

 <p>ISSN NO. 2320-5407</p>	<p>Journal Homepage: - <a href="http://www.journalijar.com">www.journalijar.com</a></p> <h2 style="text-align: center;">INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</h2> <p style="text-align: center;">Article DOI: 10.21474/IJAR01/2458 DOI URL: <a href="http://dx.doi.org/10.21474/IJAR01/2458">http://dx.doi.org/10.21474/IJAR01/2458</a></p>	 <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) ISSN 2320-5407 Journal homepage: <a href="http://www.journalijar.com">http://www.journalijar.com</a> Journal DOI: 10.21474/IJAR01</p>
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### RESEARCH ARTICLE

#### ISOLATION, CHARACTERIZATION OF ACINETOBACTER BAUMANNII RESISTANT TO CARBAPENEM AND THEIR EFFECT ON MICE HEPATORENAL FUNCTIONS.

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#### Manuscript Info

#### Manuscript History

Received: 23 October 2016  
Final Accepted: 21 November 2016  
Published: December 2016

#### Abstract

This study was carried out to illustrate multi-drug resistant *Acinetobacter baumannii* which have carbapenem resistance genes and to show its effect on mice hepatorenal functions. Forty four (4.2%) *A.baumannii* strains were isolated from 1050 positive cultures of all nosocomial pathogens isolated from Mansoura University Hospitals over one year. Sensitivity rates of *A.baumannii* were (28%) to imipinem, (66%) to meropenem, and (50%) to amikacin. Phenotypic detection of extended-spectrum  $\beta$ -lactamases (ESBLs) was done by the Modified Hodge test which showed that (54.55 %) of *A.baumannii* were positive and 45.45% were negative. The *bla*<sub>OXA</sub> genes were identified in *A.baumannii* by PCR analysis of which (72.72%) were positive for *bla*<sub>OXA-23</sub> gene and (29.54%) were positive for *bla*<sub>OXA-58</sub> gene. Statistically significant increase of mice liver function markers in serum of infected mice in group I (infected with resistant *A.baumannii* strain) and group II (infected with sensitive *A.baumannii* strain) in comparison to control group and the effect in the group I was higher than group II indicating that resistance genes may be accompanied by virulence genes. Regarding the renal function tests there were slight changes in the form decrease of creatinine and urea levels in infected mice in comparison to the control group with no statistically significant change. There were no considerable change in Na<sup>+</sup>, K<sup>+</sup>, and uric acid levels. Statistically significant decrease of Glutathione level (GSH) and in activities of superoxide dismutase (SOD) and catalase (CAT) were found in mice liver tissue in group I and II in comparison to control group.

It may be concluded that, *A. baumannii* infections in human need early detection, with restriction of the usage of carbapenems and improving infection-control procedures. The effect of *A. baumannii* infections in mice especially on liver functions, decrease immunity and disturb antioxidant balance.

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#### Introduction:-

*Acinetobacter* spp. are aerobic, non-motile non fermentative coccobacilli which occur in pairs. They have emerged as important opportunistic pathogens, frequently occurring in critically ill intensive care unit (ICU) patients with

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prolonged hospitalizations or chronic illness. Outbreaks involving multidrug-resistant strains have occurred worldwide <sup>(1,2)</sup>.

*A. baumannii* is resistant to the action of many antimicrobial drugs and spreads easily from patient to patient and survives desiccation. It persists in the environment for many days and causes extended epidemic outbreaks <sup>(3)</sup>. Severe infections by *A. baumannii* occurred in hospitalized patients such as pneumonia, skin infections, bacteremia, urinary tract infections, and meningitis, with an attributed mortality of 7.8 to 23% <sup>(4)</sup>.

Carbapenemases may be defined as  $\beta$ -lactamases that significantly hydrolyze at least imipenem and/or meropenem. They are increasingly reported worldwide among nosocomial and community acquired Gram-negative bacilli <sup>(5)</sup>. The enzymatic degradation by carbapenemases is the most prevalent mechanism of carbapenem resistance in *A. baumannii* that include OXA-type and Metallo-  $\beta$ -lactamases (MBLs) <sup>(6)</sup>.

More specifically, acquired OXA-type carbapenem-hydrolyzing class D  $\beta$ -lactamases of the OXA-23, OXA-24/40 and OXA-58 subfamilies, and the intrinsic OXA-51-type is common among *A. baumannii* isolates <sup>(7)</sup>.

During sepsis, the liver plays a key role in regulating a wide range of key metabolic, homeostatic, and host-defense activities. It is implicated in the host response, participating in the clearance of the infectious agents/products. Sepsis also induces liver damage through hemodynamic alterations or through direct or indirect assault on the hepatocytes or through both <sup>(8)</sup>.

The liver actively modulates the inflammatory processes by filtering, inactivating and clearing bacteria., bacterial product e.g endotoxin and the stimulated liver releases the highest amount of cytokines <sup>(9)</sup>. Acute renal failure (ARF) is the most common renal manifestation of sepsis and sepsis accounts wholly or in part, for more than 50% of cases of acute renal failure <sup>(10)</sup>.

Antioxidants are important in living organisms because they may delay or stop the formation of free radicals by giving hydrogen atoms or scavenging them. Severe sepsis leading to shock is the principle cause of death in ICUs. Immune cells use reactive oxygen species (ROS) in order to support their functions and need adequate levels of antioxidant defenses to avoid harmful effects of an excessive ROS production <sup>(11)</sup>.

The present work aims to evaluate carbapenem resistant genes in *A. baumannii* and to determine their toxic effect on excretory system in mice.

## **Patients and Methods:-**

**Patients and clinical samples:** Sputum, urine, blood, wound swabs were collected from August 2013 to July 2014. The samples were collected from patients who were admitted to Mansoura University Hospitals (MUHs) and showing manifestations of hospital acquired infections.

## **Processing of patient samples:-**

All collected samples were processed in the Microbiology Diagnostics and Infection Control Unit (MDICU) in medical microbiology and immunology department, Mansoura faculty of medicine according to the standard methods of microbiology and the unit policies.

## **Culture:-**

Samples were cultured on different bacteriological media, according to the type of sample. *A. baumannii* colonies on blood agar were smooth, grayish white, mucoid, non hemolytic and smaller than that of Enterobacteriaceae, on MacConkey's and CLED agar were non lactose fermenting (pale) mucoid colonies.

## **Microscopic examination:-**

Gram stained films were done from the growing colonies to detect Gram-negative coccobacilli.

## **Antibiotic sensitivity testing:-**

Was done by the disk diffusion (Kirby-Bauer) method. Using Mueller-Hinton agar and group of commercially prepared antibiotic discs <sup>(9)</sup>. The antibiotic discs used were amikacin, amoxicillin, cefuroxime, cefotaxime, gentamicin, imipenem and meropenem <sup>(12)</sup>.

**Modified Hodge Test:-**

The presence of a distorted inhibition zone around the imipenem disc after overnight incubation was interpreted as a positive Hodge test which indicated production of Metallo-  $\beta$ -lactemase (MBLs) <sup>(13)</sup>.

**Detection of bla<sub>OXA-23</sub> and bla<sub>OXA-58</sub> by PCR method:-**

Multiplex PCR was done for the detection of the two families of OXA-type carbapenamases found in *A.baumannii* <sup>(14)</sup>.

**DNA extraction:-**

Was done by using (Gene JET Genomic DNA Purification Kit, Thermo Scientific, For 50 preps, Lot. 00189391, The European Union (EU) Lithuania) according to manufacturer instructions.

**Amplification:-**

Sequences of primers were used for multiplex PCR for the detection of genes encoding bla<sub>OXA-23</sub> and bla<sub>OXA-58</sub> genes.

Primer	Primer sequence (5'-3')	Product size (bp)
blaOXA-23-F	GATCGGATTGGAGAACCAGA	501
blaOXA-23-R	ATTTCTGACCGCATTTCCAT	
blaOXA-58-F	AAGTATTGGGGCTTGTGCTG	599
blaOXA-58-R	CCCCTCTGCGCTCTACATAC	

PCR reaction was carried out in a final volume of 50  $\mu$ L containing 1x reaction buffer, 5  $\mu$ L of extracted DNA template, 100 mM each dNTP, 1  $\mu$ L of each primer and 1  $\mu$ L of Taq polymerase. Amplification was done as follows:

❖ Initial denaturation step: at 94 °C for 15min.

❖ Three steps cycling: (33 cycles)

✓ Denaturation: at 94 °C for 25 Sec.

✓ Annealing: at 53 °C for 40 Sec.

✓ DNA extension: at 72 °C for 50 Sec.

❖ Final extension: at 72 °C for 6 min.

❖ The thermal cycler program was adjusted by using PTC-100TM Programmal Thermal Controller, Peltier-Effect Cycling, MJ.

**Electrophoresis of the amplified products:-** <sup>(15)</sup>

Ethidium bromide stained bands in agarose gel (1.5%) were visualized on UV transilluminator and photographed. The expected bands were 501 bp for bla<sub>OXA-23</sub> gene and 599 bp for bla<sub>OXA-58</sub> gene in comparison to molecular size marker (100 bp DNA Ladder).

**Animal groups:-**

Mice (15 animals each weighing 25 $\pm$ 3 g) were randomly divided into three groups. The first group served as normal control. The second group was injected with 200  $\mu$ L/g body weight of resistant *A. baumannii* strain. In the third group, mice were injected with 200  $\mu$ L/g body weight of sensitive *A.baumannii* strain. The 3 groups were housed in the animal house of the Faculty of Science, Mansoura University.

**Sampling and preparation of tissue homogenate:-**

At the end of the study period 3 days after *A.baumannii* infection, all mice were placed individually in metabolic cages for 24h. Animals were overnight fasted then sacrificed under ether anesthesia by cervical dislocation. Blood samples were collected from each mouse, centrifuged at 2000 xg for 15 min, obtained sera were kept for biochemical analysis. The liver homogenization was done in ice cold saline solution and the homogenate was kept frozen at -20°C until being analyzed, as soon as possible.

**Determination of liver functions and kidney functions:-**

Serum ALT, AST and bilirubin were detected using the method of Young <sup>(16)</sup>. Serum creatinine, urea, uric acid, sodium and potassium were detected using the methods of Schirmeister <sup>(17)</sup>, Fawcett and Soctt <sup>(18)</sup>, Barham and Tindler <sup>(19)</sup>, Henry et al., <sup>(20)</sup> and Sunderman <sup>(21)</sup> respectively.

**Determination of antioxidants Glutathion (GSH), Superoxid Dismutase (SOD) and Catalase (CAT) levels:-** Were estimated using the methods of Prins and Loose <sup>(22)</sup>, Nishikimi et al. <sup>(23)</sup> and Bock et al. <sup>(24)</sup> respectively.

### Results:-

Out of 1050 positive cultures of all nosocomial samples received, 44 (4.2%) *A.baumannii* strains were isolated and characterized. *A.baumannii* strains were isolated from 15 urine (34.09%), 13 blood (29.54%), 10 sputum (22.73%) samples and 6 septic wounds (13.64%).

**Antibiotic susceptibility:-** Twelve (28%) *A.baumannii* isolates were sensitive to imipinem, 29 (66%) were meropenem sensitive and 22 (50%) were sensitive to amikacin and no sensitivity was observed with Cefuroxime (Table 1).

**Modified Hodge test:-** Carbapenemase production was confirmed by Modified Hodge tests. Out of 44 *A.baumannii* isolates, 24 (54.55%) were positive by Hodge test and 20 (45.45%) were negative.

**PCR results:-** Multiplex PCR detected 32 (72.72%) of the isolates carried bla<sub>OXA-23</sub> gene, while the bla<sub>OXA-58</sub> gene was detected in 13 (29.54%) of the isolates (Table 2).

**The effect of *A. baumannii* infection on the excretory system and antioxidant levels in mice:-** Statistically significant increase of liver function markers in serum of infected mice in group I infected with resistant *A.baumannii* strain in comparison to control group (ALT P<0.0001, AST P<0.001 and total bilirubin P<0.02). (Table 3). Regarding the renal function test there were slight changes in the form decrease of creatinine and urea levels in the infected mice in comparison to the control group with no statistically significant change. There were no considerable change in Na<sup>+</sup>, K<sup>+</sup>, and Uric acid levels between different mice groups (Table 4). Also Statistically significant decrease of GSH (P< 0.0001) and in activities of SOD (P< 0.0001) and CAT (P< 0.0001) were noticed in mice liver tissue of group I and group II in comparison to control group (Table 5).

**Table 1:** Antibiotic susceptibility pattern of 44 nosocomial *A.baumannii* strains.

Antibiotics	Total no. of strains = 44					
	Sensitive strains		Intermediate strains		Resistant strains	
	No	%	No	%	No	%
Amikacin (AK)	22	50	10	23	12	27
Amoxycillin (AX)	6	14	34	77	4	9
Gentamicin (CN)	12	27	14	32	18	41
Cefuroxime (CXM)	0	0	0	0	44	100
Cefotaxim (CTX)	6	14	10	23	28	63
Imipenem (IPM)	12	28	3	6	29	66
Meropenem (MEM)	29	66	6	14	9	20

**Table 2:** PCR results for 44 *A.baumannii* isolates.

Isolates	bla <sub>OXA-23</sub>		bla <sub>OXA-58</sub>	
	Positive	Negative	Positive	Negative
	No	No	No	No
Urine	11	4	3	12
Blood	9	4	5	8
Sputum	7	3	3	7
Wound swabs	5	1	2	4
Total Positive	32 (72.72%)		13 (29.54%)	

**Table 3:-** Serum ALT, AST and total bilirubin in control group and *A.baumannii*. infected mice.

Liver functions		Animal Groups				
		Control (n = 15)	Group I (n = 15)	Test of significance	Group II (n = 15)	Test of significance
ALT (U/L)	Mean ±SD	34.9±2.4	298.3±0.6	t = 45.1 P < 0.0001	52.6±4.5	t = 3.7 P = 0.002
AST (U/L)	Mean ±SD	172.3±8.2	274.9±7.4	t = 4.5 P = 0.001	230.1±9.3	t = 2.7 P = 0.02
Total Bilirubin (mg/dl)	Mean ±SD	0.7 ±0.04	1.9 ±0.16	t= 2.9 P = 0.02	0.6 ± 0.03	t= 0.7 P = 0.5

ALT:alanine aminotransferase and AST:aspartate aminotransferase

**Table 4:-** Serum kidney markers in control group and *A.baumannii* infected mice.

Kidney functions						
		Control (n = 15)	Group I (n = 15)	Test of significance	Group II (n = 15)	Test of significance
Cr (mg/dl)	Mean±SD	0.69±0.03	0.68±0.03	t= 0 P=1	0.6 ±0.03	t= 0.3 P=0.8
BU(mg/dl)	Mean±SD	23 ± 0.6	23.2 ±0.5	t= 0.03 P=1	21.2 ±0.5	t= 0.3 P=0.8
UA(mg/dl)	Mean±SD	3.8± 0.4	3.7± 0.3	t = 0.4 P = 0.7	3.9± 0.4	t = 0.3 P = 0.8
Na <sup>+</sup> (m.Eq/L)	Mean±SD	147.2± 5.9	148.5± 5.2	t = 0.4 P = 0.7	147± 5.02	t = 0.05 P = 0.9
K <sup>+</sup> (m.Eq/L)	Mean±SD	5.7± 0.45	5.7± 0.6	t = 0.1 P = 0.9	6.3± 0.7	t = 1.8 P = 0.1

Cr: creatinine, BU:blood urea, UA: Uric acid, Na<sup>+</sup>: Sodium and K<sup>+</sup>:Potassium.

**Table 5:-** liver glutathione contents, Superoxide dismutase activity and Catalase activity in tissue of control group and *A.baumannii* infected mice.

		Control n = 15	Group I n = 15	Test significance of	Group II n = 15	Test significance of
GSH (mg/g)	Mean ±SD	11.3 ± 0.5	**3.5 ± 0.6	t = 22.6 P < 0.0001	**7.7 ± 0.6	t = 10.58 P < 0.0001
SOD (U/g)	Mean ±SD	420.3 ±13.8	**254.3 ± 28.4	t = 12.9 P< 0.0001	**342.2 ± 17.5	t = 8.6 P < 0.0001
CAT, (U/g)	Mean ±SD	782.7 ± 7.3	**649 ± 14.6	t = 20.04 P< 0.0001	**705.5 ± 20.5	t = 8.7 P< 0.0001

GSH: Glutathion. SOD: Superoxid Dismutase. CAT: Catalase

## Discussion:-

*A.baumannii* is a Gram-negative bacterium associated with nosocomial infections. While this organism can be found in soil and water, there is evidence that the most of the recent infections are caused by strains that populate the hospital environment <sup>(25)</sup>.

Multi-drug resistant (MDR) *A.baumannii* clones are spread throughout many different geographic areas and treatment options for MDR *A.baumannii* infections are limited in most cases to carbapenems <sup>(26)</sup>.

The frequency of *A.baumannii* isolation in this study was 4.2% of all hospital pathogens, these results were in agreement with Seifert et al., (1995) who found that *A.baumannii* accounted for 4.6% of isolates <sup>(27)</sup>. Our results were not coordinated with the results obtained by Abul-Ella et al., (1997) who reported 7.9% of *Acinetobacter* spp. among nosocomial isolates <sup>(28)</sup>.

This study reported 12 (28%) of *A.baumannii* were sensitive to imipinem and 29 (66%) were sensitive to meropenem and 22 (50%) of isolates were sensitive to amikacin. These results were in agreement with **Corrêa et al., (2012)** who found 28% of *A.baumannii* isolates were sensitive to imipinem but found 30% were sensitive to meropenem<sup>(29)</sup>. On the other hand **Unal and Garcia-Rodriguez, (2005)**<sup>(30)</sup> registered 73.1% of *A.baumannii* isolates were susceptible to meropenem and 69.8% were susceptible to imipinem. In contrast to our results, **Gales et al., (2001)**<sup>(31)</sup> reported 88% of *Acinetobacter* spp. were imipinem sensitive, and 87% of *Acinetobacter* spp. were meropenem sensitive.

*A.baumannii* have OXA-type carbapenemases enzymes ( $\beta$ -lactamases bacterial enzymes) which is the main enzymes that catalyze the hydrolysis of  $\beta$ -lactam antibiotics and make *A.baumannii* resistant to carbapenems. The Modified Hodge test is a phenotypic method for detection of carbapenemases<sup>(32)</sup>. During our study we showed that 54.55 % of *A.baumannii* isolates were positive for carbapenemase production by the modified Hodge test and 45.45 % were negative. **Khajuria et al.,(2014)**<sup>(33)</sup> found 60% of *A.baumannii* isolates were positive by the modified Hodge Test, but **Aksoy et al., (2015)**<sup>(34)</sup> observed 96% positive by the modified Hodge test among 52 *A.baumannii* strains. Carbapenem resistance occurred due to OXA type carbapenemases is a growing problem and metallo- $\beta$ -lactamase production along with co- production of OXA enzymes are considered to be the important reason for resistance to imipinem in *A.baumannii*.

We obtained 32 (72.72%) isolates carried bla<sub>OXA-23</sub> and 13 (29.54%) isolates carried bla<sub>OXA-58</sub>. These results were in agreement with **Quiñones et al., (2015)**<sup>(35)</sup> who found that, from 30 *A.baumannii* strains 76% were positive for bla<sub>OXA-23</sub> gene and 3% were positive for bla<sub>OXA-58</sub> by multiplex PCR, on the other hand, **Shoja et al., (2013)**<sup>(36)</sup> detected bla<sub>OXA-23</sub> in 85% of isolates but the bla<sub>OXA-58</sub> was not found, resistance to carbapenems in these isolates may be related to other mechanisms including decreased permeability, alteration of penicillin-binding proteins, AmpC stable derepression and overexpression of efflux pump<sup>(37)</sup>.

The liver actively modulates inflammatory processes by filtering, inactivating and clearing bacteria and bacterial product e.g: endotoxin and the stimulated liver releases the highest amount of cytokines<sup>(9)</sup>. We found Statistically significant increase of liver function markers of infected mice in group I infected with resistant *A.baumannii* strain in comparison to control group [serum ALT (P < 0.0001), AST (P = 0.001) and total bilirubin (P = 0.02)]. These results were in agreement with **Nesselere al., (2012)**<sup>(8)</sup> who found that serum total bilirubin, AST and ALT levels were significantly higher in infected rats than control rats after injection of rats with endotoxins of *Salmonella anatum*. As liver and spleen macrophages clear bacteria, viruses and endotoxins and they secrete chemical mediators and cytokines such as prostaglandins, leukotrienes, TNF- $\alpha$  that may leads to increase liver enzymes and bilirubin levels in the blood<sup>(8)</sup>.

Disturbance in the serum levels of creatinine, urea, Na<sup>+</sup> and K<sup>+</sup> may be as a result of disturbance of the filtration capacity of glomeruli due to dysfunction of the endothelium as a result of the production of pro-inflammatory humoral mediators by sepsis in kidney<sup>(38)</sup>. In addition, inflammatory mediators may lead to vasoconstriction and vasodilatation and no synthesis in septic shock which may affect kidney functions process<sup>(38)</sup>.

Antioxidant molecules work as scavengers, this way reducing Reactive oxygen species (ROS) bioavailability<sup>(39)</sup>. In the present study statistically significant decrease in GSH level (P < 0.0001), SOD (P< 0.0001) and CAT (P< 0.0001) activity in liver tissue after *A.baumannii* infection in comparison to control group. These results may be attributed to the role of sepsis in which liver dysfunction is considered an early event during sepsis and endotoxin injection resulted in membrane damage in the liver, which causes decrease levels of free radical scavengers and leads to an imbalance in the hepatic vasoregulatory gene expression, that causes reduced concentrations of GSH, as well as SOD and CAT activities, an explanation which in accordance with **Sakaguchi and Furusawa, (2006)**<sup>(40)</sup>.

### In Conclusion:-

The infection with *A.baumannii* causes damage of liver functions, renal functions and immune system which may lead to death so, the infection with this type of bacteria must be avoided in ICUs. This needs improving basic and extended knowledge on hygiene, the reinforcement of infection control measures, and the early detection, with restriction of the usage of carbapenems, to control the spread of these multidrug resistant organisms.

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