Bacterial resistance challenged by binary antimicrobial combinations

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Resistance or multi-resistance to antibiotics by microorganisms has generated one of the most emerging problems of public health due to the loss of susceptibility to drugs. The World Health Organization (WHO) publishes the groups of bacteria considered antibiotic-resistant "priority pathogens", and for these microbes new antimicrobials or strategic combinations are urgently needed.

A novel strategy to control resistance is the application of combined antimicrobials with synergistic effect to destroy priority pathogens, but also against parasites, fungi, even viral particles.

Therefore, it is important to expand the scientific knowledge regarding to antimicrobial combinations and the pharmacological treatments to preserve human health because paradoxically, the former solution to the spread of infectious diseases found in antibiotics, is now the main cause of a new problem: antimicrobial resistance.

Keywords: Resistance; synergism; antimicrobial combinations

1. Introduction

Antibiotic resistance (AR) has been defined as a bacterial change in response to the exposure of one or multiple antibiotics to treat bacterial infections. However, based on the acute and even uncontrollable problem of resistance worldwide, the term antimicrobial resistance (AMR) is commonly used because its breadth encompasses the description of response to different antimicrobial compounds (not only antibiotics) for more diverse microbes, that is to say, not only bacteria, also against parasites, fungi, even viral particles.

Antibiotics and therapeutic drugs, in general called antimicrobial agents, today are ineffective against microbial resistance, but the efforts of scientific community have driven to the systematic search of strategies and new active compounds to combat diverse mechanisms responsible of resistance or multi-resistance to therapeutic agents [1].
In this regard the researches are giving priority to eradication of etiological agents causatives of infections that affect the health of the population worldwide.

Recently, the World Health Organization (WHO) publishes the list of bacteria for which new antibiotics are urgently needed because are considered antibiotic-resistant "priority pathogens" [2]. The list includes twelve groups of bacteria classified in three categories of priority and their antibiotic resistance, and below in Table 1 the groups are indicated by critical, high and medium priority:

Table 1 Bacterial groups classified by priority categories of need for new antibiotics

Priority	Family of bacteria	Antibiotic-resistance
Critical (1)	*Acinetobacter baumannii*	Carbapenem
	Pseudomonas aeruginosa	Carbapenem
	Enterobacteriaceae	Carbapenem
	*ESBL-producing**	
High (2)	*Enterococcus faecium*	Vancomycin
	Staphylococcus aureus	Methicillin
		Vancomycin
	Helicobacter pylori	Clarithromycin
	Campylobacter spp.	Fluoroquinolone
	Salmonellae	Fluoroquinolone
	Neisseria gonorrhoeae	Cephalosporin
		Fluoroquinolone
Medium (3)	*Streptococcus pneumoniae*	Penicillin-non-susceptible
	Haemophilus influenzae	Ampicillin
	Shigella spp.	Fluoroquinolone

*Extended Spectrum Beta-Lactamase (ESBL) producing bacteria.

The above list will serve to define the steps to be followed by groups of researchers at a worldwide level, taking into account priorities framed by the established levels according to the infection hazards and risks for threat for the human health.

In this regard, the urgent need to disclose new antimicrobial agents as well as the development of efficient treatments to combat the increase of antimicrobial resistance leads to different strategies around the world.

2. Susceptibility to antimicrobials, *in vitro* and *in vivo* assays

2.1 Susceptibility to antimicrobials

Until a few years ago, the monotherapy has been the typical approach to address the infective diseases, but currently, there is enough evidence that combined antimicrobials or multitherapy are more effective than single-drug-based treatments [3].

The most novel studies in the fight against resistance includes the discovery of new pharmaceuticals as well as tests of combinations of antibiotics or combined therapies to obtain products with a broader spectrum that show a synergistic effect between two antimicrobials (A and B) [4].

2.1.1 Susceptibility to antimicrobials, *in vitro* assays

Evidence for the synergic behaviour between combined antimicrobials is obtained by antibiotic sensitivity tests through diffusion techniques (disk susceptibility test, antibiogram) and dilution techniques (microbroth dilution).

The diffusion techniques *in vitro* provide information about of the sensitivity to antimicrobials against target bacteria and are defined as resistance (R), intermediate susceptibility (I) or susceptibility and Susceptible (S) [5].

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Resistance (R) occurs if the microorganism is not affected by high doses of antibiotic. Bacteria, viruses and some parasites develop resistance as a defense or competition mechanism and are immune to the effects of antibiotics, antivirals or antimalarials respectively [6].

Intermediate susceptibility (I) is a variable response to an antimicrobial and is clinically susceptible and/or resistant but is eliminated if the antibiotic is concentrated at the site of infection or if the dosage is increased. [6].

Susceptible (S) or sensitive refers to microbe affected by antimicrobials as result of the loss or inactivation of their defense mechanisms.

The agar disk-diffusion techniques, antibiogram and well diffusion method are the most used techniques for *in vitro* determination of antimicrobial susceptibility.

The antibiogram test consists in the use of sterile disks impregnated with a known concentration of the antimicrobial compounds. The disks are deposited on petri dish agar inoculated with the bacterial culture. If the bacterial culture is resistant to antibiotic, it will grow on the agar surface, but on the other hand, sensitive bacterial growth and will develop a specific halo corresponding to antibiotic inhibition effect.

A variation of antibiogram test is the well-diffusion method in which, wells of specific diameter are punched on agar inoculated with bacterial culture. Subsequently, each well is filled with a known amount and concentration of the antimicrobial agent. After a specific incubation temperature and time, the results are interpreted after measuring the diameter of inhibition of bacterial growth [7].

Both agar disk-diffusion techniques antibiogram and well diffusion in Figure 1 shows halos of microbial growth inhibition caused by different minimum inhibitory concentrations of antimicrobials.

Fig. 1 Agar-disk diffusion methods. Antibiogram for gram-positive bacteria indicating resistance (R) sensitivity (S) and indifference (I) to antibiotics (left). Well-diffusion method showing inhibition of bacterial growth detected by halo formation around the wells (right).

In the terminology adopted for the study of different antimicrobial agents, the Minimum Inhibitory Concentration (MIC) is considered the lowest concentration, expressed in mg/L, required to inhibit the growth of a microorganism under specific conditions *in vitro* within a set period of time.

A widely used *in vitro* dilution technique to determine the behavior of combined antimicrobials is the checkerboard broth microdilution method assay and results are plotted indicating indifference, additive, synergism or antagonism effects (Figure 2). Synergy testing method performed by the checkerboard used to compare *in vitro* efficacy of antimicrobial combinations are performed according to the recommendations of Clinical and Laboratory Standards Institute Administrative Procedures guidelines (CLSI) for broth microdilution [8, 9].

Prior to testing, serial twofold dilutions of each antimicrobial at least double the MIC are prepared. Usually 50 µL of Müeller-Hinton broth (MHB) are pipetting into each well of the microdilution plates. The antimicrobial A of the combination serially diluted along the ordinate, while the antimicrobial B is diluted along the abscissa (Figure 2a). An inoculum of 0.5 McFarland turbidity standard is prepared in MHB and each microtiter well is inoculated with 100 to150 µL of pathogen inoculum. Finally the plates are incubated at optimum temperature and time.

The resulting checkerboard contains each combination of two antimicrobials, with tubes that contain the highest concentration of each antimicrobial at opposite corners (Figure 2a, b).

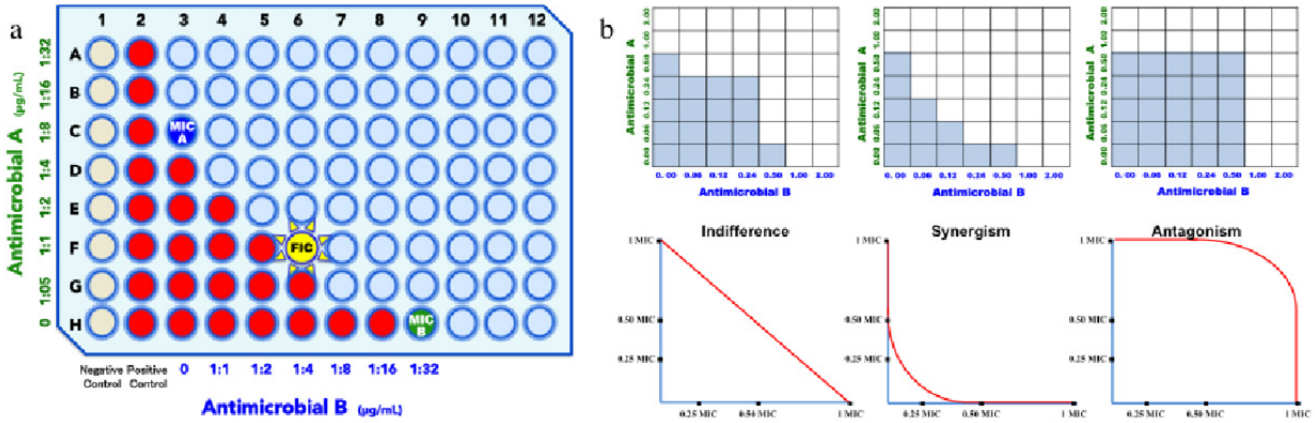

Fig. 2 Synergy testing method performed by the checkerboard assay. a) Serial twofold dilutions of each antimicrobial at least double the MIC, b) Graphical representation of the possible effects exerted by antimicrobial combination (Indiference, Synergism, antagonism).

The effect of the antimicrobial combination can be established as indifference, addiction, synergism or antagonism. *Indifference* (I) expresses the combined antimicrobial activity and is not different to the activity of the most active antimicrobial when acting alone. *Additive* (A) effect is equal to the sum of the activities of two antimicrobials acting separately.

In *synergism* (S), the activity of two antimicrobials is greater than the sum of individual activities. *Antagonism* (A) is the activity of two antimicrobials together and significantly less than the sum of the activities of the antimicrobials when they act alone, contrary to synergism. Examples of numerical values for each mentioned effect shown in Table 2.

Additionally, checkerboard assay determines the effect on potency of the combination of antimicrobials in comparison to their individual activities, represented as the Fractional Inhibitory Concentration Index (FICI) value. The FIC index value may be calculated according to the following equation:

$$\Sigma \text{ FICI} = \text{FICI}_A + \text{FICI}_B$$

The FICI is the sum of the FIC of each antimicrobial:

$$\text{FICI}_A = \frac{\text{MIC}_A \text{ in presence of B}}{\text{MIC}_A \text{ alone}} + \text{FICI}_B = \frac{\text{MIC}_B \text{ in presence of A}}{\text{MIC}_B \text{ alone}}$$

The FIC index was established to understand the effect of the combination of two antimicrobials under investigation as shows in Table 2.

Table 2 Fractional Inhibitory Concentration Index and effect of the combination of antimicrobials.

Index	Synergism	Additive	Indifferent	Antagonism
FIC	$\leq 0.5 - 1$	$>0.5 \pm 1$	$>1 - <2$	$\geq 1 - 2$

After review the microdilution assay it is important to establish that diffusion and dilution techniques are comparable since there is a direct correlation between the diffusion halo diameter of microbial growth measured by diffusion and the MIC determined by dilution [9, 10].

2.1.2 Susceptibility to antimicrobials, *in vivo* assays

Comparing the effect of combined antimicrobial using only *in vitro* methods is unfortunately not enough for emulating real life conditions, and for this reason, *in vivo* complementary assays are required. The proper selection of experimental models has been the key to success in infectious disease research for more than a century to study the relationship between drug exposure and *in vivo* efficacy. In models, the antimicrobials efficacy can be measured directly in the focus of infection and therefore, detect the physiopathological consequences of the infection [11].

A wide variety of *in vivo* models have been used to characterize the pharmacodynamics of antimicrobials, classifying them in human models (groups of volunteers), and non-human models using various animals such as rats, pigs, mice, guinea pigs and rabbits [10]. Mammalian infection models are necessary to obtain data regarding appropriate dosage, potential toxicity and efficacy of combination therapy because the generated data is relevant to human infections for similarity in response [12]. The study of combined therapy *in vivo*, includes two antimicrobials assayed themselves (as monotherapies) and together, and synergisms can be observed using only particular model systems. Here are some examples of successful experimental models for *in vivo* research.

Reports about the model of larva of wax moth (*Galleria mellonella*), a combination of telavancin-colistin was tested, after obtaining positive results against *Acinetobacter baumannii* (a multidrug resistant pathogen). The combination displayed rapid and constant bactericidal activity, and significantly more active than the two monotherapies in the invertebrate model of infection [13].

The *Haemophilus ducreyi*, the causative agent of chancroid sexually transmitted disease (STD), shows epidemiological synergy with HIV infections. The combination of streptomycin and ceftriaxone assayed in rabbit models of chancroid, showed unexpected synergism against *H. ducreyi*. This synergy between an aminoglycoside and β-lactam, is theoretically explained by the increased uptake of aminoglycoside in the presence of the β-lactam in the cell wall [14, 15].

3. Combination antimicrobials, an emerging option against antimicrobial resistance

The imminent threat caused by the crisis of antimicrobial resistance of pathogenic organisms worldwide has generated a frenetic search for new strategies to contain and/or eliminate this condition that represents a high risk for human health. Some strategies are related to efforts to generate new drugs and pharmacological treatments against bacterial groups considered antibiotic-resistant "priority pathogens". Below will see some interesting strategies such as the development of innovative therapeutic schemes, established to verify outstanding treatments to eradicate diseases. In addition, the design of binary antimicrobial combinations and some combinations of antibiotics with new antimicrobial compounds are briefly commented.

3.1 Innovative therapeutic schemes

The Beta-lactam are the most used broad-spectrum antibiotics group. The production of β-lactamases (a group of enzymes that cleave amide bond in beta lactam rings of beta lactam antibiotics, rendering them harmless) is the most frequent cause of resistance to β-lactam antibiotics, by gram-negative bacteria [16]. It has been reported that when bacteria express extended spectrum beta lactamases (ESBLs), they become resistant to many different β-lactam antibiotics, such as penicillins, aztreonam and cephalosporins [17].

Recently, the effect of regular antibiotics on infections has decreased due to the increasing number of antibiotic-resistant disease-causing bacteria, thus, new therapies are being researched. Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococci* (VRE), *Klebsiella, Clostridium difficile* and *Pseudomonas* antibiotic resistant bacteria, are becoming more and more common [18, 19]. The principal new developed therapies are binary antimicrobial combinations (using combinations of two traditional antibiotics, or an antibiotic and a compound with antimicrobial effect), and the use of nanoparticles (as it's been shown that when materials change size to a nanometric scale, new properties emerge) [20].

Nanotechnology is a modern and innovative approach to the development of new formulations based upon metallic nanoparticles with antimicrobial properties [21]. There are several materials for which nanoparticles bactericidal effect has been investigated. Metal oxides (with silver, gold, platinum and zinc) are the most researched ones [19]. Among the inorganic ones, silver nanoparticles (AgNPs) have been extensively studied, its antibacterial activities have been reported and it's know that in small concentrations, silver is nontoxic to human cells. AgNPs have also been used in the treatment of infections in open wounds, with positive results AgNPs also have different targets on microorganisms, so emerging resistance to this kind of material, it's unlikely to happen.

For *Escherichia coli* (a gram-negative bacteria), silver nanoparticles interactions have been demonstrated to be shape-dependent, in relation to the active surface area (although the relation hasn't been fully understood), truncated triangular nanoparticles have showed to have the highest biocidal effect, compared with spherical and rod-shaped ones. [22]. Silver has also shown the capacity to eliminate MRSA [23]. Since 2016, it is estimated that 320 tons of nanosilver are used annually, representing 30% of the nanoproducts [24, 25].

Therapeutic roles for zinc nanoparticles in different diseases have been established. The antibacterial effect of zinc oxide (ZnO) has been assigned to a reaction of ZnO with water; aqueous suspensions produce increased levels of reactive oxygen species [26]. Studies have revealed improved activity of ZnO nanoparticles when used in combination with cephalosporins, beta lactams and aminoglycosides against different pathogenic microorganisms [21].

However, the use of nanoparticles has its disadvantages. Principally, in most of the methods hazardous chemicals are used, a high amount of energy is required and there's a low ratio conversion of material to nanoparticles. To solver this issue, green synthesis of nanoparticles has been researched with promising *in vitro* results, animal models need to be tested so the green-synthetized nanoparticles can be used as antibiotics [19].

The blue light (400-500nm) is increasing attention due to significant antimicrobial activity against a broad range of bacterial and fungal pathogens and the minor chance to resistance emergence compared to antibiotics. It has also been shown to be effective against MRSA and other bacterial pathogens [27]. The principal advantage of light-based therapies is the equal killing effectiveness regardless of antibiotic resistance. When comparing blue light to ultraviolet irradiation (another light-based therapy), blue light is less detrimental against mammalian cells [28].

3.2 Binary antimicrobial combinations

3.2.1 Combination of traditional antibiotics

A therapy with a combination of antibiotics for multidrug-resistant bacteria is frequently used, but this kind of therapy is controversial and has been a highly debatable topic. Among the potential benefits of combination therapies against monotherapies are: synergistic bactericidal effects, broader antibacterial spectrum (antibiotics against same bacteria specie, but different cell target) and a reduced risk for emerging resistance during treatment (bacteria have a lower chance of develop emerging resistance to the method of action of both antibiotics) [29]. Synergism has been defined as a phenomenon in which two different compounds are combined to enhance their individual activity. Regarding antibiotic efficiency, synergism can be stated as a fractional inhibitory concentration indices (FIC) derived from a checkerboard titration [21]. Mechanisms of synergy aren't fully understood, but plausible explanations include increasing permeability to other antibiotics (counteracting decreased permeability, like porin loss) and hydrolyzed beta-lactams acting as competitive inhibitors of beta-lactamases [30]. Superinfections, selection for resistant strains (survivals to the therapy are resistant to not only one, but both antibiotics used), increased risk for toxicity and increased costs for the whole treatment are, however, the risks involved in this kind of therapy [29].

Combination antibiotic therapy (for the treatment of gram-negative infections) usually consists of a beta-lactam supplied with aminoglycoside or a fluoroquinolone. This combination is used due to the broad coverage of both antimicrobial agents and their different spectra of activity. The combination of antibiotics to use is determined by *in vitro* synergistic effects seen in two agents, although *in vitro* effects doesn't necessarily equal to an *in vivo* effect, but when there's no information is available, *in vitro* results are a good starting point to combination therapy [31].

Any of the three points below justifies the use of combination therapy:

1. The improved effect of using both antibiotics against only one observed *in vitro*.
2. Preventing or delaying the emergence of new antimicrobial-resistant bacteria.
3. Having two different antimicrobial agents with different targets improves the coverage to eliminate the pathogen.

An ongoing debate exists over if combination therapy works or not. There are several reviews discussing this topic, and the consensus seems to be that the second agent added doesn't make a significant difference to the effect of the first agent, some of the reviews mention that all the new second agents discovered should be saved for when they're actually vital in the war against antimicrobial resistant bacteria, and speculate that optimization of dose, frequency of administration and duration over which the antibiotic is infused is likely more important in the prevention of emergence resistant, than the use of a combination therapy [31]. Nevertheless, when the appropriate antibiotic treatment is delayed in patients with septic shock, the broad spectra of combination therapy seems to be the appropriate therapy (due to the probability of finding a working antibiotic) to avoid the increase in mortality due to the patient's condition [29].

3.3 Combination of antibiotics with new antimicrobial compounds

Nowadays the discover of compounds with antimicrobial activity of plant or bacterial origin offers an alternative to the treatment of infections with traditional antibiotics which as already mentioned before, are being used in high doses. Herbs and spices have demonstrated antimicrobial properties principally attributed to the presence of flavonoids and their derivates as in the case of wild marigold (*Tagetes minuta*), sulfur compounds like allicin in the garlic (*Allium sativm*) or phenols like carvacrol and thymol in oregano (*Origanum vulgare*) and more. Of the antimicrobial compounds of bacterial origin the bacteriocins of lactic acid bacteria (LAB) like nisin (*Lactococcus lactis*) main bioconservative of dairy foods, or pediocin (*Pediococcus acidilactici*) stand out. Other bacteriocins with a broad antimicrobial potential are

those from *Bacillus thuringiensis* which have demonstrated a wide range of inhibitory activity against gram-positive and gram-negative bacteria [31, 32, 33, 34, 35, 36, 37].

A strategy that currently seeks to improve the effect of antibiotics in the treatments of infections is the combination of these with natural antimicrobial compounds. As already mentioned, there are a number of substances that have the potential to be used in the treatments of infections due to their activity against pathogenic microorganisms or act in synergy with traditional antibiotics in order to reduce the dose used, it means reduce the minimal inhibitory concentration (MIC) that it´s the lowest concentration (mg/mL) required of some antimicrobial agent to inhibit the growth of some microorganism after an incubation. Usually the interaction between two or more drugs is represented by the fractional inhibitory concentration (FIC) which indicates the level of synergy between the agents, where values of FIC ≤. 0.5 represents synergy, by the other hand values > 4 indicates antagonism between the agents and a value between 0.5 and 4 means indifference [38, 39, 40].

Table 3 shows some combinations of traditional antibiotics with antimicrobial compounds and the values of MIC and FCI index.

Table 3 Combination between natural antimicrobial compounds and antibiotics against pathogenic bacteria

Natural compound	Antibiotic	Microorganisms	Activity or effect	Reference
Thymol	Vancomycin	*E. coli*	FIC < 0.5	[41]
Pomegranate extract	Chloramphenicol, gentamicin, ampicillin, tetracycline, oxacillin	*Staphylococcus aureus* methicillin-resistant (MRSA) methicillin-sensitive *Staphylococcus aureus* (MSSA)	FIC <0.5	[42]
Clove extract	Tetracycline, ampicillin, chloramphenicol	*P. aeruginosa* (antibiotic resistant)	MIC 10 mg/mL	[42]
Eugenol	Tetracycline, ampicillin, chloramphenicol	*Proteus* spp. (antibiotic resistant)	MIC 5 mg/mL	[42]
Carvacrol	Ampicillin, tetracycline, penicillin, bacitracin, erythromycin, novobiocin	*Salmonella typhimurium* SG1 (antibiotic resistant)	FIC < 0.4	[43]
Isothiocynate	Erythromycin	*Streptococcus pyogenes*	FIC < 0.3	[43]
Eucalyptus essential oils	Ciprofloxacin	*Acinetobacter baumannii* multi drug resistant (MDR)	FIC < 0.5	[44]
Curcumin	Oxacillin, ampicillin, ciprofloxacin, norfloxacin	Methicillin-resistant Staphylococcus aureus (MRSA).	FIC 0.07-0.75	[45]
Manuka honey	Rifampicin	*S. aureus* (MRSA)	FIC <0.5	[46]
Nisin	Colistin	*E coli* O157:H7	MIC 0.01 µg/mL	[47]
Pediocin	Colistin	*E coli* O157:H7	MIC 0.03 µg/mL	[47]
Enterocin DD28 and DD 93	Kanamycin, erythromycin	*Staphylococcus aureus* methicillin-resistant (MRSA)	FIC ≤ 0.375	[48]
Durancin 61A	Vancomycin.	*Staphylococcus aureus*	FIC = 0.3	[49]

Conclusions

Pathogens resistant to most conventional antibiotics considered antibiotic-resistant "priority pathogens" by urgent need for new antibiotics options, are the reality of the uncontroling crisis of bacterial resistance. Efforts to implement renew research strategies to manage the crisis are obviously needed worldwide, as well as new agents to treat bacterial infections. Significant progresses in combination of antimicrobials compared to the single antibiotics, is the strategy with the highest expectation to control microbial resistance in the short or medium term.

Globally, in all sciences, strategies are planned for the future, but with respect to critical antimicrobial resistance the future is ahead, it is here.

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