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Synergistic combination of colistin with imipenem, amikacine or ciprofloxacin against *Acinetobacter baumannii* and *Pseudomonas aeruginosa* carbapenem-resistant isolated in Annaba hospital Algeria

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Key words: Carbapenemases, Colistin, Combination, GNB-NF, Sub-inhibitory concentration

Abstract: **Objective:** The aim of this study is to detect in vitro the synergetic activity of colistin in combination with imipenem, amikacin or ciprofloxacin, at sub-inhibitory concentrations, against carbapenems-resistant (CR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains isolated from various wards in Annaba teaching hospital in eastern Algeria.
Materials and Methods: The minimal inhibitory concentrations (MIC) were determined by broth macrodilution (BMD). Carbapenemase encoding genes were screened using polymerase chain reaction (PCR). The activity of colistin in combination with second antibiotic was evaluated by the Checkerboard Technique.
Results: 39 CR *P. aeruginosa* and 21 CR *A. baumanni* strains where collected. The MIC values ranging from (0.25 to 4 µg/ml) to colistin, ≥16 µg/ml for imipenem, ≥4 µg/ml to amikacin and ≥8 µg/ml ciprofloxacin. The PCR reveals the presence of the genes bla_{OXA23} (n = 12), bla_{OXA24} (n = 6), bla_{NDM1} (n = 3) in *A. baumannii* and bla_{VIM2} (n = 12) in *P. aeruginosa*. The combination of colistin with imipenem showed synergistic effect on 57.14% and 46.15% of *A. baumannii* and *P. aeruginosa* isolates, respectively. For colistin and amikacin, the synergistic effect is detected in 28.6% of *A. baumannii* and 30.8% of *P. aeruginosa*. While colistin and ciprofloxacin showed synergy on 14.29% and 15.38% of *A. baumannii* and *P. aeruginosa* isolates, respectively.
Conclusion: CR *A. baumannii* and *P. aeruginosa* remain the most prevalent infection agents in patients from high-risk wards at Annaba Hospital. Colistin associated with imipenem or with amikacin at sub-inhibitory concentrations gives very encouraging results allowing better management of infections caused by this type of bacteria.

Introduction

Non-fermenting Gram negative bacilli such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are pathogens emerging as frequent causes of nosocomial infections, such as pneumonia, septicemia, skin, wound and urinary tract infections, with mortality rates of 18% to 61% in critically ill patients (Mohanty *et al.*, 2013; Mathlouthi *et al.*, 2015).

The treatment of these infections is often difficult because of the natural and acquired resistance of these organisms to multiple classes of antibiotics, essentially extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones. This is what severely limits therapeutic options (Mohanty *et al.*, 2013). Carbapenems have been often used as treatment for infections caused by these multidrug-resistant (MDR) bacteria.

However, in recent years this situation has changed, with the emergence of carbapenem-resistant Gram-negative bacilli (GNB), reported from different regions of the world, including mediterranean countries (Mellouk *et al.*, 2017).

The most common mechanism of resistance is the acquisition of genes encoding for carbapenemases particularly, Metallo-β-lactamases (MBLs) in *P. aeruginosa* such as VIM, IMP, GIM, FIM, and SPM. The bla_{VIM-2} gene is the dominant Metallo-β-lactamase in all over the world (Toumi *et al.*, 2018). In Algeria, this gene was reported in

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Annaba, and Oran (Toumi *et al.*, 2018; Touati *et al.*, 2013; Sefraoui *et al.*, 2014).

For *A. baumannii* carbapenem resistance is mostly associated with oxacillinases, especially OXA-23, OXA-24, OXA-58, which have a higher dissemination worldwide, including African countries. Resistance by NDM-1 is also increasingly reported (Mathlouthi *et al.*, 2015). In Algeria, several studies describe these enzymes in *A. baumannii* strains isolated in Annaba (Touati *et al.*, 2012; Mellouk *et al.*, 2017; Toumi *et al.*, 2018). Setif, Tizi-Ouzou (Bakour *et al.*, 2012), Tlemcen, Sidi Bel Abbes, Oran (Mesli *et al.*, 2013), Algiers and Bejaia (Bakour *et al.*, 2014).

The colistin, is a polypeptide antibiotic of the group E polymyxin family. It exhibits rapid and concentration-dependent bactericidal activity by destroying the outer membrane of Gram-negative bacteria (Kipnis and Guery, 2010). This antibiotic has been used in recent years as an alternative to the treatment of MDR BGN, such as CR *A. baumannii* and *P. aeruginosa*. However, resistances to colistin have been reported recently in all across the globe (Ahmed *et al.*, 2016). With limited therapeutic options, clinicians are increasingly forced to turn to combination of antibiotics in the hope that these may be efficacious against these MDR bacteria. Many colistin-based combinations were tested have been shown synergistic activity *in vitro* (Wareham *et al.*, 2011). These synergies could prevent the emergence of resistant strains and also use lower doses of colistin, reducing its toxicity (Daoud *et al.*, 2013).

The aim of the present study is to search *in vitro* the synergetic activity of colistin in combination at sub-active concentrations with antibiotics usually used as first-line drugs in Algeria such as imipenem, amikacin or ciprofloxacin against CR *A. baumannii* and *P. aeruginosa* strains isolated from various wards in Annaba teaching hospital in eastern Algeria.

Materials and Methods

Bacterial isolates

A total of 60 strains *Acinetobacter baumannii* and *Pseudomonas aeruginosa* CR were collected on 240 non-fermenting Gram-negative bacilli (NF-GNB) made responsible for infections during the period from September 2015 and April 2017. These strains were isolated from not redundant 900 pathological specimens: distal protected sampling (DPS), pus and urine taken from patients hospitalized in the various departments (intensive care units, burns units, endocrinology and urology) at Annaba hospital-Algeria. The isolates were identified using API 20NE galleries (Bio-Mérieux), and confirmed by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Seng *et al.*, 2009).

Antibiotic susceptibility testing

Antibiotic susceptibility to was determined for thirteen antibiotics using the Muller-Hinton (MH) agar diffusion method, according to the recommendations of Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI), 2018).

Phenotypic and genotypic characterization produced carbapenemases

All the isolates resistant to imipenem were subjected to modified Hodge test (MHT) as was described by CLSI. A dilute suspension of 1/10 of *E. coli* ATCC 25922 (0.5 Mc Farland) was prepared and inoculated the surfaces of MH agar plates. An imipenem disk (10 µg) is placed in the center to the plate. Test organism was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated for 18 hours at 35°C. The MHT was interpreted as positive based on the presence of a distorted inhibition zone between of the strain tested, and the culture of *E. coli* (Toumi *et al.*, 2018).

The detection of MBL was also performed by the EDTA test (Toumi *et al.*, 2018). Two imipenem discs (10 µg), deposit with 25 mm one of the other, one containing 10 µl of EDTA (solution 0.5 M, pH = 8). After 18 hours of incubation at 35°C, the diameters of inhibition zones around the disks are measured and compared. An increase superiors 6 mm in the zone diameter around the imipenem-EDTA disc compared with that of the imipenem disc alone was considered as positive for MBL production.

The search for genes encoding for carbapenemases (bla_{OXA-23}, bla_{OXA-24}, bla_{NDM}, bla_{VIM}, bla_{KPC}) was realized by real-time Polymerase Chain Reaction (PCR) and standard PCR, using starters and probes specific to various reference strains. The amplified PCR obtained products have been sequenced using Big Dye terminator chemistry on an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, California, United States). The nucleotide and deduced protein sequences were analyzed using the ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta *et al.*, 2014).

Determination of the minimal inhibitory concentrations (MICs)

The MICs of colistin, imipenem, ciprofloxacin and amikacin were determined by broth macrodilution according to the recommendations of CLSI (Rahal, 2011). Antibiotic stock solutions are prepared in sterile distilled water at a concentration of 1024 µg/ml. Thus semi-logarithmic dilutions of reason 2 can be made to obtain concentrations ranging from 512 µg/ml to 0.063 µg/ml. In parallel bacterial suspensions are prepared at densities of 0.5 MF (10^8 CFU/ml). Then, they are diluted to $1/10^{th}$. Inoculation is carried out by culturing 50 µl of the bacterial suspension in 700 µl of cation-adjusted Mueller-Hinton broth (CAMHB) at which 250 µl of the antibiotic at given concentration is added. One control tube of culture without antibiotic and another without inoculums are included in the study. All the prepared tubes are incubated for 18 hours at 35°C. The MIC corresponds to the first tube without bacterial growth visible to the naked eye. Two reference strains (*Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 25953) used for quality control is tested under the same conditions.

Type of colistin based combination activity

The activity of colistin based combination was determined by broth macrodilution checkerboard method (Humphries, 2016). In 700 µl of CAMHB we add 50 µl of the 0.5 MF

bacterial suspension diluted 1/10. Then we add 125 µl of each stock solution of the two associated antibiotics prepared in a checkerboard configuration at adequate concentration. *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 25953 are used for quality control.

The fractional inhibitory concentration index (FICI) for each of the three combinations was calculated according to the following formula: **FICI** = FICI A + FICI B, FICI A = MIC of drug A in combination/MIC of drug A alone, and FICI B = MIC of drug B in combination/MIC of drug B alone.

The FICI results for each combination were interpreted as follows: synergistic (FICI ≤ 0.5); indifferent 0.5 < FICI ≤ 4; antagonistic (FICI > 4) (Pillai *et al.*, 2005).

Results

A total of 240 strains of NF-GNB responsible for infections were isolated from 900 non-redundant pathological samples. Among them 60 strains (25%) showed resistance to imipenem and met the BMR criterion. These carbapenem-resistant strains are derived mainly from pus (n = 44; 73.33%), urine (n = 9; 15%), site of operation (n = 9; 10%), and PPD (n = 3; 5%) *P. aeruginosa* predominated with 39 strains (65%) followed by *A. baumannii* (n = 21; 35%) (Tab. 1).

All CR *A. baumannii* strains are fully resistant to ticarcillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, aztreonam, ceftazidime, cefepime, cefotaxime, gentamicin and tobramycin (Fig. 1). It produces carbapenemases and only three strain posses MBL revealed by the EDTA. The detection of the genes of resistance to carbapenem permits to highlight 12 (57.14%) genes bla_{OXA-23}, 6 (28.57%) bla_{OXA-24} and 3(14.29%) bla_{NDM-1} (Tab. 2). The MICs values are high for imipenem and ciprofloxacin, ranging from 16 to 32 µg/ml and 16 to 128 µg/ml, respectively (Figs. 2 and 3). While for amikacin the MIC ≥8 µg/ml and for colistin is ranging from 0.25 to 2 µg/ml (Figs. 4 and 5).

The situation is different for CR *P. aeruginosa* strains, it show less resistance to ticarcillin, ticarcillin/clavulanic acid and ceftazidime (61.5%), piperacillin/tazobactam (46.2%), cefepime and aztreonam (76.92%), cefotaxime (100%), gentamicin (100%), tobramycin (69.23%) (Fig. 1). Twelve (30.77%) strains produce carbapenemases that have been shown to be MBL. Four CR *P. aeruginosa* strains possess the bla_{VIM-2} gene (Tab. 2).

For the remaining strains, carbapenem resistance may be the result of a type OprD mutation or the presence of an efflux pump. The absolute values of MICs of the imipenem vary within a range of 16 to 128 µg/ml (Fig. 2). Ciprofloxacin and Amikacin exhibit MICs ≥8 µg/ml and ≥4 µg/ml, respectively (Figs. 3 and 4). Colistin which remains active shows MICs ranging from 1 to 4 µg/ml (Fig. 5).

The results of the activity of different colistin-based combinations on CR *A. baumannii* showed a synergy rate of the order of 57.14% when it is associated with imipenem.

TABLE 1

Distribution of carbapenem resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains related to clinical characteristics of patients

Parameter	Carbapenem-resistant bacteria	
	***A. baumannii* (n = 21)**	***P. aeruginosa* (n = 39)**
Gender, n (%)		
Male	15 (71.43)	24 (61.54)
Female	6 (28.57)	15 (38.46)
Source: n (%)		
Pus	15 (71.43)	30 (76.92)
Urine	3 (14.29)	3 (7.69)
PPD	00	3 (7.69)
Operative sites	3 (14.29)	3 (7.69)
Service, n (%)		
Intensive care unit	00	6 (15.38)
Burns	3 (14.29)	24 (61.54)
Endocrinology	12 (57.14)	3 (7.69)
Urology	6 (28.57)	6 (15.38)
Previous use of antimicrobials, n (%)		
Cefotaxime	3(14.29)	9 (23.08)
Imipenem	8 (38.09)	16 (41.03)
Amikacin	3 (14.29)	5 (12.82)
Ciprofloxacin	4 (19.04)	9 (23.08)
Imipenem + Amikacin	3 (14.29)	00

Note: DPS = distal protected sampling; C3G = third-generation cephalosporin.

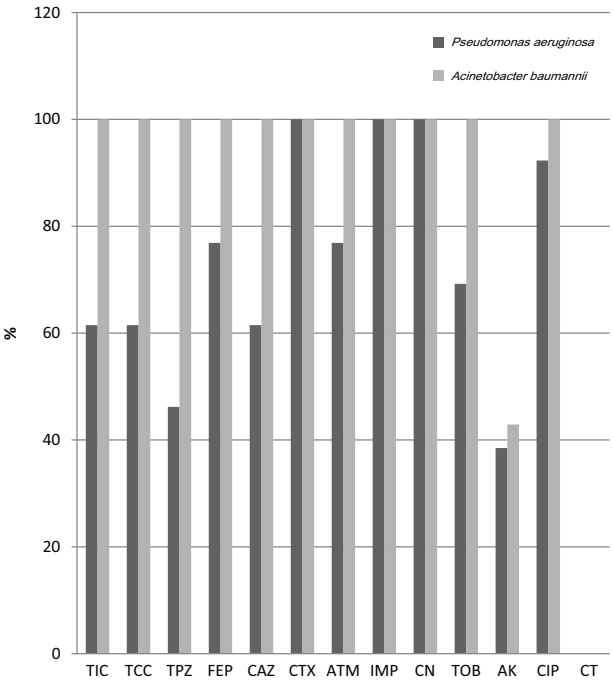

FIGURE 1. Antibiotic susceptibility of carbapenem resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical strains.
TIC = ticarcillin; TCC = ticarcillin/clavulanic acid; TPZ = piperacillin/tazobactam; FEP = cefepime; CAZ = ceftazidime; CTX = cefotaxime; ATM = aztreonam; IMP = imipenem; CN = gentamicin; TOB = tobramycin; AK = amikacin; CIP = ciprofloxacin; CT = colistin.

TABLE 2

Genotypic characterization by PCR of carbapenem-resistant *Pseudomonas aeruginosa and Acinetobacter baumannii* strains

Isolates		*bla* genes				Total
		Class B		Class D		
		bla_{VIM}	bla_{NDM}	bla_{OXA-23}	bla_{OXA-24}	
A. baumannii	N	0	3	12	6	21
	(%)	0	(14.29)	(57.14)	(28.57)	(100)
P. aeruginosa	N	12	0	Nd.	Nd.	12
	(%)	(30.77)	0	Nd.	Nd.	(30.77)

Note: N = number of isolats; Nd = not determined.

More preferably, the concentrations required for this synergistic activity range from 0.25 to 0.5 µg/ml for colistin and from 2 to 8 µg/ml for the imipenem. On these same strains, the combination of colistin with amikacin also gives encouraging results with a synergistic effect up to 42.86% at sub-inhibitory concentrations of 0.25 µg/ml for colistin and from 1 to 4 µg/ml for amikacin. While the combination of colistin with ciprofloxacin is indifferent type for most strains (85.71%). In this case the synergistic effect was observed in only three strains (14.29%) at sub-inhibitory concentrations of 0.25 µg/ml for colistin and 4 µg/ml for ciprofloxacin (Tab. 3).

For CR *P. aeruginosa* strains, the combination of colistin with imipenem acts synergistically at 46.15% at sub-inhibitory concentrations ranging from 0.25 to 1 µg/ml for colistin and from 4 to 8 µg/ml for imipenem. However, the combination of colistin with amikacin shows a synergistic effect of 30.77% at sub-inhibitory concentrations ranging from 0.5 to 1 µg/ml for colistin and from 4 µg/ml for amikacin. A weak synergistic effect (15.38%) is observed for the combination of colistin with ciprofloxacin at sub-inhibitory concentrations ranging from 0.25 to 1 µg/ml for colistin and from 4 to 8 µg/ml for ciprofloxacin, while 84.62% of isolates have an indifferent effect. It should be noted that no antagonistic effect was observed for the three combinations tested on both CR *A. baumannii* and *P. aeruginosa* strains (Tab. 3).

Discussion

Like many other regions of the world, Algeria has experienced a worrying emergence of carbapenem resistance in recent years, particularly among GNBs such as *A. baumannii* and *P. aeruginosa* (Bakour *et al.*, 2014). This phenomenon is a major public health problem, given that these molecules are often the only effective treatment against these particularly multiresistant microorganisms (Mellouk *et al.*, 2017). In our study, the resistance rate to imipenem of *A. baumannii* and *P. aeruginosa* tested strains is very high with MIC values ranging from 16 to 128 µg/ml. They all have an MDR profile and 55% of them produce carbapenemases.

They are all susceptible to colistin with MIC values ranging from 0.25 to 2 µg/ml for CR *A. baumannii* and

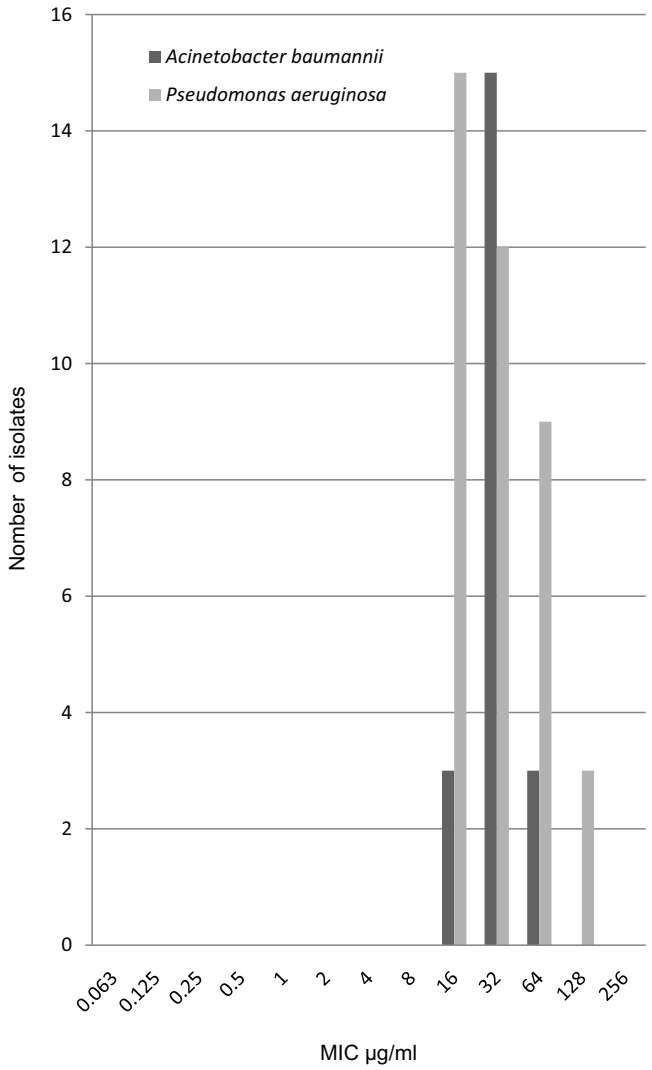

FIGURE 2. MIC of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolats to imipenem.

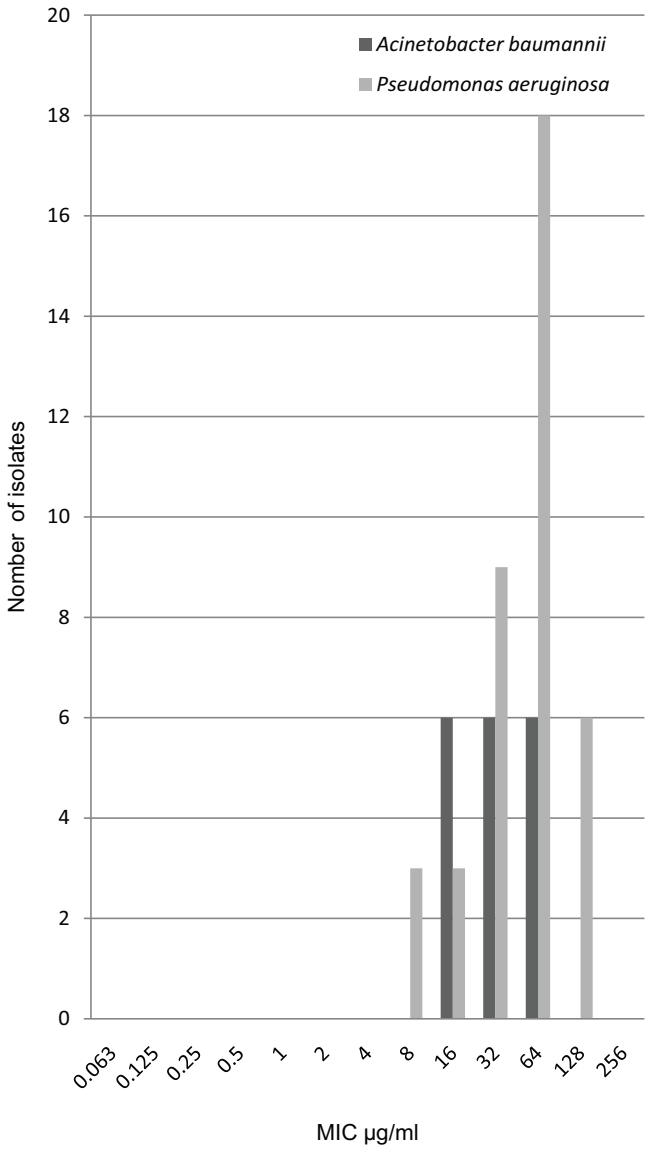

FIGURE 3. MIC of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolats to ciprofloxacine.

from 1 to 4 µg/ml for CR *P. aeruginosa*. This molecule is a last resort therapeutic option to fight against these strains. However, in recent years they acquired a growing resistance in addition to its toxicity, which leads to many therapeutic failures (Ahmed *et al.*, 2016).

Previous, in vitro studies have shown that the combination of the colistin with carbapenems has good antibacterial activity on GNB especially on *A. baumannii* because it presents high rates of synergy and bactericidal activity with a less antagonism and less development of resistance. Zusman *et al.* (2013) showed that the synergy rate of the colistin-carbapenems combination is >80% for *A. baumannii* and >60% for *P. aeruginosa*. Ramadan *et al.* (2018) describes similar results for this type of combination where the rate of synergy was 83.3% and 63.6% for CR *A. baumannii* and *P. aeruginosa*, respectively.

Our results showed that colistin associated with imipenem is the most active combination on carbapenem resistant *A. baumannii* (57.14%) and *P. aeruginosa* (46.15%) strains. In addition there is a significant decrease in the MIC of the imipenem. The synergy is obtained especially at a modal sub-inhibitory concentration of colistin of 0.5 µg/ml associated with a modal sub-inhibitory concentration of the imipenem of 8 µg/ml. This situation is observed mainly in *A. baumannii* producer strains of oxacillinase $bla_{OXA\text{-}23}$. These strains are isolated from diabetic patients under antimicrobial therapy based on imipenem and/or amikacin, cefotaxime.

On the other hand, in *P. aeruginosa* strains, synergistic activity is observed mainly in imipenem resistant strains with no production of carbapenemases. The modal sub-inhibitory concentrations of colistin and imipenem are equal to 0.25 µg/ml and 8 µg/ml, respectively. The majority of these strains are isolated from pus samples in patients hospitalized in the service of large burns antibiotics based on imipenem or amikacin.

This improvement may be due to the destabilization action of the outer membrane of GNB by colistin facilitating membrane permeability and increasing the intracellular concentrations of imipenem. Thus, it enlarges the possibilities of treatment and especially overcome the issue of resistance carbapenems and decreased the risk of colistin toxicity (Daoud *et al.*, 2013).

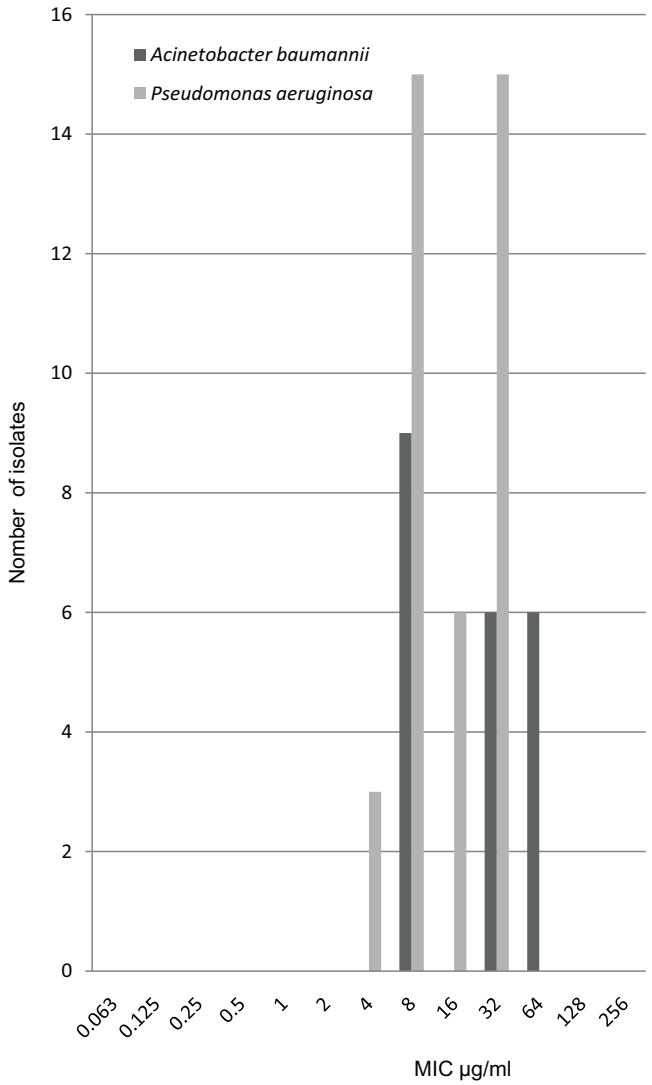

FIGURE 4. MIC of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolats to amikacin.

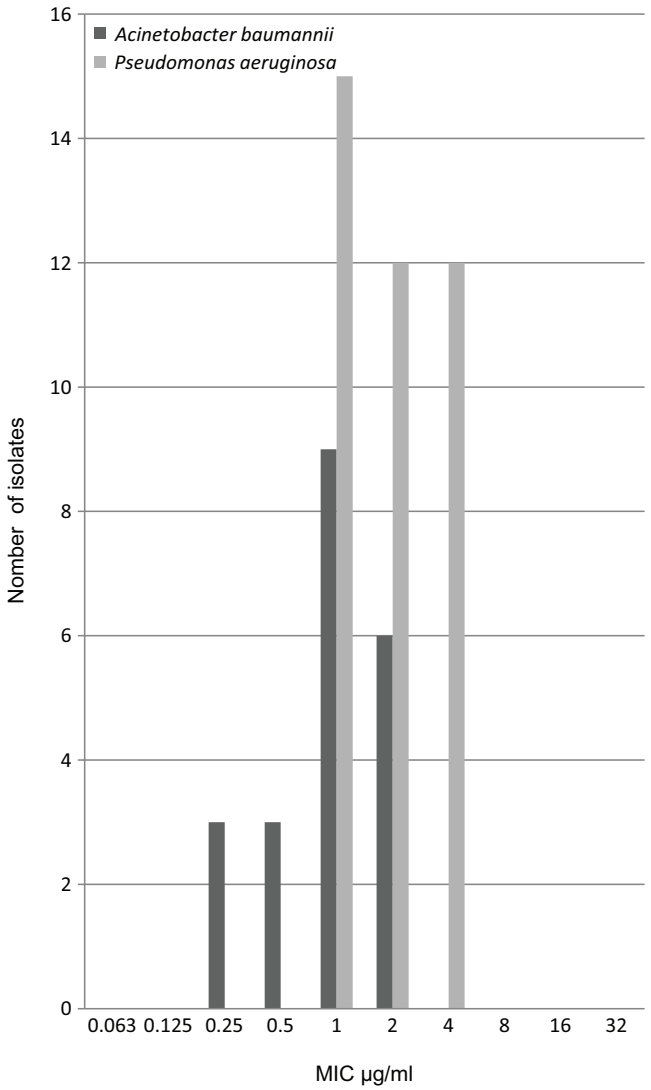

FIGURE 5. MIC of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolats to colistin.

In therapeutic practice the combination of colistin with amikacin is not recommended because of the toxicity of both antibacterial families (Martis *et al.*, 2014; Zhou *et al.*, 2017). However this combination allowed the treatment of meningitis caused by *A. baumannii* MDR (Fulnecky *et al.*, 2005). The same result is reported by in vitro and in vivo studies on strains of *P. aeruginosa* MDR (Tascini *et al.*, 2000). In this study, we recorded an encouraging synergistic effect of around 42.86% and 30.77% on CR *A. baumannii* and *P. aeruginosa* strains, respectively. Note that the synergy detected at modal sub-inhibitory concentrations of colistin is amikacin equal to 0.25 µg/ml and 4 µg/ml respectively, for strains of *A. baumannii* producing bla_{OXA-23}. While for *P. aeruginosa*, only strains harbouring bla_{VIM-2} show synergy at modal sub-inhibitory concentrations of 0.5 µg/ml and 4 µg/ml for colistin and amikacin, respectively.

The combination of colistin with ciprofloxacin is poorly documented and the available data show variable results

TABLE 3

Activity of antimicrobial combinations against carbapenem resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates

Antimicrobial agents	*Acinetobacter baumannii* (n = 21)			*Pseudomonas aeruginosa* (n = 39)		
	Synergy FICI ≥ 0.5	Indifferent 0.5 < FICI ≤ 4	Antagonism FICI > 4	Synergy FICI ≥ 0.5	Indifferent 0.5 < FICI ≤ 4	Antagonism FICI > 4
Colistin-imipenem	12 (57.14%)	9 (42.86%)	00	18 (46.15%)	21(53.85%)	00
Colistin-ciprofloxacin	3 (14.29%)	18 (85.71%)	00	6 (15.38%)	33 (84.62%)	00
Colistin-amikacin	9 (42.86%)	12 (57.14%)	00	12 (30.77%)	27 (69.23%)	00

depending on the considered species (Martis *et al.*, 2014). Thus, Rynn *et al.* (1999) and Richards and Xing (1993) report a total synergistic effect for colistin with ciprofloxacin on *P. aeruginosa* strains MDR. On the other hand, the synergistic effect of the same combination is of little importance on *A. baumannii* MDR (Kheshti *et al.*, 2019). This is shown by both CR *A. baumannii* and *P. aeruginosa* (14.29% and 15.38% respectively). Moreover, in case of synergistic effect the modal MIC is very low. This combination has an activity mostly indifferent 85.71% for CR *A. baumannii* and 84.62% for CR *P. aeruginosa*.

Although the clinical significance of our results for these combinations has not yet been determined, the combination of colistin with imipenem or with amikacin may be a therapeutic alternative to explore for the control of carbapenem-resistant NF-GNB by production of carbapenemases.

Conclusion

The results of this study allow us to say that colistin at low concentrations not only restores but also improves the antibacterial activity of imipenem or amikacin on carbapenem-resistant *A. baumannii* and *P. aeruginosa* strains. Such associations represent a therapeutic alternative that can contribute to the resolution of the problem of emergence and dissemination of OXA-23-type oxacillinase and metallo-β-lactamase type VIM-2-producing NF-GNB strains frequently isolated in intensive care unit in Algeria. However, additional in vivo investigations are needed to judge the efficacy of such combinations.

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