In Vitro Effiacy Of Different Antibiotic Combinations On Aminoglycoside-Resistant *Acinetobacter Baumannii*

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Abstract :

The potential synergy of combination of β-lactams(ceftriaxone, cefixime, carpabenem, and impenim)and aminoglycosides were tested against multidrug resistant *Acinetobacter baumannii*. Two-hundred bacterial pathogens were collected from Egyptian hospitals from various infection sites.One hundred and twentytwo isolates (60%) were resistant for aminoglycosides.Out of two-hundred strains,130 *Acinetobacter baumannii* strains (65%), were impenim resistant and nearly 180 *Acinetobacter baumannii* strains (90%),were resistant to cephalosporin. The MIC was determined for *Acinetobacter baumanni i*strains(32 to >512 mg/ml). In the checkerboard method, 38 combination from 45 combinations showed synergism for more than60% of the tested strains but only two demonstrated antagonism against 5 of tested strains. i.e. the ratio of synergy were detected for gentamycin with impenim, ceftriaxone and cefixime was 100%. Also synergism observed in case of combination between amakacin and tobramycin with ceftriaxone and cefixine with 100%,but in case of combination of tobramycin with imipenem showed ratio of synergy is 50% Whereas, combination between amakacin/impenim showed antagonism .

Keywords: *Acinetobacter baumannii*, aminoglycosides, β-lactams combination. Synergy.

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Introduction:

Acinetobacter baumannii is considered as one of the major causes of nosocomial outetbreaks and is resistant to most available antibiotics. Aminoglycosides wasatreatment options for*Acinetobacter* infections but their resistance has increased in the recent years. Antimicrobial resistance in Gram-negative bacteria are one of the three greatest threats to human health(Allen, et al 1995, Bergogne-Berezin, et al., 1987). *Acinetobacter bamanunii* is one of the three most challenging Gram-negative pathogens, especially in intensive care units. Approximately 14,000 critically ill patients with*A. baumannii* infections were highly associated with increased mortalityand high morbidity rates(Bouvet, and. Grimont. 1986).

It is often causes a multiple infections likebloodstream, respiratory tract, and wound infections (Mortensen et al., 2014; Peleg et al., 2008;Allen, et al 1995, Anstey, et al 1992,Bouvet, et al 1987, Bouvet., et al1990). Multidrug-resistant *A.baumannii* strains are a critical concern, resulting in a major outbreaks worldwide.Traditionally, β-lactams and aminoglycosides were successfully used to treat susceptible*A. baumannii* (Chopade, et al., 1985), but unfortunately, with increasing abuse, strains have emerged resistant to virtually all antibiotics in monotherapy (Crombach,,et al 1989). Nowadays carbapenems were hitherto considered the treatment of choice against severe *A. baumannii* infections, carbapenem-resistant *A. baumannii* isolates are rapidly increasing (Devaud, et al 1982). Aminoglycoside monotherapy was caused significant killing of *A. baumannii* but followed by rapid and extensive resistance emergence *in vitro* and in patients (Douboyas,., et al 1994, Drusano,. 1991, Eliopoulos, and Eliopoulos. 1989). β-Lactam antibiotics are widely used and very safe, as well as clinicians are well trained on the safe use of aminoglycosides (Joly-Guillou, et al 1987). Aminoglycoside and β-lactam antibiotics have different mechanisms of action and resistance; there is no efflux pump which affects both of these antibiotic classes in *A. baumannii* (Joly-Guillou et al 1990). This suggests that β-lactams may kill aminoglycoside-resistant bacteria and vice versa (Klastersky, J. et al 1977,Marques, et al 1995) Additionally, β-lactam disrupt the outer membrane of *A. baumannii*in which enhance the target site penetration of aminoglycoside, since the outer membrane of *A. baumannii* is approximately 2- to 7-fold less permeable than that of *Pseudomonas aeruginosa* and approximately 50-fold less permeable than that of *Escherichia coli* (Martinez-Martinez, et al 1995, Meyers, et al 1991). The high rates of resistance in *A. baumannii* highlight the necessary need for an alternative treatment options, such as rationally optimized combination therapies.Therefore, we conducted in this study to check the susceptibility pattern of resistant *Acinetobacter baumannii* against commonly available antibiotics in our set up and identify synergistic

bacterial killing and overcome of resistance for combinations of a β-lactam with an aminoglycoside against *A. baumannii* as substantial treatment options.

MATERIALS AND METHODS:

1. **Collection and identificationof bacterial isolates.**

Two hundred bacterial isolates were collected from clinical samples (blood, urine, stool, sputum, wound and endotracheal tube infection)from microbiological laboratories belonging to four hospitals in Cairo, Egypt (Nasser Institute, El-Kasr Al-Ainy Hospital, Abou El-Reesh, El-Haram Hospital, and Hussaini Hospital) along the period from November2016 to December 2017.All bacterial isolates were identified by conventional methods confirmed using OXA-51 gene that is intrinsic to the species, using the primers sequences as following(Woodford, N**,**et al 2006).:

5”-TAATGCTTTGAT CGGCCTTG-3”

3”-TGGATTGCACTTCATCTTGG-5”

2. **Antimicrobial Susceptibility testing**

Antimicrobial susceptibility testing of identified *Acinetobacter*strains was carried out by disk diffusion method using the Kirby–Bauer technique1966 (Meyers, B. R., et al 1991) and as the recommendations of CLSI document M2-A41 (NCLSI 1994). Antibiotics to be tested; were selected referring to CLSI document M100-S28 (CLSI, 2018), and they included the first and second line antibiotics commonly used for treatment of *Acinetobacter* infections. The tested antibiotics included; gentamicin, tobramycin, amikacin, meropenem , imipenem , Amoxycillin Clavulanate, cefixime, Ampicillin, Cefoperazone, Cefoperazone-Sulbactam, Cefotaxime, Cefoxitin, Ceftazidime, Ceftriaxone, Cefturoxime, Ciprofloxacin, Co-trimoxazole, Levofloxacin, Piperacillin, Oflaxacin, Norfloxacin.

3. **Determination of Minimum Inhibitory concentrations (MICs)of antibiotics against *Acinetobacter baumannii* isolates:**

Minimum Inhibitory Concentrations (MICs)of different antibiotics againstclinical *A. baumannii* strains(1.5×10 ˆ8 CFU/ml)were determined by broth microdilution method in Mueller-Hinton broth MHB (Oxoid, USA) according to Clinical and Laboratory Standards Institute methods (CLSI 2014).The different antibiotic standards include: cefixime, ceftriaxone, imipenem, Gentamicin, Tobramycin and Amikacin .The stock solutions of antibiotic were prepared using following equation(Eucast,2013 ,Anderws,2001.):

Weight of powder (mg) = [Volume of solution (ml) × Concentration (mg/L)]/ Potency of powder (mg/g)

4. **Combinations of antibiotics using Checkerboard method:**

Combination of antibiotics was done by using checkerboard method(Eliopoulos and moellering, 1996) for five selected multidrug-resistant*Acinetobacter baumannii* strains namely, ACN1N, ACN4, ACN12, ACN15 and ACN18.The checkerboard dilution test is widely used *in vitro* for the evaluation of combination potential synergetic effect of both individual and combined antibiotics as represent by FIC index. The concentration range of each used antibiotic combination tested in range from 1/4 XMIC up to 2X MIC dilution.Each test was performed in triplicate with starting inoculum at concentration of 5×10 ˆ5 CFU/ml.

The fractional inhibitory concentration (FIC) index is a mathematical expression used to represent the interaction of antibiotics, and was calculated for each antibiotic in each combination using the following formula.

FIC index=FICA + FICB

1. **FICA =** MIC of drug A in combination/ MIC of drug A alone
2. **FICB =** MIC of drug B in combination/ MIC of drug B alone

The FIC indices were interpreted as:

Synergy (was defined when) =FIC≤0.5.

Additive or indifferent (was defined when) =FIC>0.5≤4.0.

Antagonism (was defined when) = FIC >4.0.

The checkerboard method (Microtitre method) was performed in 96 well microtitre plates containing Cephalosporins plus aminoglycosides and Carbapenem plus aminoglycosides antibiotics.

Result:

In the present study,we collected two hundred bacterial pathogens from Egyptian hospitals from different infection sites. However, the most common clinical specimen were endotracheal infections followed by sputum, blood ,urine and wounds .The isolates were identified using conventional methods depending on cultural and biochemical characteristics on blood and MacConkey agar medium and as oxidase negative and catalase positive isolates. The positive 180 *Acinetobacter* isolates were confirmed using PCR detection of *bla-oxa-51* gene with amplicon size 353 bp that is characteristic for *Acinetobacter baumannii*. The phenotypic resistance patterns represented in Table (1),which showed that *A. baumannii* stains are resistant to aminoglycoside,β-lactam, Fluroquinolons and Sulfa drugs in a variable degrees of resistance as assessed by disk diffusion methods. Out of two hundred *A. baumannii*

strains, one hundred and twenty two strains (60%) were resistant to aminoglycosides,130strains was impenim resistant and nearly 170 strains were resistant to cephalosporin. *A.baumannii*strains were exhibited maximal resistance against β-lactam91%, and minimal degree resistance against ofloxacin 46.5% and intermediate degree resistance against aminoglycosides (53%).Additionally,the resistance rate of*A. baumannii*was ranged from 35.5% to 99% and sensitivity rate was from 1% to 60.5% (Figure 1).

It is proposed that the phenotypic pattern of the selected five *A.baumannii*strains, all five strains were resistant to aminoglycosides (100%) as well as five strains were resistant to fourteen antibiotics (100%) except norfloxacin that showed 80% degree of resistance.

In order to study the overcome of resistance problem, it was decided to focus on evaluate the MICs of selected antibiotic alone and in combination. In table (3) showed the MIC values of antibiotics belonging to aminoglycoside and β-lactam groups. All strains showed high MICs for all antibiotics tested in a range from 128 to ≥512 µg/ml for aminoglycoside groups and β-lactam groups in a range from 64 to ≥512 µg/ml.

In vitro antibacterial activity of tested antibiotics combination against multidrug resistances*A.baumannii* showed in table (4) by employing checkerboard method. Synergism was achieved in all combination using gentamycin (100%) for five tested strains. However, 93.33% and 66.66% was found for all combination using tobramycin and amikacin, respectively. In addition, according to FICindex, cephalosporins antibiotics were found to have synergistic effect when used with aminoglycosides other than carbapeneme (imipenem). Among combinations, antagonism was seen in 40% of selected strain in combination between amikacin and imipenem(AK &IMP) according to FIC index while additive or indifferent effect was observed in 60% and 40 % of selected strains with amikacin and imipenem (AK plus IMP) and tobramycin and imipenem (TOB plus IMP), respectively i.e. Synergism was most observed in all antibiotic combination against tested strains whereas the least effective combination was to amikacin plus imipenem.

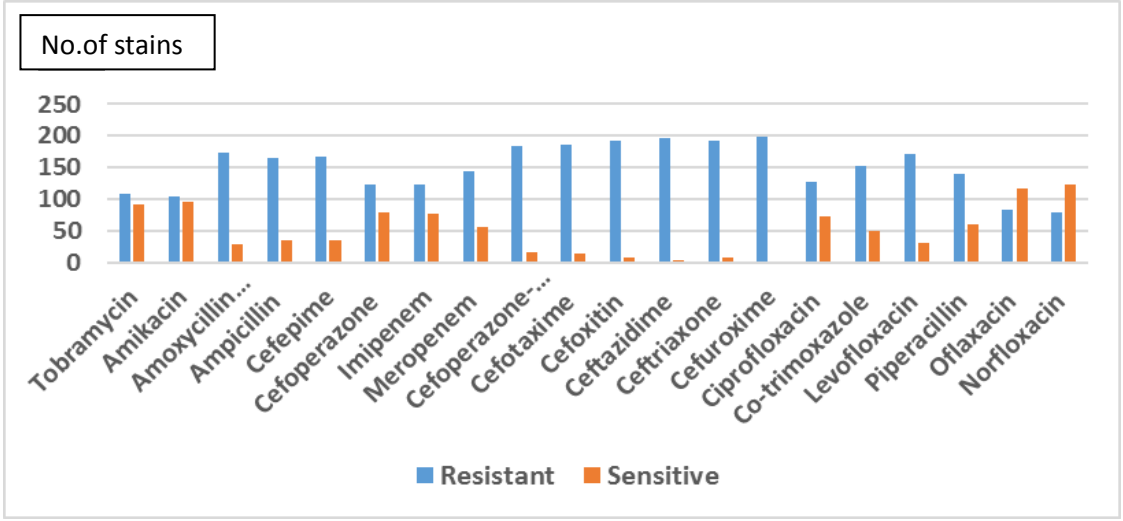

Figure (1) Phenotypic resistant index of *Acinetobacter baumannii* strains.

Table (1): percentage of resistance patterns of aminoglycosides resistant *Acinetobacter*strains

Antibiotic groups		Antibiotics	Sensitive(S)		Resistance (R)	
			%	No.	%	No.
Aminoglycosides		Gentamicin (GN)	39.5	79	60.5	121
		Tobramycin (TOB)	45	90	55	110
		Amikacin (AK)	47	94	53	106
β-lactam	Penicillins	Amoxycillin/clavulanate (AMC)	12.5	25	87.5	175
		Ampicilin (AMP)	17.5	35	82.5	165
		Pipracillin (PRL)	43	86	57	114
	Cephalosporins	Cefepime (FEP)	17	34	83	166
		Cefoperazone (CEP)	39	78	61	122
		Cefoperazone-Sulbactam	8.5	12	91.5	183
		Cefotaxime (CTX)	12	24	88	176
		Cefoxitin (FOX)	4	8	96	192
		Ceftriaxone (CRO)	2	4	98	196
		Cefturoxime (CXM)	4	8	96	192
		cefixime (CFM)	1	2	99	198
	Carbapenem	Imipenam (IMP)	35	70	65	130
		Meropenom (MEM)	27	54	73	146
Quinolones &Fluroquinolons		Ciprofloxacin (CIP)	23.5	47	76.5	153
		Levofloxacin (LEV)	9.5	19	90.5	181
		Ofaxacin (OFX)	53.5	107	46.5	93
		Norfloxacin (NOR)	60.5	121	39.5	79
Sulfa drugs		Co-trimoxazole (STX)	24.5	49	75.5	151

Table (2) phenotypic resistance patterns of selected *Acinetobacter* strains

Strain No.	CFM	AMP	AMC	AK	TOB	CTX	MEM	IMP	CEP	NOR	PRL	LEV	STX	CIP	CXM	CRO	CAZ	FOX	GN
ACN 1N	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
ACN 4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
ACN 12	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
ACN 15	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
ACN 18	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R: Resistance S: Sensitive, GN: gentamicin, TOB: tobramycin, AK: amikacin, MEM: meropenem, IMP: imipenem, AMC: Amoxycillin Clavulanate, CFM: cefixime, CRO: Ceftriaxone, AMP: Ampicillin, CEF: Cefoperazone, Cefoperazone-Sulbactam, CTX: Cefotaxime, FOX: Cefoxitin, CAZ: Ceftazidime, CXM: Cefturoxime, CIP: Ciprofloxacin, STX: Co-trimoxazole, LEV: Levofloxacin, PRL: Piperacillin, OFX: Oflaxacin, NOR: Norfloxacin.

Table (3): MICs of tested antibiotics against selected *Acinetobacter baumannii* strains

***Acinetobacter* baumannii Strains**	**Concentration of antibiotics (mg/L)**					
	Aminoglycosides group			**β-lactam group**		
	Gentamicin	Tobramycin	Amikacin	Impenim	ceftriaxone	cefixime
ACN1N	≥512	≥512	≥512	64	512≥	512≥
ACN4	≥512	128	128	512≥	>512	>512
ACN12	256	≥512	>512	128	512≥	512≥
ACN15	≥512	128	128	256	>512	256
ACN18	≥512	256	256	128	256	>512

Table (5) : Combination of aminoglycosides and β-lactamsagainst*Acinetobacterbaumannii*strains.

Antibiotic Comb.	1N			4			12			15			18		
	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity
GN/IMP	32/8	0.158	S	16/4	0.03	S	16/4	0.09	S	16/4	0.04	S	16/4	0.06	S
GN/CRO	4/4	0.015	S	4/4	0.011	S	32 /128	0.37	S	32	0.188	S	4/4	0.01	S
GN/CFM	8/64	0.14	S	4/4	0.013	S	8/ 128	0.28	S	8	0.5	S	4/4	0.10	S
AK/IMP	512/ 256	5	Ag	128/	0.625	Ad	128/64	0.628	Ad	512/128	4.5	Ag	64/	0.75	Ad
AK/CRO	16/4	0.038	S	8/128	0.158	S	8/ 128	0.25	S	32/32	0.282	S	8/	0.28	S
AK/CFM	16/128	0.28	S	8/128	0.04	S	16/ 32	0.078	S	16/32	0.25	S	4/16	0.03	S
TOB/IMP	16/128	2.03	Ad	32/64	0.375	S	32 /64	0.56	Ad	32/64	0.5	S	8	0.28	S
TOB/CR	4/4	0.01	S	4/4	0.034	S	4/32	0.07	S	4/32	0.06	S	8	0.28	S
TOB/CF	4/32	0.07	S	4/4	0.035	S	16/4	0.037	S	16/4	0.14	S	8	0.2	S

FIC: fractional inhibitory concentration, S : **Synergy ,** Ad : **Additive ,** Ag : **Antagonism.**

Discussion:

Aminoglycosides resistance in *Acinetobacter* spp. has emerged as a significant health problem due to the therapeutic option was very limited. Aminoglycoside resistance is common in *Acinetobacterspp.* and that was in agreement with Lambert *etal.*,(1997).Who mentioned that the inactivation of the antibiotic was carried out by specific modifying enzymes such as acetyltransferases, phosphotransferases, and adenylyltransferases. *Acinetobacter* spp are frequently resistant to multiple antimicrobial agents; there are several reports on strains resistant to most clinically relevant drugs *(Lu 2008,Giamarellou, etal., 2008).* Differences in antibiotic susceptibility have been observed between countries, probably as a result of environmental factors and different patterns of antimicrobial usage. Gaur and co-works reported more than 80% of isolates to be resistance to cephalosporin, aminoglycosides, and quinolones especially second and third-generation (Gaur *et al*., 2008).The present study showed the resistance rate to imipenem, ampicillin/tobramycin, ceftazidime, cefixime, gentamicin, amikacinand ciprofloxacin were more than 90% in the selected multidrug-resistant *Acinetobacterbaumannii* this observation is consistent with those of (Livermore, 2002).

The obtained result indicate that, endotracheal infections were the most common clinical specimen of *Acinetobacter spp.* The frequency of isolation and variety of bacteria found in clinical specimens in different countries widely varies *(*Shiri et al, 2005; Van Looveren&Goossens 2004). Potential risk factors for colonization or infection of hospitalized patients with multidrug-resistant *Acinetobacter*strains include length of ICU stay, underlying diseases, or conditions, exposure to carbapenems or third-generation cephalosporin, hospitalization and using urinary catheterization (Cisneros et al. 2005; Prashanth & Badrinath,2006). The findings showed that clinical isolates of *Acinetobacter* spp. in our hospital carrying various kinds of aminoglycoside resistance. Once of the common ways to overcome antibiotic resistance was combination of gentamycin, amikacin and tobramycin with imipenem, ceftriaxone and cefixime. The results virtually extend to the results of previous studies on aminoglycosides in combination with beta lactam against *Acinetobacter baumnnii*, the checkerboard method was used to assess the synergy between antimicrobials against *Acinetobacterspp*. in many of these studies antibiotic combinations have demonstrated the synergistic or bactericidal effects against bacteria that have been resistant to the individual drugs by using checkerboard methods. For example, synergistic effects have been demonstrated for double and triple antibiotic combinations including an aminoglycoside, an

anti-pseudomonal beta-lactam, colistin, a fluoroquinolone, a macrolide, or rifampin against multidrug-resistant *Pseudomonas* spp. (Fish et al., 2008;Saiman et al., 2002;Aoki et al., 2009). Double and triple antibiotic combinations including an aminoglycoside, ampicillin/sulbactam, a carbapeneme, colistin, rifampin, tigecycline, or vancomycin have been effective against multidrug-resistant *Acinetobacter* spp. (Urbanet al., 2010;Kiffer et al. 2005, Hornsey &Wareham, 2011) each drug combination was evaluated in duplicate,this study revealed that various antimicrobial combinations could be synergistically *in vitro* against multidrug-resistant of most *Acinetobacter* spp. The checkerboard method is employed for this purpose. The results obtained in the study showed the overall rate of synergy in most antibiotic combination.

The combinations of imipenem, ceftriaxone and cefixime with a second group (gentamycin, amikacin and tobramycin) mostly resulted in synergy. Combinations of these antibiotics with gentamycin exhibited synergy in 100% of the performed tests with the five *Acinetobacter* spp. in combination between amikacin and β-lactams (AK plus IMP, CRO and CFM) was 100% and also in case combination between tobramycin with β-lactams (TOB plus IMP, CRO and CFM) was 100%. While in 40% of selected strains antagonism was seen. This observation is consistent with the experience of others (Lim et al., 2008;Prashanth&Badrinath,2006).

In another study,Tod et al., 2000 by assessing ceftazidime plus tobramycin and piperacillin/tazobactamplus tobramycin combinations against multidrug-resistant *P.aeruginosa* were evaluated, and synergy ratios of 50% and 67%, respectively were observed. With respect to fosfomycin, synergistic interactions with other antibacterial drugs were verify in 57% of the tests, rate similar to that reported previously for multidrug-resistant *P.aeruginosa***.** Fosfomycin enhances the active transport of tobramycin in *P.aeruginosa*; *in vitro* synergic actions were also demonstrated for polymyxin E, imipenem, ceftazidimeand ciprofloxacin (Obara &Nakae, 1991. Landersdorfer et al., 2013).As observed in other studies, the rate of synergy of antibacterial combinations varies according to isolate and is not strictly associated with susceptibility or resistance to imipenem. Comparison of the two multidrug-resistant *P.aeruginosa* revealed more frequent and significant drug MIC reductions for the 46R isolate than for the 72R isolate. Thus, it is advisable to test each multidrug-resistant isolate with the different drugs in combination (Shiri et al., 2005).

Among the synergy results, only a few antibacterial combinations have led to sufficient MIC reductions (Chastre et al., 2000). Other authors also noted synergism between third and fourth generation cephalosporin and aminoglycosides (often gentamicin, amikacin and tobramycin) against 30% to90% of *Enterobacteriaceae* (Eliopoulos &Eliopoulos 1988; Cha, 2008).

Conclusion:The present study showed that the emergence of *Acinetobacter* spp. resistance to antimicrobial agents in Eyption hospitals is associated with the spread of more than 60% of MDR *Acinetobacterspp.*Bacterial isolates from patients were resistant to aminoglycosides, broad-spectrum cephalosporin, gentamycin, amikacin and tobramycin with imipenem, ceftriaxone, and cefixime and trimethoprim/sulphamethoxazole. Antimicrobial synergy was observed against clinical isolates of MDR *Acinetobacter spp*. Some drug combinations resulted in sufficientMIC reductions, which suggest that these combinations may be of clinical use for infections of MDR *Acinetobacterspp*as an alternative to antibiotic therapy, suggesting its potential as an among alternative tested aminoglycosides.Therefore, *in vitro* data must be validated by assessing the clinical performance of combinations of antimicrobial agents before specific recommendations to modify existing treatment guidelines for*Acinetobacter* infections are possible.

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المخلص العربي

تاثير امتزاج المضادات الحيوية المختلفة علي بكتريا الاسينيتوباكتر بومنييا المقاومة للامينوجليكوسيد بالمختبر.

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استاذ دكتور مساعد بالهيئة القومية للرقابة والبحوث الحيوية بقسم الميكروبيولوجي.

تم عزل مائتين عزلة بكترية من اماكن مختلفة من المستشفيات المصرية وبدراسة حساسية هذة العزلات وجد منهم حوالي مائة وعشرون سلالة مقاومة للامينوجليكوسيد بنسبة ٦٠% ,وحوالي مائة وثلاثون عزلة بنسبة ٦٥% مقاومة للمضاد الحيوي ايمبينم وحوالي مائة وثمانون عزلة بنسبة ٩٠% مقاومة للسيفالوسبورين .

وبقياس الحد الادني للعزلات وجد ان التركيزات الثبطة تتروح ما بين ٥١٢ الي ٣٢ ملي جرام وباستخدام هذة الطريقة لوحظ ان ٦٠% كانت تأثير متعاون بين ايمبينيم وجينتاميسين و ١٠٠% مع سيفترياكسون وسيفيكسيم.

حوالي اثنين من اربعه واربعين لوحظوا انه في تأثير سلبي . اي لايوجد سمة تعاون بين المضادين الحيوين ايمينيم وايماكسين بينما كان العكس .

مزج المضادات الحيوية في العدوي الخاصة بالمستشفيات التي تسببها بكتريا الاسينتوباكتر بومنييا باستخدام طريقه الشطرنج (checkboard).

في اختبار امتزاج مجموعات المضادات الحيوية وهي البيتالكتام (سيفترياكسون , سيفيكسيم , كاربابينيم , وايمينيم) مع الامينوجليكوسيد ضد عزلات الاسينتوباكتر بومنييا المقاومة لمعظم المضادات الحيوية التي تسبب العدوي بالمستشفيات.

وكذلك ايضا اظهرت النتائج اعلي تأثير تعاون بين اماكيسين مع سيفيرياكسون وسيفكيم بنسبة ١٠٠%ولكن في حالة التوبراميسين مع ايمينيم بنسبة ٥٠% وبنسبة ١٠٠%في حالة التوبراميسين مع سيفترياكسون،سيفيكسيم.