted from overnight cultures by using the boiling method **(Higgins et al., 2010).** PCR was performed for the following carbapenemase-encoding genes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{NDM-1-like}}$, $bla_{\text{GES-like}}$) **(Cicek et al. 2013; Fallah et al., 2013; Lean et al., 2014).** Primers used in PCR are listed in **(table 1).** The PCR protocol used was as previously described by **Fallah et al. (2013).** Briefly 5µL of DNA was added to PCR reaction mixture (final volume of 50 µL) then amplified with the following PCR protocol: 95 ℃ for 3 min; 30 cycles at 95 ℃ for 1 min; 45-55℃ (according to the primer) for 45s **(Table1);** 72 ℃ for 1 min and finally 72 ℃ for 5 min using a T-personal PCR Thermal Cycler (Biometra, Uk). Resulting PCR products were visualized in gel documentation system (Cleaver Scientific, UK) after agarose gel (2%) electrophoresis and staining with ethidium bromide (Promiga-USA).

Table 1: Primers used for different carbapenem resistant genes

Primer name	Sequence (5'-3')	Tm*	Amplicon size (bp)	Reference
bla$_{OXA-51}$	F: TAATGCTTTGATCGGCCT R: TGGATTGCACTTCATCTTGG	55 ℃	353	***Lean et al., 2014*** **[18]**
bla$_{OXA-23}$	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	45℃	501	***Lean et al., 2014*****[18]**
bla$_{OXA-24}$	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	52 ℃	246	***Lean et al., 2014*** **[18]**
bla$_{OXA-58}$	F: AAGTATTGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	52 ℃	599	***Lean et al., 2014*** **[18]**
bla$_{NDM}$	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	52 ℃	621	***Fallah et al., 2014*** **[17]**
bla$_{GES}$	F: ATGCGCTTCATTCACGCAC R: CTATTTGTCCGTGCTCAGGA	55 ℃	863	***Cicek et al., 2013*** **[16]**

2.4. Molecular typing using PFGE

All *A. baumannii* isolates were analyzed for clonal relationship using the standard PulseNet PFGE protocol at US Naval Medical Research Unit 3 (NAMRU -3). Overnight culture of *Acinetobacter* isolates on blood agar was suspended in cell suspension solution **(100 mM Tris: 100 mM EDTA, pH 8.0)**. Equal volumes of bacterial suspension and 1% Seakem® Gold Agarose (Lonza,Rockland, USA) were mixed and dispensed in a plug mould. Agarose plugs were lysed in the cell lysis solution (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl, 20 mg/ml

proteinase K), washed and digested with restriction enzymes Apa1 (New England BioLabs Inc., USA) for *Acinetobacter* and Xba1 for *Salmonella braenderup* as molecular standard (New England BioLabs Inc., USA). ApaI. Electrophoresis was conducted in 1% Seakem® Gold Agarose and 0.5X TBE Buffer (PH 8.0) using CHEF-DR II system (Bio-Rad Laboratories, USA) at 6 V/cm2 voltage, switch angle 120°, with switch times ranging from 5 s to 35 s at temperature of 14° C, for 20 h.

A *Salmonella* serotype *Braenderup* strain (H9812) (NAMRU-3 Laboratories), was chosen as the universal size standard and was incorporated twice per gel to allow standardization of all fingerprints **(Hunter et al., 2005)**. Gel images were scanned by Gel Doc 1000 imaging system (Bio-Rad, USA) and analyzed with BioNumerics software version 6.1 (Applied Maths, Belgium) for dendrogram construction. Isolates were considered of the same PFGE clone (pulsotype), if their Dice similarity index was ≥ 85% **(Durmaz et al., 2009).**

The protocol of work was approved by Theodor Bilharz Research Institute (TBRI) institutional review board (FWA00010609), and the work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans.

2.5. Statistical analysis

Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. Results were expressed as mean, range, number and percent. Comparison between categorical data [number (%)] was performed using either Chi-square test or Fisher Exact test whenever it was appropriate. P values less than 0.05 were considered significant.

3. Results

3.1. Bacterial isolates

A total of 54 *A. bamannii* clinical isolates were recovered during the study period 26/54 (48.1%) were carbapenem resistant. The majority of the isolates were recovered from respiratory samples (n=11; 42.3%) followed by pus (n=8; 30.8%), urine (n=6; 23.1%) and blood (n=1; 3.9%). ICU was the main source of specimen recovery with isolation rate (10/26; 38.5%) followed by Surgery (23.1%) and Urology (19.2%) units**.** The distribution of *A. baumannii* isolates according to gender showed that 15 (57.7%) of the isolates were recovered from males and 11 (42.3%) were recovered from females with no significant difference (p >0.05).

3.2. Antimicrobial susceptibility profile

Results of VITEK2 compact system showed that all *A. baumannii* isolates were resistant to at least three classes of antibiotics (MDR). They were absolutely resistant to imipenem and meropenem. All isolates were resistant to piperacillin, piperacillin-tazobactam, and cefepime. High rates of resistance were observed for ceftazidime, ciprofloxacin, ticarcillin (96.2%) and trimethoprim-sulphamethaxole (92.3%). Colistin and minocycline showed the highest sensitivity of 100% and 61.1% respectively **(Table 2).**

Table (2): Susceptibility profile of *A. baumannii* isolates to the tested antibiotics by VITEK2 Compact system

Antibiotics	*A. baumannii* isolates N=26		
	Sensitive N (%)	**Intermediate N (%)**	**Resistant N (%)**
Ticarcillin	0 (0%)	1(3.8%)	25 (96.2%)
Imipenem	0 (0%)	0 (0%)	26 (100%)
Meropenem	0 (0%)	0 (0%)	26 (100%)
Ceftazidime	1 (3.8%)	0 (0%)	25 (96.2%)
Cefipime	0 (0%)	0 (0%)	26 (100%)
Piperacillin/tazobactam	0 (0%)	0 (0%)	26 (100%)
Piperacilin	0 (0%)	0 (0%)	26 (100%)
Amikacin	7 (27%)	3 (11.5%)	16 (61.5%)
Gentamycin	4 (15.4%)	0 (0%)	22 (84.6%)
Tobramycin	4 (15.4%)	5 (19.2%)	17 (65.4%)
Ciprofloxacin	1 (3.8%)	0 (0%)	25 (96.2%)
Minocycline	16 (61.1%)	7 (27%)	3 (11.5%)
Colistin	26 (100%)	0 (0%)	0 (0%)
Trimethoprim-Sulphamethaxole	2 (7.7%)	0 (0%)	24 (92.3%)

3.3. Phenotypic Detection of MBL Production

MBL IP/IPI E-test strips showed that 20/26 (76.9%) of *A. baumannii* isolates were MBL positive.

3.4. Distribution of carbapenemase genes

In addition to the intrinsic *bla*$_{\text{OXA-51-like}}$ that was found in all isolates, PCR results showed that *bla*$_{\text{OXA-23-like}}$ was the main carbapenem resistance gene detected in 16 (61.5%) of the isolates followed by *bla*$_{\text{NDM-1-like}}$ in 7 (26.9%) isolates and *bla*$_{\text{GES-like}}$ in 2 (7.7%) isolates. Neither *bla*$_{\text{OXA-58-like}}$ nor *bla*$_{OXA\text{-}24\text{-}like}$ were identified in any isolate. The coexistence of *bla*$_{NDM\text{-}1\text{-}like}$/*bla*$_{OXA\text{-}23\text{-}like}$and*bla*$_{GES\text{-}like}$/*bla*$_{OXA\text{-}23\text{-}like}$ was found in 4 (15.4%) and 2 isolates (7.7%) respectively **(Table 3).**

3.5. PFGE analysis

All the studied *A. baumannii* isolates were fingerprinted for clonal relationship, the results show high diversity as most of the isolates were < 80% similar **(Figures. 1, 2).**

Table (3): Distribution of carbapenemase genes in carbapenem resistant *A. baumannii* (CRAB) isolates

Carbapenemase Genes	***A. baumannii* Isolates N (%)**
OXA-51	26/26 (100%)
OXA-23	16/26 (61.5%)
NDM	7/26 (26.9%)
GES	2/26 (7.7%)
OXA-58	0(0%)
OXA-24	0 (0%)

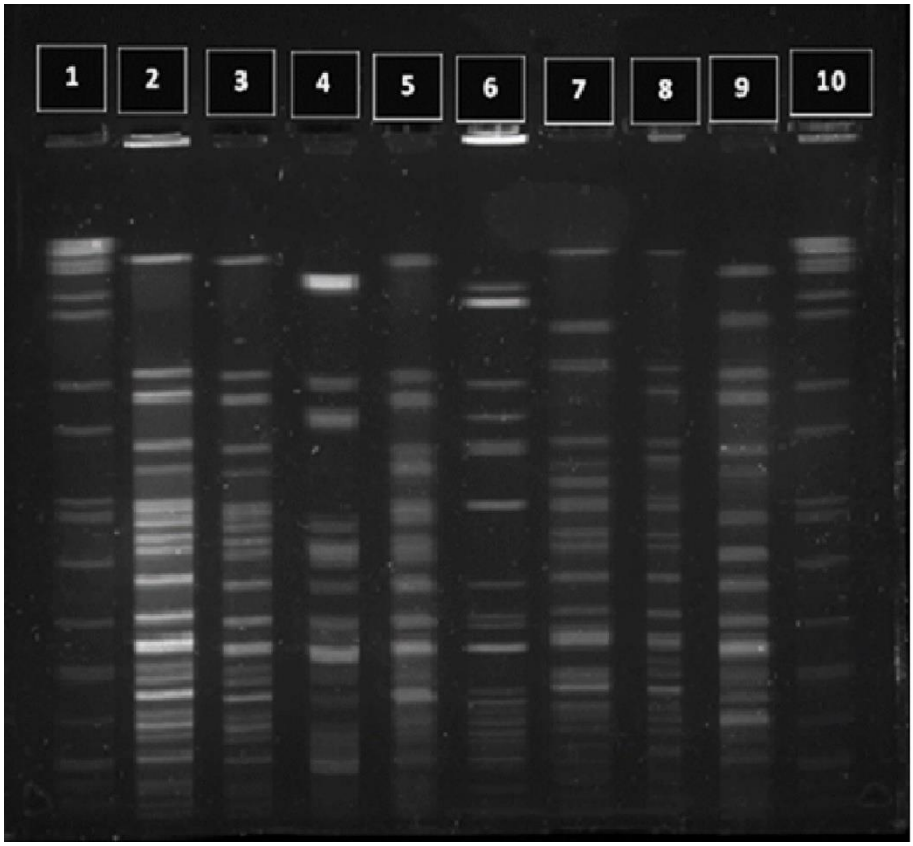
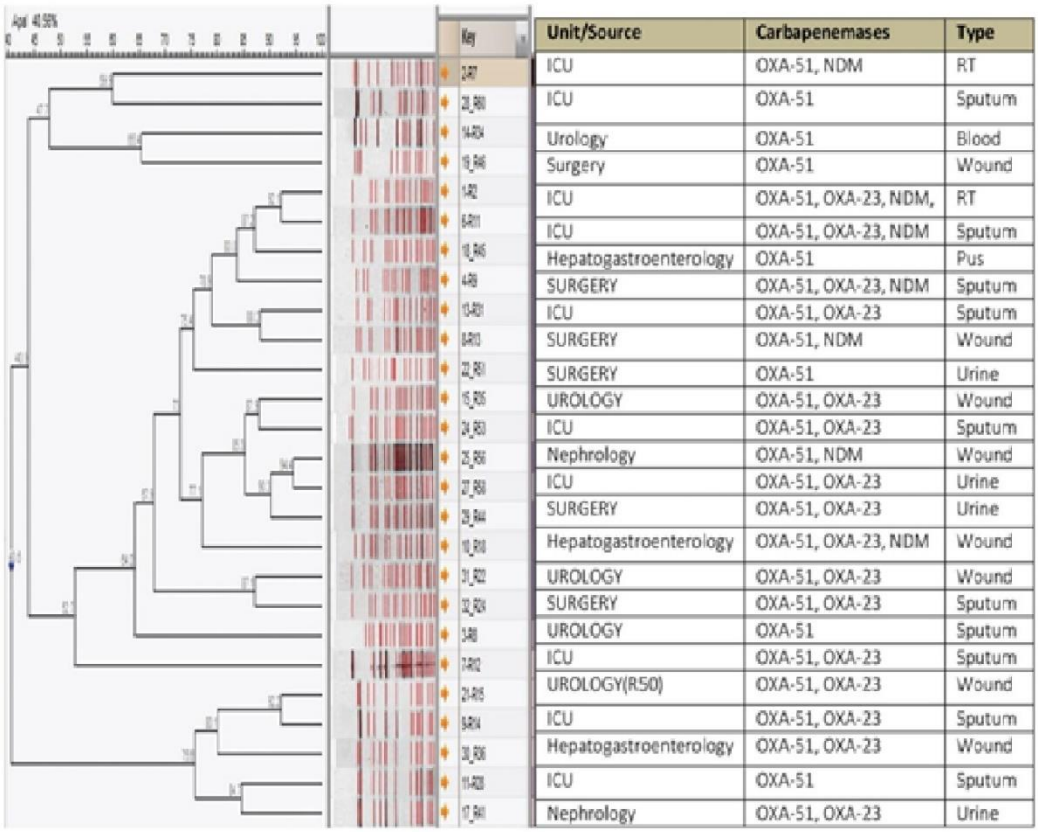


Figure 1: Representative agarose gel showing PFGE patterns of ApaI- digested genomic DNA of *A. baumannii* isolates. Lanes 1 and 10: *Salmonella* serotype Braenderup strain (H9812) as a molecular weight marker. Lanes 2 – 9: Different *A. baumannii* isolates.



Unit/Source	Carbapenemases	Type
ICU	OXA-51, NDM	RT
ICU	OXA-51	Sputum
Urology	OXA-51	Blood
Surgery	OXA-51	Wound
ICU	OXA-51, OXA-23, NDM,	RT
ICU	OXA-51, OXA-23, NDM	Sputum
Hepatogastroenterology	OXA-51	Pus
SURGERY	OXA-51, OXA-23, NDM	Sputum
ICU	OXA-51, OXA-23	Sputum
SURGERY	OXA-51, NDM	Wound
SURGERY	OXA-51	Urine
UROLOGY	OXA-51, OXA-23	Wound
ICU	OXA-51, OXA-23	Sputum
Nephrology	OXA-51, NDM	Wound
ICU	OXA-51, OXA-23	Urine
SURGERY	OXA-51, OXA-23	Urine
Hepatogastroenterology	OXA-51, OXA-23, NDM	Wound
UROLOGY	OXA-51, OXA-23	Wound
SURGERY	OXA-51, OXA-23	Sputum
UROLOGY	OXA-51	Sputum
ICU	OXA-51, OXA-23	Sputum
UROLOGY(R50)	OXA-51, OXA-23	Wound
ICU	OXA-51, OXA-23	Sputum
Hepatogastroenterology	OXA-51, OXA-23	Wound
ICU	OXA-51	Sputum
Nephrology	OXA-51, OXA-23	Urine

Figure 2: Dendrogram of PFGE patterns of 26 *A. baumannii* isolates from different clinical samples. PulseNet PFGE protocol was implemented using the restriction enzyme Apa I. Cluster analysis was conducted using BioNumerics software (version 6.1) recording high diversity.
ICU: Intensive Care Unit, RT: Respiratory Tube.

4. Discussion

Within the past three decades, antimicrobial resistance rates among *A. baumannii* have escalated dramatically worldwide. In some countries, more than 90% of *A. baumannii* are MDR. In this study we aimed to address the magnitude of CRAB infections in our tertiary care hospital in Egypt, and to study the genetic and epidemiologic diversity of our isolates by studying the carbapenemase resistance genes and the clonal relatedness of those clinical isolates.

Our overall isolation rate of CRAB isolates was 48.1% which is comparable to other studies from our geographic area, reporting resistance to carbapenems ranging from 50%-60% **(Al-Agamy et al., 2014; Ramadan et al., 2018),** Other studies even reported higher resistance rates reaching up to 100% cabapenem resistance among *A. baumannii* isolates **(Mohamed and Raafat, 2011; Alkasaby and Zaki, 2017).**

In the Middle East and North Africa the resistance rate of *A. baumannii* to imipenem was variable reaching 25.2% in Kuwait **(Al-Sweih et al., 2011),** 47.9% in Algeria **(Bakour et al., 2013) ,** 95% in Turkey **(Cicek et al., 2013),** 92.2% in Saudi Arabia **(Alsultan et al., 2014) ,** 75.7% in Morocco **(El Kettani et al., 2017),** 87% in Tunisia **(Cheikh et al., 2018).**

The present study documented a high prevalence of MDR-AB in ICU (38.5 %). This is consistent with recent studies from Nepal (33.68%) **(Banerjee et al., 2018)**. In Moroccan ICUs, *Acinetobacter spp*. represented 24.85% of all isolates and 31.5% of all Gram-negative rods **(Uwingabiye et al., 2016).** Higher rates of 60% have been reported in surgical ICU of Zagazig University Hospitals in Egypt **(Ramadan et al., 2018).** High resistance of *Acinetobacter* species in ICUs is related to various risk factors, such as immune compromised persons, major trauma or surgery, previous or prolonged duration of stay in hospitals, the use of invasive devices, broad-spectrum of antibiotics therapy, possible contaminations and transmission of these bacteria through hospital environment and hands of healthcare workers **(Uwingabiye et al., 2016).** Dissemination of MDR-AB harboring OXA-type carbapenemase has been progressively reported worldwide **(Luo et al., 2015).** In this study *bla*$_{\text{OXA-51-like}}$ gene was detected in all isolates, as they are considered as intrinsic genes in *A. baumannii* species, whereas *bla*$_{\text{OXA-23-like}}$ was retained by 61.5% of isolates, and neither *bla*$_{\text{OXA-58}}$ nor *bla*$_{OXA\text{-}24\text{-}like}$ were identified in any isolate from this study. Correspondingly, ***Al-Agamy et al.*** **(2014)** detected *bla*$_{OXA\text{-}23\text{-}like}$in 50% of their isolates**.** Researchers from Lebanon, Iran and United Arab Emirates also reported the predominance of *bla*$_{OXA\text{-}23\text{-}like}$ gene **(Al Atrouni et al., 2016; Dehbalaei et al., 2016; Mohammadi et al., 2016).**

The *bla*$_{NDM\text{-}1\text{-}like}$ gene was not previously identified as playing a major role in carbapenem resistance among *A. baumannii* clinical isolates in Egypt **(Al-Agamy et al., 2014).** However 26.9 % of our CRAB isolates tested positive for the class B carbapenemase *bla*$_{NDM\text{-}1\text{-like}}$.The presence of *bla*$_{NDM\text{-}1\text{-like}}$ has been recently documented by **Ramadan et al. (2018)** and **Gomaa et al. (2017).** These results indicate that *bla*$_{NDM\text{-}1\text{-}like}$ harboring *A. baumannii* strains are exhibiting a considerable spread in hospital settings in Egypt. The *bla*$_{NDM\text{-}1\text{-}like}$ carrying *A. baumannii* has recently emerged in many countries, including France **(Decousser et al. 2013),** Germany, Spain, Switzerland (**Joshi et al., 2017),** Libya **(Mathlouthi et al., 2016)** India **(Rahman et al., 2018),** Pakistan **(Sartor et al., 2014)** and Nepal **(Shrestha et al., 2015).** In the current study, 76.9% of our CRAB isolates were phenotypically detected as MBLs by E-test, while *bla*$_{NDM\text{-}1\text{-like}}$ was only detected in 26.9%. This discrepancy could be explained by the presence of other MBL encoding genes (IMP-like, VIM-like or SIM-1), which were not investigated in the current study.

GES variants are from Ambler class A β- lactamases, which have relatively low carbapaenemase activities, but have been reported in the last 5 years in *A. baumannii* **(Al-Agamy et al., 2014).** In the present study, *bla*$_{\text{GES-like}}$ was detected in 7.7% of the CRAB isolates. This is lower than that detected by **Ramadan et al. (2018)** (50%) and in accordance with the findings of Turkish and Saudi studies with prevalence of 23.8% and 18.5%, respectively **(Cicek et al., 2013**; **Al-Agamy et al., 2014).**

In the 7 strains, where *bla*$_{OXA\text{-}51\text{-}like}$ was the only gene detected, resistance can be explained by non-enzymatic mechanisms or insertion of *ISAba1* sequences in *bla*$_{OXA\text{-}51\text{-}like}$ rendering it resistant to carbapenems **(Hu et al., 2007).**

PFGE typing was applied for our CRAB strains as it is a useful epidemiological tool for outbreak analysis. Antimicrobial susceptibility testing of our isolates

displayed similarities that could suggest a possibility of horizontal dissemination rather than the dissemination of a certain clone that resulted in increased rates of resistance especially in our ICU. Results indicated that the genetic similarity of the studied *Acinetobacter* isolates by PFGE was extremely variable. It showed high diversity as most of the isolates were < 80% similar. It is verified that the $bla_{OXA\text{-}23\text{-}like}$ can be either plasmid or chromosome borne, while $bla_{NDM\text{-}like}$ is often plasmid mediated. In addition, **Bonnin et al. (2013)** declared that the dissemination of $bla_{GES\text{-}like}$ gene is rather due to plasmid dissemination. These factors have increased the risk of horizontal dissemination, resulting in increased rates of resistance in healthcare settings, hence diverse *A baumannii* isolates are extensively circulating in hospitals **(Almasaudi, 2018).**

5. Conclusion

carbapenem resistance among *A. baumannii* isolates is increasing dramatically. The results of the current study revealed that $bla_{OXA\text{-}23\text{-}like}$, $bla_{NDM\text{-}like}$ and $bla_{GES\text{-}like}$ genes are the most common genes in carbapenem resistant *A. baumannii*. In our hospital setting. The clonal diversity of our CRAB isolates suggest that it could be due to horizontal dissemination of mobile genetic elements rather than propagation of a certain clone.

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Conflict of interest

Authors declare that there is no conflict of interest exists.

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