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Original article

ANTIBIOTIC COMBINATIONS WITH COLISTIN AGAINST CARBAPENEM-RESISTANT *KLEBSIELLA PNEUMONIAE* - *IN VITRO* ASSESSMENT

Yuliya Marteva-Proevska[1,2], Tzvetan Velinov[1], Rumyana Markovska[2], Diliyana Dobrikova[1], Ivan Pavlov[1], Lyudmila Boyanova[2], Ivan Mitov[2]
1) Central Laboratory of Clinical Microbiology, University Multiprofile Hospital for Active Treatment Alexandrovska, Sofia, Bulgaria.
2) Department of Medical Microbiology, Faculty of Medicine, Medical University of Sofia, Bulgaria.

SUMMARY

Purpose: The treatment of infections, caused by highly resistant strains of Gram-negative bacteria is extremely difficult. A potentially valuable option is combination antibiotic therapy. The aim of this study was to evaluate three different in vitro methods for synergy testing and to assess the effect of different combinations with colistin against carbapenem-resistant K. pneumoniae strains.

Matherial/methods: A screening test for synergy with colistin (developed in the laboratory) and the microdilution method of El-Azizi were used on 50 carbapenem-resistant strains of K. pneumoniae. Additionally, time-kill assays (TKA) were performed for one antibiotic combination.

Results: A total of 16 combinations were tested with the screening test. Synergy and probable synergy with colistin were observed mainly with azithromycin (18% of the isolates), rifampicin (16%), meropenem (14%) and doxycycline (12.8%). The combinations colistin-rifampicin, colistin-meropenem and colistin-gentamicin, were synergistic in 36%, 8% and 20%, respectively, according to the microdilution method of El-Azizi. The observed synergy was detected mainly against some of the colistin resistant strains. Agreement between the two methods was found in 80% for the combinations colistin-rifampicin and colistin-meropenem and in 84% for colistin-gentamicin. Agreement between the three methods used was observed for four strains (80%).

Conclusions: The screening test may represent a rapid and cost effective screening of a large number of combinations with colistin. The microdilution method of El-Azizi may provide an opportunity for rapid testing of three double and one triple antibiotic combinations in one plate. There is an urgent need for standardization of the methods for synergy testing and guidelines for diagnostic laboratories.

Key words: combination therapy, in vitro assessment, colistin, *K.pneumoniae*, microdilution method El-Azizi,

INTRODUCTION

The treatment of infections, caused by highly resistant Gram-negative bacteria is extremely difficult [1]. A potentially valuable option is combination antibiotic therapy, even when the resistant pathogens are not susceptible to any of the individual agents [2]. The microbiologists are expected to guide clinicians and to attempt to determine the appropriate antibiotic combinations. There are different methods for *in vitro* assessing of the combined effect of the antibiotics such as the checkerboard method, the multiple-combination bactericidal test (MCTB), methods, based on E-tests and time-kill assays (TKA) - most of which are very time consuming, expensive and not easy to perform in routine diagnostic work [3]. Recently, a novel microdilution method for testing of three double and one triple antibiotic combinations in one plate was developed by El-Azizi [4].

The aim of this study was to evaluate three different *in vitro* methods for synergy testing (a screening test for synergy, the microdilution method of El-Azizi and TKA) and to assess the effect of different combinations with colistin against carbapenem-resistant *K. pneumoniae* strains. The time-kill assays were performed on five strains for the colistin-rifampicin combination .

MATHERIALS AND METHODS

Bacterial strains

A total of 50 carbapenem-resistant strains of *K. pneumoniae* were investigated. The strains were isolated in the period 2013 - 05/2018 (90% of them from 01/2017 to 05/2018) from specimens of patients of Alexandrovska University Hospital in Sofia, Bulgaria – from urine (64%), respiratory samples (28%), wounds and related samples (6%) and blood cultures (2%). The species identification was done by using BBL Crystal E/NF identification system (BD).

Susceptibility testing

The susceptibility testing of all antibiotics tested, except for colistin was performed by the disk-diffusion

method, according to the EUCAST recommendations [5, 6]. The colistin MICs were determined with the broth microdilution method (BMD) in cation-adjusted Mueller-Hinton II broth (MHB II, BioLab ZRt) with colistin sulfate (Sigma-Aldrich), according to the current joint CLSI-EUCAST working group recommendations and the ISO standard 20776-1 [7, 8]. *E.coli* ATCC 25922 and *E.coli* NCTC 13846 were used as quality control strains.

Phenotypic detection of carbapenemase production was done by using the MBL, KPC and OXA-48 Confirm kit (Rosco diagnostica A/S, Denmark), according to the manufacturer's instructions.

Real-time PCR group specific polymerase-chain reaction

The genetic confirmation of carbapenemase production was done by real-time PCR (RT-PCR) with the MDR KPC/OXA Real-TM kit and the MDR MBL (VIM, IMP, NDM) Real-TM kit of *Sacace Biotechnologies Sri, Italy*, according to the manufacturer's instructions.

Screening test for synergy with colistin with pre-diffusion

A disk-diffusion based test for synergy, developed in the laboratory, was used for initial screening of a large number of combinations for synergy and probable synergy with colistin. It was performed with the standard antibiotic-containing disks for disk-diffusion (BD), and the pre-diffusion technique was applied. The following antibiotic disks were used: colistin 10 μg, rifampicin 5 μg, meropenem 10 μg, gentamicin 10 μg, tobramycin 10 μg, amikacin 30 μg, ciprofloxacin 5 μg, azithromycin 15 μg, doxycycline 5 μg, tigecycline 15 μg, clindamycin 2 μg, chloramphenicol 30 μg, vancomycin 5 μg, piperacillin/tazobactam 30/6 μg, aztreonam 30 μg, cefoperazone/sulbactam 105 μg and fosfomycin 200 μg.

Colistin disks (up to 9 disks with one of them in the center of the plate) were placed on non-inoculated Muller-Hinton agar (MHA) (HiMedia Laboratories) and incubated at 35±1°C for two hours (first period of pre-diffusion), and then the plates were maintained at room temperature for an additional two hours (second period of pre-diffusion). Then the disks were removed. From a pure culture of the tested strain, using the direct colony suspension method, a 0.5 McFarland suspension was prepared. For each strain tested, as for the disk-diffusion method, two plates of MHA were inoculated with a swab - one of them with pre-diffusion of colistin (plate 1) and the other without pre-treatment (plate 2). On the inoculated plates, in parallel, disks, containing the antibiotics of choice were put, and on the plate with the pre-diffusion, they were placed exactly upon the places of the removed colistin disks. The place of one colistin disk was left empty in order to assess the inhibition of colistin alone after pre-diffusion. The plates were incubated overnight at 35±1°C in ambient air. The next day, the diameters of zones of inhibition of all antibiotics alone (on the plate 2) and from the pre-diffusion of colistin alone (plate 1) and in combination with colistin (plate 1) were measured. For each combination, the difference (Δ, mm) between the zone of inhibition of the combination and that of inhibition of the more active antibiotic alone, was recorded. The interpretation was done as follows: Δ of (-2), (-1), 0, 1 or 2 mm → indifference; Δ = (-3) or (-4) mm → probable antagonism; Δ of ≤ (-5) mm → antagonism; Δ = 3 or 4 mm → probable synergism; Δ of ≥5 mm → synergism.

Microdilution Method of El-Azizi

The method was used to assess the following combinations with colistin: colistin - rifampin, colistin - meropenem, colistin - gentamicin, colistin - rifampin - meropenem, colistin - meropenem - gentamicin, colistin - rifampicin - gentamicin. The experiments were performed in sterile 96-well plates (Dinatech S.A.) with Muller-Hinton II broth (Biolab ZRt, Hungary), colistin sulfate (Sigma-Aldrich, U.S.), meropenem trihidrate (Sigma-Aldrich, U.S.), gentamicin sulfate (Sigma-Aldrich, U.S.) and rifampicin (HiMedia Laboratories, India). The concentrations of the antibiotics were as follows: colistin, 0.007 - 8 μg/ml (for colistin susceptible strains) and 0.125 - 128 μg/ml (for colistin resistant strains), meropenem, 0.06 - 64 μg/ml, gentamicin, 0.03 - 32 μg/ml and rifampicin, 0.125 - 128 μg/ml.

The experiments were performed according to the original test protocol of *El-Azizi* (2016) [4] but without the determination of the minimum bactericidal concentrations of the antibiotics and the combinations [4]. The first 3 rows of each plate (A to C) were used for each of the antibiotics alone. The next three rows (D, E, F) were used to test the double combinations, while row G was used for the triple combination of these antibiotics [4]. The MICs of antibiotics alone and in combinations were determined and were assessed with respect to the most potent antibiotic with lowest MIC value, alone and in double and triple combinations [4]. For each combination an Interaction code (IC) was generated, an Interaction type (IT) was determined, and the results were interpreted, according to the original test protocol of El-Azizi [4].

Time-kill assays (TKA)

The experiments were performed on five *K. pneumoniae* strains for the combination colistin - rifampicin as described elsewhere [9] with little modifications, in a total volume of 10 ml of MHB II. The final antibiotic concentrations of colistin and rifampicin were 1.2 mg/l and 1.7 mg/l, respectively. They were chosen in order to represent the mean non-protein bound plasma concentrations of these antibiotics at steady state [9]. The starting bacterial inoculum was about 1- 5 x 10^7 CFU/ml. Samples were taken at 0, 2 and 24 h, serially diluted, spread on plates and incubated at 35±1°C. Colonies were counted after 24h of incubation. The effect of the combination was considered synergistic if there was ≥2 $\log_{10}$ decrease in cfu/ml between the combination and the more active antibiotic alone [10]. The effect of the antibiotics and their combination was measured with the difference in the inoculum ($\log_{10}$ cfu/ml (24h) - $\log_{10}$ cfu/ml (0h)). A bacteriostatic effect was considered when this difference was between 0 and (-3), a

bactericidal effect - when it was below (-3) and when this difference was above 0 - there was no bacteriostatic or bactericidal effect. [9].

Statistical analyses

The statistical analyses were performed using the methods of descriptive statistics. The distribution of the observed results in the groups of colistin susceptible and colistin resistant strains was compared using the Mann-Whithney two sample rank test with SPSS software, version 16.0. A p-value of <0.05 was considered statistically significant. For the calculations of categorical agreement between the tests, the number of strains with synergy/probable synergy and antagonism/probable antagonism from the screening test was combined. They were compared with the number of the strains with synergy and antagonism from the microdilution test, respectively.

RESULTS

Antibiotic susceptibility and genetic testing

All strains were resistant to broad-spectrum cephalosporins, ciprofloxacin, piperacillin/tazobactam and co-trimoxazole. Non-susceptible (I+R) to imipenem and meropenem were 94% and 100% of the strains, respectively. Of them, a small percentage was susceptible to gentamicin (16%), amikacin (4%) and chloramphenicol (16%). Seventeen strains of K.pneumoniae (34%) were also resistant to colistin. The genetic testing revealed that 96% of the strains (48/50) were NDM-producers. One strain of K. pneumoniae was OXA-48 positive. The production of a putative carbapenemase was not confirmed for one of the tested strains, as the RT-PCR was negative for bla NDM, KPC, OXA-48, IMP and VIM.

Screening test for synergy with colistin with pre-diffusion

A total of 16 double combinations of colistin with different antibiotics were tested (Table 1). In most cases these combinations were indifferent. Synergy and probable synergy were observed mainly with azithromycin (in 18% of the isolates), rifampicin (in 16%), meropenem (in 14%) and doxycycline (in 12.8%), (Table 1). In most of the antibiotic combinations tested, the observed synergy was mainly against colistin resistant strains (Table 2). The distributions in the groups of colistin susceptible and colistin resistant differed significantly in the combinations colistin-rifampicin (Mann-Whithney U=148.5, p<0.01), colistin-azithromycin (Mann-Whithney U=154, p<0.01), colistin-doxycycline (Mann-Whithney U=144, p<0.01), colistin-aztreonam (Mann-Whithney U=56, p<0.01), colistin-tigecycline (Mann-Whithney U=56, p<0.01), colistin-gentamicin (Mann-Whithney U=231, p<0.05), colistin-chloramphenicol (Mann-Whithney U=58, p<0.05) and colistin-ciprofloxacin (Mann-Whithney U=214.5, p<0.05).

Table 1. The results obtained for 16 double and 3 triple antibiotic combinations with colistin by the screening test for synergy with colistin and the microdilution method of El-Azizi.

Combination	Screening test for synergy with colistin with pre-diffusion						Microdilution method of El-Azizi[1]			
	n	A	PA	I	PS	S	n	A	I	S
Colistin - Rifampicin	50	0	0	42 (84%)	2 (4%)	6 (12%)	50	0	32 (64%)	18 (36%)
Colistin - Meropenem	50	1 (2%)	1 (2%)	41 (82%)	5 (10%)	2 (4%)	50	1 (2%)	45 (90%)	4 (8%)
Colistin - Azithromycin	50	0	0	41 (82%)	3 (6%)	6 (12%)	-	-	-	-
Colistin - Doxycycline	47	0	0	41 (87.2%)	3 (6.4%)	3 (6.4%)	-	-	-	-
Colistin - Tigecycline	34	0	0	32 (94.1%)	1 (2.9%)	1 (2.9%)	-	-	-	-
Colistin - Gentamicin	50	0	0	47 (94%)	3 (6%)	0	50	1 (2%)	39 (78%)	10 (20%)
Colistin - Tobramycin	34	0	0	33 (97.1%)	0	1 1(2.9%)	-	-	-	-
Colistin - Amikacin	34	0	0	32 (94.1%)	2 (5.9%)	0	-	-	-	-
Colistin - Ciprofloxacin	49	0	0	46 (93.9%)	2 (4.1%)	1 (2%)	-	-	-	-
Colistin - Clindamycin	44	0	0	41 (93.2%)	0	3 (6.8%)	-	-	-	-

Colistin - Chloramphenicol	34	0	0	31 (91.2%)	1 (2.9%)	2 (5.9%)	-	-	-	-
Colistin - Vancomycin	39	0	0	38 (97.4%)	1 (2.6%)	0	-	-	-	-
Colistin - Piperacillin/tazobactam	34	0	0	34(100%)	0	0	-	-	-	-
Colistin - Cefoperazone/sulbactam	35	0	0	33(94.3%)	2(5.7%)	0	-	-	-	-
Colistin - Fosfomycin	40	0	1 (2.5%)	37 (92.5%)	1 (2.5%)	1 (2.5%)	-	-	-	-
Colistin - Aztreonam	34	0	0	32 (94.1%)	2 (5.9%)	0	-	-	-	-
Colistin-Meropenem-Gentamicin*	-	-	-	-	-	-	50	3 (6%)	47 (94%)	0
Colistin-Meropenem-Rifampicin*	-	-	-	-	-	-	50	1 (2%)	47 (94%)	2 (4%)
Colistin-Rifampicin-Gentamicin*	-	-	-	-	-	-	50	0	50 (100%)	0

A - antagonism, PA - probable antagonism, I - indifference, PS - probable synergism, S - synergism; n - number of strains tested

[1,] According to the original protocol of El-Azizi (2016): For each combination an Interacion Code (IC) was generated, based on the two-fold increase or decrease in the MICs of the most potent antibiotic in the combination; A (antagonism) - the MIC of the more potent antibiotic increased by 2-fold or more in combination with other antibiotics, I (indifference) - the MIC of the more potent antibiotic was unchanged or increased/decreased by 1-fold concentration; S (synergism) - the MIC of the most potent antibiotic decreased by 2-fold or more compared to the MIC of antibiotic alone.

*For triple combinations - the same rule was applied, but the comparison was made with any of the double combinations that contained the more active antibiotic, *El-Azizi, (2016).*

Microdilution method of El-Azizi

Three double and three triple combinations with colistin were tested (Table 1). As with the screening test, in most cases the combinations were indifferent. Synergy was observed in 36% of the strains with rifampicin (18/50), in 20% with gentamicin (10/50) and only in 8 % with meropenem (4/50). The distributions in the groups of colistin susceptible and colistin resistant differed significantly in the combination colistin-rifampicin (Mann-Whithney U=58.5, p<0.01) and colistin-gentamicin (Mann-Whithney U=161, p<0.01).

The three triple combinations were almost in all cases indifferent when compared with the corresponding double combinations. Against two colistin susceptible strains, the combination of colistin-meropenem-rifampicin was synergistic when compared to the combination colistin - rifampicin (Table 2).

Table 2. Distribution of the results obtained by the screening test and the microdilution method of El-Azizi for selected antibiotic combinations, based on the colistin susceptibility of the strains

Combination (test/method)	Type of strains		Antagonism	Probable Antagonism	Indifference	Probable synergy	Synergy
			N, %	N, %	N, %	N, %	N, %
Colistin - Rifampcin (Screening test)	Colistin (n_1=33)	S	0	0	33 (100%)	0	0
	Colistin (n_2=17)	R	0	0	9 (52.9%)	2 (11.8%)	6 (35.3%)
	Total (n=50)		**0**	**0**	**42 (84%)**	**2 (4%)**	**6 (12%)**
Colistin - Rifampcin (microdilution test El-Azizi)	Colistin (n_1=33)	S	0		30 (90.9%)		3 (9.1%)
	Colistin (n_2=17)	R	0		2 (11.8%)		15 (88.2%)
	Total (n=50)		**0**		**32 (64%)**		**18 (36%)**

Colistin-Meropenem (Screening test)	Colistin (n_1=33)	S	0	1 (3%)	30 (90.9%)	2 (6.1%)	0
	Colistin (n_2=17)	R	1 (5.9%)	0	11 (64.7%)	3 (17.6%)	2 (11.8%)
	Total (n=50)		**1 (2%)**	**1 (2%)**	**41 (82%)**	**5 (10%)**	**2 (4%)**
Colistin-Meropenem (microdilution test El-Azizi)	Colistin (n_1=33)	S	1 (3%)		31 (94%)		1 (3%)
	Colistin (n_2=17)	R	0		14 (82.4%)		3 (17.6%)
	Total (n=50)		**1 (2%)**		**45 (90%)**		**4 (8%)**
Colistin-Gentamicin (Screening test)	Colistin (n_1=33)	S	0	0	33 (100%)	0	0
	Colistin (n_2=17)	R	0	0	14 (82.4%)	3 (17.6%)	0
	Total (n=50)		**0**	**0**	**47 (94%)**	**3 (6%)**	**0**
Colistin-Gentamicin (microdilution test El-Azizi)	Colistin (n_1=33)	S	1 (3%)		30 (90.9%)		2 (6.1%)
	Colistin (n_2=17)	R	0		9 (52.9%)		8 (47.1%)
	Total (n=50)		**1 (2%)**		**39 (78%)**		**10 (20%)**
Colistin - Azithromycin (Screening test)	Colistin (n_1=33)	S	0	0	32 (97%)	1 (3%)	0
	Colistin (n_2=17)	R	0	0	9 (52.9%)	2 (11.8%)	6 (35.3%)
	Total *(n=50)*		**0**	**0**	**41 (82%)**	**3 (6%)**	**6 (12%)**
Colistin - Doxycycline (Screening test)	Colistin (n_1=32)	S	0	0	32 (100%)	0	0
	Colistin (n_2=15)	R	0	0	9 (60%)	3 (20%)	3 (20%)
	Total (n=47)		**0**	**0**	**41 (87.2%)**	**3 (6.4%)**	**3 (6.4%)**
Colistin - Tigecycline (Screening test)	Colistin (n_1=28)	S	0	0	28 (100%)	0	0
	Colistin (n_2=6)	R	0	0	4 (66.7%)	1 (16.7%)	1 (16.7%)
	Total (n=34)		**0**	**0**	**32 (94.1%)**	**1 (2.9%)**	**1 (2.9%)**
Colistin - Ciprofloxacin (Screening test)	Colistin (n_1=33)	S	0	0	33 (100%)	0	0
	Colistin (n_2=16)	R	0	0	13 (81.2%)	2 (12.5%)	1 (6.2%)
	Total (n=49)		**0**	**0**	**46 (93.9%)**	**2 (4.1%)**	**1 (2%)**
Colistin - Aztreonam (Screening test)	Colistin (n_1=28)	S	0	0	28 (100%)	0	0
	Colistin (n_2=6)	R	0	0	4 (66.7%)	2 (33.3%)	0
	Total (n=34)		**0**	**0**	**32 (94.1%)**	**2 (5.9%)**	**0**
Colistin-Chloramphenicol (Screening test)	Colistin (n_1=28)	S	0	0	27 (96.4%)	1 (3.6%)	0
	Colistin (n_2=6)	R	0	0	4 (66.7%)	0	2 (33.3%)
	Total (n=34)		**0**	**0**	**31 (91.2%)**	**1 (2.9%)**	**2 (5.9%)**

n - total number of strains tested; n_1 - number of colistin susceptible strains; n_2 - number of colistin resistant strains

Time-kill assays

Time-kill assays for the combination colistin - rifampicin were performed with five strains. The combination was more active than colistin alone against four of them. In all cases, after the initial inhibition from colistin and from the combination colistin-rifampicin, a substantial re-growth was observed after 24h of incubation (data not shown). The number of bacteria at 24 h exceeded the starting inoculum, and despite the observed synergy, the combination didn't lead to bacteriostatic or bactericidal effect (Table 3).

Table 3. Comparison of the results of the 3 methods for the combination colistin-rifampicin

	Result from the screening test for synergy with colistin with pre-diffusion	Result from the microdilution El-Azizi	Time-kill experiments			
			Δ (24h-0h) between the combination and colistin alone* log_{10} CFU/ml	Δ for the combination at 24h against the starting inocula (0h)**, log_{10} CFU/ml	Bacteriostatic effect	Bactericidal effect
24K¶	I	I	-7.475 (S)	1.813	no	no
32K¶	S	S	-4.208 (S)	2.139	no	no
70K¶	S	S	-3.497 (S)	2.185	no	no
81K	I	I	1.002 (I)	3.328	no	no
92K¶	S	S	-5.572 (S)	1.378	no	no

* represents the difference " (24h-0h) in the bacterial inocula (in log_{10} CFU/ml) between the combination and the more active antibiotic in it alone (colistin)

** represents the difference (") in bacterial inocula (in log_{10} CFU/ml) between 24h and the starting inoculum (0h) for the combination

I = indifference; S=synergism; ¶ -colistin resistant strain

Comparison of the results, obtained from the methods used

Three combinations were tested against all strains with two of the methods used (the screening test for synergy with colistin and the microdilution method of El-Azizi). Agreement between them was observed in 80% in the combinations colistin-rifampicin and colistin-meropenem (40/50) and in 84% in colistin-meropenem (42/50). For five of the strains, time-kill assays for the combination colistin-rifampicin were performed. Agreement between the 3 methods was observed in 4 of the strains (80%) (Table 3).

DISCUSSION

A total of 50 carbapenem-resistant strains of *K. pneumoniae* were included in the present study, 90% of them being isolated over a period of 17 months (from 01/2017 - 05/2018). Most of them (96%) were NDM-producers. The first NDM producing bacteria in our country were detected in a hospital outbreak, caused by *E.coli* in 2012 [11]. Soon after, NDM-positive *K.pneumoniae* strains were isolated in three hospitals in Sofia [12, 13] and a hospital outbreak was reported [14]. The large number of NDM producing *K.pneumoniae* strains, included in the present study and the fact that they were isolated over a period of several months highlight the great concern of their large and rapid dissemination [14]. The strains included in this study were highly resistant to almost all antibiotics tested. The most worrying fact is that 34% of them were resistant to colistin, too.

Polymyxins are regarded as last-line agents for the treatment of infections caused by carbapenemase producing Gram-negative bacteria [15]. However, the reports for colistin heteroresistance in *K. pneumoniae* [16, 17] and the high mutant prevention concentrations determined for this pathogen [18, 19] stress the risk of emergence of colistin resistance during mono-therapy with colistin. The prevention or at least slowing the emergence of colistin resistance could be achieved with the use of combination therapy [19]. Colistin is frequently used as a component of effective combinations, as it increases the permeability of other antibiotics through the bacterial outer membrane [20].

There are a number of different approaches to synergy testing, with no consensus about which is the best one of them [3]. According to Doern (2014), there are a lot of in vitro data analyzing synergistic antimicrobial combinations, but almost none of the information can be linked to treatment outcomes, and as if there is no true gold standard for synergy, it is difficult to know which results are correct [3]. At the same time, the rapidly growing antibiotic resistance and the lack of therapeutic options for the treatment of infections caused by highly resistant Gram-negative bacteria force diagnostic laboratories to perform synergy testing. The existing methods are very complex, expensive and time consuming [1, 3].

The screening test for synergy with colistin, used in this study, gives an opportunity for quick screening of large number of antibiotic combinations. The pre-diffusion technique, used in some E-test based methods for synergy testing [21] was applied in the test, but instead of E-tests, standard disks for disk-diffusion susceptibility testing were

used. The longer period for pre-diffusion (four hours) allows better diffusion of high molecular drugs such as colistin. The test is simple and easy to perform in routine diagnostic laboratories. It could guide microbiologists upon choosing the antibiotic combinations to be evaluated with some of the other synergy testing methods.

In our study, when the screening test was used, synergy and probable synergy was observed mainly with azithromycin (18%), rifampicin (16%), meropenem (14%) and doxycycline (12.8%).

Based on the results of the screening method, literature review and preliminary testing, we choose three combinations (colistin-rifampicin, colistin-meropenem and colistin-gentamicin) to be tested with the microdilution method of El-Azizi. This method is based on broth microdilution and gives an opportunity for testing of the susceptibility profile of a pathogen against 3 antibiotics and concurrently of three double and one triple combination among them in one plate [4]. Compared with the checkerboard and time-kill assays, the method is simpler, faster and less expensive [4]. If the protocol is performed without modifications, the method also provides information about the inhibitory and the bactericidal effects of the antibiotic combinations [4]. In the present study, the synergy of the combination colistin-rifampicin was observed in 36% of the strains tested (18/50) with the microdilution method of El-Azizi. The combination colistin-meropenem was synergistic against four strains (8%), while colistin-gentamicin was active against 10 (20%) strains. However, the combination colistin-gentamicin should be used with great caution, because of the nephrotoxicity of the two antibiotics. As a whole, the agreement between the two methods was observed in 80% for the combinations colistin-rifampicin and colistin-meropenem (40/50) and in 84% for colistin-gentamicin (42/50). The effect of the combination colistin - rifampicin in our study was explored also with TKA against five of the strains. Synergy was observed against four of them. Agreement between the 3 methods used in this study was observed in 4 (80%) strains.

Synergy between polymyxins and rifampicin against multidrug resistant Gram-negative bacteria was reported in many studies using different synergy testing methods [22, 23, 1, 10, 9]. Synergy between colistin and azithromycin against *A. baumannii, K. pneumoniae* and *P. aeruginosa* was demonstrated by Lin et al. (2015) [24]. Interestingly, in our study, the synergy of colistin with most of the antibiotics tested was observed mainly against some colistin resistant strains. Elemam et al. (2010) found that the combinations polymixin B-rifampicin and polymyxin B-doxycycline were synergistic against polymyxin-resistant KPC-producing strains of *K. pneumoniae* [1]. Indifference of the combination colistin - rifampicin against colistin susceptible, but synergistic effect against colistin-resistant strains was reported for KPC-producing *K. pneumoniae* by Gaibani et al. (2014) [25]. The combination was also found to have evident and potent *in vitro* post-antibiotic effect (PAE) against colistin-resistant strains of *K. pneumoniae* and this effect was further prolonged by tigecycline [25]. The exact mechanism of synergy in the present study and why it is observed mainly against some of the colistin resistant strains should be further investigated.

There is limited data about the effects of different triple combinations against carbapenem-resistant strains of *K. pneumoniae*. Tangdän et al., (2014) found that the combination colistin-rifampicin-meropenem demonstrated synergistic and bactericidal effects in TKA and was the most effective against four carbapenemase-producing strains of *K. pneumoniae* (VIM and NDM) [10]. The combination of polymyxin B-rifampicin-meropenem was found synergistic against two KPC-producing strains of *K. pneumoniae* by Diep et al. (2017) [26]. However, Lagerbäck et al. (2016), when evaluating the combined effect of colistin, rifampicin and meropenem with TKA against eight NDM-1 producing *K. pneumoniae* strains, found that the combination colistin and rifampicin was effective and should be explored *in vivo* and considered for clinical evaluation, but meropenem had little additive effect to it [9]. The same finding was observed in our study, too. We evaluated the combination colistin - meropenem - rifampicin against 50 carbapenem-resistant strains of *K. pneumoniae* with the microdilution method of El-Azizi - the effect was synergistic in comparison with the combination colistin-rifampicin only in two cases.

The main limitation of our study is the small number of strains and combinations tested with the TKA, which doesn't give an opportunity for better evaluation of the performances of the two other methods used.

CONCLUSION

With the rapid uncontrolled spread of carbapenemase-producing highly resistant Gram-negative bacteria worldwide, the clinicians would increasingly require synergy testing in the microbiological laboratories. A large number of combinations have to be tested with the hope that the appropriate one will be found. The screening test, used in this study enables rapid screening of a large number of combinations with colistin and could assist the laboratories in choosing antibiotic combinations to be explored with some of the other traditionally used methods. Our results showed that colistin combinations with rifampicin, azithromycin, meropenem, doxycycline, gentamicin could be of benefit in case of infections, caused by highly resistant strains of *K. pneumoniae*.

There is an urgent need for standardization of the methods for synergy testing and guidelines for diagnostic laboratories in order the microbiologists not only to register antibiotic resistance but also to be able to assist clinicians in the difficult task of choosing the best treatment for infections caused by highly resistant Gram-negative bacteria.

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Conflict of interests:

No conflict of interests is declared

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Address for correspondence:
Yuliya Marteva-Proevska, MD.
Central Laboratory of Clinical Microbiology, UMHAT Alexandrovska Sofia, 1, St Georgi Sofiiski Str., Sofia, Bulgaria; Tel: +359 2 92 30 436
Email: proevska@abv.bg