Journal of Applied and Natural Science
14(1), 148 - 162 (2022)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Review Article

A review of antibiotic synergy in carbapenemase-producing bacteria

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Article Info
https://doi.org/10.31018/jans.v14i1.3248
Received: December 29, 2021
Revised: February 19, 2022
Accepted: February 25, 2022

How to Cite
Vamsi, K. S. *et al.* (2022). A review of antibiotic synergy in carbapenemase-producing bacteria. *Journal of Applied and Natural Science*, 14(1), 148 - 162. https://doi.org/10.31018/jans.v14i1.3248

Abstract
The problem of antibiotic resistance has garnered too much attention over the last few decades for posing a global hazard to the clinical handling and the inhibition of several deadly infections caused by bacteria. It burdens the world not only clinically but also economically... Antibiotic agents known as carbapenems are a very effective and typically designated for the treatment of multidrug-resistant (MDR) bacterial infections. To identify a suitable antibiotic combination to be used *in vivo*, one must be able to determine the synergism between the antibiotics *in* vitro. Several methods, such as the checkerboard method, multiple-combination bactericidal test, time-kill and E-test, have been used for this purpose. However, the lack of proper standardization procedures, types of bacterial agents, bacterial load, stage of infection and other factors make it very difficult to reproduce or correlate the results with other methods.Carbapenem-destroying lactases, which have recently emerged as mechanisms of resistance, are increasing in number and decreasing the treatment alternatives available. These infections are treated with colistin and tigecycline, but monotherapy may result in clinical breakdown because of a variety of factors. To control these infections, clinicians often choose combinations of drugs over monotherapy. There is an extreme lack of information on synergistic antibiotic combinations accounting for the diverse mechanisms of GNB resistance commonly encountered. The incidence of carbapenem-resistant GNB in Indian articles is also unknown. Therefore, we anticipate that this study may provide methodology for the selection of an appropriate antibiotic combination.

Keywords: Antibiotic; Carbapenem, Carbapenemase, Checkerboard assay, Synergy, Time kill assay

INTRODUCTION

Antibiotics, antimicrobial substances active against bacteria, are key to fighting potential bacterial infections. Over a century ago, the first antibiotic, “salvarsan”, was used in 1910 (Hutchings *et al*., 2019). Globally, they are still being prescribed to treat and prevent a variety of infections caused by bacteria (Katz and Baltz, 2016). Antibiotics help fight infections by either inhibiting or killing the pathogen that causes the infection. However, the inappropriate prescription and overuse of antibiotics for treating humans, animals and agriculture has resulted in infectious bacteria becoming resistant to antibiotics (Ventola, 2015). This mechanism termed antimicrobial (antibiotic) resistance has seen an alarming global increase, significantly endangering the antibiotic-based

treatment and outcomes of several bacterial infections (Giamarellou and Poulkau, 2009). Even though the rise of antibiotic resistance is a natural process, the excessive and untimely use of antibiotics can speed up the process of gaining resistance. Some of the ways to combat antibiotic resistance would be to prevent the occurrence of infections and spread of resistance, effective use of antibiotics only when required and prescribed by the physician and the development of new drugs (antibiotics). However, the ability of bacteria to gain resistance to the new antibiotic in a short period makes the whole process of antibiotic development futile. Therefore, this option is no longer considered profitable because of the economic and regulatory challenges faced in developing and bringing a new antibiotic to the market for use. Examples of some of the notable drug-resistant bacteria are MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin-resistant enterococci), CRE (carbapenem-resistant Enterobacteriaceae), drug-resistant variants *of Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae,* MDR *Pseudomonas* aeruginosa and Acinetobacter as well as extended-spectrum beta-lactamase (ESBL)-producing members of Enterobacteriaceae. (Pfaller *et al.,* 2018).

With the option of developing new antimicrobials being ruled out, the only promising strategy to break the spread of antibiotic resistance would be to combine antibiotics to achieve better treatment efficacy. This is termed “combination therapy” instead of using a single antibiotic, “monotherapy”. If used in combination, antibiotics may show three varying degrees of response: additive, synergistic or antagonistic. If the effect of the combination equals the sum of their individual effects, they are additive. If the effect is higher than the sum of the individual components, it is “synergistic”, whereas the interaction will be considered “antagonistic” when the effect of the combination is less than the individual antibiotic effect. This review focuses on antibiotic synergy with a special emphasis on the synergy observed against carbapenemase-producing bacteria and the methods of measuring this synergy. (Briceland *et al.,* 1988).

Even though the debate about whether combination antimicrobial therapy is better than monotherapy is still ongoing, some research and studies have shown that combination therapy could provide better results (Ratner *et al.,* 2008). The overall mortality rate has been lower in patients following combination therapy than in those on single antibiotic therapy (Schmid *et al*., 2019). For infections with no effective antimicrobial agents, combination therapy has proven to be highly effective. Several reports have studied the effect of combination therapy in treating multidrug-*resistant Pseudomonas aeruginosa* (Dundar and Otkun, 2010; Xipell*et al.,* 2017)*, Acinetobacter baumannii (Sands et al*., 2007; Wareham and Bean, 2006; Li *et al.,* 2021) *Klebsiella pneumoniae*(Lee *et al*., 2009, Nordman*et al*., 2009), and some of the most fatal drug-resistant pathogens. The study concluded that combination therapy is the best therapy when colistin and tigecycline are not accessible.

CARBAPENEMS

Beta-lactams, antibiotics whose structure has a beta-lactam ring, are routinely prescribed antibiotics for clinical infections. They can be categorized into several classes:penicillins, cephalosporins, carbapenems, monobactams and beta-lactamase inhibitors (Thakuria and Lahon, 2013).Letourneau and Calderwood (2020) studied the actions of acombination of beta lactamase inhibitors of carbapenamase and monobactams. Carbapenems, an important class of beta-lactams identified in the late 1970s, exhibit good safety and efficacy. They have a broad spectrum of action with utmost effectiveness against Gram-positive and Gram-negative bacteria (Bradley *et al*., 1999). This makes them the antibiotics used as a last alternative for infections caused by highly drug-resistant bacteria.The literature suggests that imipenem and panipenem are more potent in treating gram-positive infections, whereas biapenem, meropenem, and ertapenem can effectively fight infections caused by gram-negative bacteria. Doripenem, the carbapenem least susceptible to hydrolysis, targets both Gram-positive and Gram-negative bacteria. Carbapenems such as imipenem and meropenem have the widest spectrum of action (Bassetti*et al*., 2009). Imipenem–Relebactam and Meropenem–Vaborbactam, which are two novel carbapenem-β-lactamase inhibitors, have been studied in combination by Zhanel et al. (2018), and relebactam and vaborbactam have been reported to broaden the spectrum of imipenem and meropenem, respectively, against β-lactamase-producing Gram-negative bacilli.Both combinations appear to be well tolerated in healthy subjects and hospitalized patients, with few serious drug-related treatment-emergent adverse events reported to date. (Giannella*et al., 2018*) assessed the effect of high-dose (HD) carbapenem-based combination therapy on clinical outcome in patients with monomicrobial carbapenem-resistant Klebsiella pneumoniae (CR-KP) BSI and discovered that when patients receive combination therapy for CR-KP BSI, the use of HD carbapenem appears to be associated with a better outcome, even when high-level carbapenem resistance is present.

Carbapenem controls bacterial infections by inhibiting cell wall synthesis, specifically peptide cross linking. They enter Gram-negative cells through outer membrane transport proteins and porins and irreversiblyacylate penicillin-binding proteins (PBPs). The acylation of PBP inhibits itstransglycosylase, transpeptidase and

carboxypeptidase activities. This results in the synthesis of very weakened peptidoglycan and altered osmotic pressure, resulting in bursting of the cell. The ability of carbapenems to bind to multiple types of PBPs (Hashizume*et al*., 1984) makes them more effective against infectious Gram-negative bacteria. To treat more serious infections, they can be combined with other antimicrobials (Cha, 2008). Comparison of combination therapy with monotherapy is reported by (Peri *et al.,* 2019), World Health Organization (WHO) stated that the superiority of combination treatment (most often including meropenem, colistin, gentamicin, or tigecycline) versus monotherapy has been limited due to the observational nature of the studies. This is of supreme importance due to the continuous emergence of multidrug-resistant (MDR) bacterial strains, which cannot be controlled with antibiotic monotherapy.

Bacteria can develop resistance to carbapenems due to mechanisms that may be intrinsic, extrinsic or both. If the bacteria are naturally resistant to certain antibiotics, they are intrinsically resistant and often complicate drug selection for treatment. Extrinsic or acquired resistance is when a sensitive bacterium gains the gene responsible for the resistance from resistant bacteria (Forsberg *et al*., 2010). They may also develop strategies to stop the antibiotic from causing any harm to the host bacteria. Some of the mechanisms include inactivation of the antibiotic using enzymes, mutating the target site of the antibiotic or rapid efflux of the antibiotics as soon as they enter the cell (Levy and Marshall, 2004). Gram-negative bacteria are more dangerous, as they are gaining resistance against all available antibiotics, leading to a scenario similar to when there were no antibiotics. Among the Gram-positive bacteria, drug-resistant variants of *Staphylococcus aureus* and Enterococci pose the biggest threat (Rossolini*et al*., 2014).

CARBAPENEMASE

Carbapenemase, a class of beta-lactamases, can hydrolyse carbapenems as well as other beta-lactams, such as penicillin, cephalosporins and monobactams. Drug-resistant bacteria producing carbapenemase can nullify the effect of carbapenem, the antibiotic used as the last resort to treat infections. Moreover, these enzymes are too resilient to be destroyed by beta-lactamase inhibitors. Based on their amino acid homology, carbapenemase can be classified into three major classes, class A, class B and class D. Of these, members of classA and class D have at their active site the presence of a serine residue, which in class B enzymes is replaced by zinc. Therefore, class A and D carbapenemases are serine carbapenemases, while class B enzymes are metallo-carbapenemases. In terms of the ability to hydrolyze carbapenem and geographical spread, the most effective ones are NDM, IMP, KPC, OXA-48 and VIM (Poirel*et al*., 2012). Members of SME, KPC, IMI, GES and NMC comprise class A, members of IMP, SPM, VIM, SIM and GIM form the class B metallo-carbapenemase, while the class D enzymes are OXA- type.(MUSA and D. N. B. (2018) stated that according to the Ambler classification, there are four molecular classes of β-lactamases, namely, A, B, C and D. The genes that encode thebeta-lactamase enzyme AmpCtype,an Ambler class C,are present in Serratia, Citrobacter and Enterobacter. It can rapidly hydrolyze penicillin, cephalosporins, and monobactams but is not inhibited by beta-lactamase inhibitors. The metallo β-lactamase subtype generated by carbapenem-resistant Pseudomonas aeruginosa isolates in Japan was classified by (Nakayama *et al.,* 2021), and it was discovered that all MBL-positive CRBP-r isolates were extremely resistant to carbapenems, which were dominant in IMP-1 synthesis. Sun *et al. (*2018) evaluated the reliability of the inhibitor-enhanced carbapenem inactivation method (CIM) for the detection and preliminary classification of carbapenemase in gram-negative rods and concluded that the CIM produces consistent results with the carbapenemase gene detection method and thus may be used to detect and classify carbapenemase in clinical microbiology laboratories. Table 1 highlights some of the key features of the different classes of carbapenemase.

Carbapenemasewas first reported in the 1980s from *Aeromonas hydrophila*, followed by reports in 1982 (SME-1 from *Serratia marcescens*), 1984 (IMI-1 from *Enterobacter cloacae*) and 1990 (NMC from *E. cloacae*). This global spread hascreated many serious concerns, as bacterial strains are gaining resistance against one of the most potent classes of beta-lactams, carbapenems (Garcia, 2013).

Class A carbapenemases (penicillinases)

They are serinecarbapenemases reported from *Serratia marcescens, Enterobacter cloacae*(Nordmann*et al*., 1993)*, Klebsiella*spp. (Yigit*et al*., 2001), etc. Class A carbapenemase can hydrolyze penicillin, cephalosporin, carbapenem, and aztreonam and is inhibited by compounds such as clavulanate and tazobactam. They comprise three major enzyme families: NMC/IMI, KPC and SME. NMC (nonmetalloenzymecarbapenemase) and IMI (imipenem hydrolyzing beta-lactamase) genes are chromosomally encodedand have been detected in *Enterobacter cloacae* isolates (Nordmann*et al*., 1993) with an internal amino acid identity of 97% (Rasmussen *et al*., 1996).*Klebsiella pneumoniae* carbapenemase (KPC) genes are not chromosomally encoded but are present on extrachromosomal elements called plasmids. They are not limited to *Klebsiella pneumoniae* (Yigit*et al*., 2001) but are also present to a lesser extent in *Salmonella* spp. and *Enterobacter* spp.

Table 1. Characteristics of different classes of carbapenemase

	Class A	Class B	Class D
Alternate name	Penicillinase	Metallo-beta-lactamase	Oxacillinase
Genes encoded on	Both chromosomes and plasmids	Mainly plasmids	Both chromosomes and plasmids
Inhibited by	Clavulanic acid, tazobactam	EDTA	*In vitro* by NaCl, variable inhibition by clavulanic acid
Examples	KPC, SME, IMI, NMC	VIM, GIM, SIM, IMP, NDM-1	OXA-23, OXA-40, OXA-48, OXA-50
Organisms	*P. aeruginosa*, *S. marcescens*, *Enterobacter-o*spp., Enterobacteriaceae	*Pseudomonas* spp., *Acinetobacter* spp., Enterobacteriaceae	*P. aeruginosa*, *Acinetobacter baumanii*

Source : various Scopus/PUBMED journals and web search

(Bratu*et al*., 2005). The gene for SME (*Serratia marcescens* enzyme) is encoded on the chromosome and has been identified in several isolates of several *Serratia marcescens.* They show 70% amino acid identity with members of the NMC/IMI family (Naas *et al*., 1994).Classification based on amino acid homology has resulted in four major classes that correlate well with the functional scheme but lack the detail concerning the enzymatic activity of the enzyme Soeung*et al.* (2020) mentioned molecular class A, including β-lactamases with serine at their active site. In the study of (Bush, 2018), it was mentioned how the serine active site forms.

Among the resistance conferred by several carbapenemase-producing bacteria, the frequency with which the resistance is shown by bacteria producing KPC poses the maximum threat (Nordmann*et al*., 2009; Srinivasan and Patel, 2008). Their rates of propagation, even on an international level, are fairly widespread and have been reported in countries such as the United States, Greece, China and Israel (Gupta *et al*., 2011; Nordmann*et al*., 2009; Bratu*et al*., 2005a), resulting in endemic and epidemic situations. They are a larger threat, as their carbapenemase can hydrolyze a very broad spectrum of antibiotics, such as fluoroquinolones, tetracycline and aminoglycosides (Bratu*et al*., 2005b; Endimiani*et al*., 2009), in combination with other mechanisms, such as changes in the transporter and enzyme modification. This compounds the problem of KPCproducers often comprising up to 57% of some outbreaks (Woodford *et al*., 2004; Bratu*et al*., 2005a). With the limited data available, it remains unknown which antibiotic combination can effectively treat infections caused by KPC-producing bacteria (Hirsch and Tam, 2010).

Class B carbapenemases (metallo-beta-lactamases)

They are metallo-carbapenemases (zinc), usually resistant to commercial beta-lactam inhibitors but susceptible to EDTA and other metal chelators. The gene for the enzyme is present on the chromosome and has been identified thus far in *Bacillus cereus* (Kuwabara and Abraham, 1967)*, Stenotrophomonas maltophila* (Saino*et al*., 1982) and *Aeromonas* spp. (Iaconis and Sanders, 1990). Localized imipenemasessuch as GIM (German imipenemase), SIM (Seoul imipenemase), VIM (Verona integron encoded Metallo beta-lactamase, commonly present in *Pseudomonas* sp.) and IMP (active on imipenem, commonly found in *Acinetobacter* sp. and *Pseudomonas* sp.) are common families of metallo-carbapenemase. VIM and IMP have been spotted worldwide in*Enterobacteriaceae,* while SIM and GIM have not spread beyond their place of origin.

NDM (NewDelhi metallo-beta-lactamase, widely present in *Enterobacteriaceae, Acinetobacter* spp. and *Vibrio cholerae* (Nordmann*et al*., 2011; Patel, 2012), is one of the classes of metallo-beta-lactamases. The infection caused by NDM was endemic to India and Pakistan. However, tourism has resulted in its spread to several countries (Kus*et al*., 2011). A key concern regarding NDM is thatit is not restricted to hospital-acquired infections but is also spread in the environment (Wu *et al*., 2010; Poirel*et al*., 2011). Such organisms often carry genes responsible for resistance to other classes of antibiotics but are susceptible to polymyxin and tigecycline (Livermore *et al*., 2011; Albur*et al*., 2012).These enzymes are currently divided into three subclasses based on a combination of structural features, zinc affinities for the two binding sites, and hydrolysis characteristics.The different active site requirements on different classes were studied by (Sun *et al.,* 2018). Subclasses B1 and B3, divided by amino acid homology, bind two zinc atoms for optimal hydrolysis, while enzymes in subclass B2 are inhibited when a second zinc is bound. Subclass B2 also differs in hydrolysis spectrum, as it preferentially hydrolyzes carbapenems, in contrast to the broad hydrolysis spectrum observed for B1 and B3 enzymes (Devkota*et al.,*2020)

Class D carbapenemase (oxacillinases)

They are OXA (oxacillin hydrolyzing) beta-lactamases and have been recognized in *Acinetobacter* sp. (Afzal-

Shah and Livermore, 1998), more specifically in *A. baumannii.* They are also present to some extent in *P. aeruginosa* and members of *Enterobacteriaceae* (Naas and Nordmann, 1999). They are present on the chromosomes and on the plasmids of several bacterial classes (Sanschagrin*et al*., 1995; Poirel*et al*., 2012). They can hydrolyze oxacillin and cloxacillin and inhibit clavulanate and EDTA (Bush *et al*.,2011). Class D carbapenemase hydrolyses carbapenems very weakly. This class has seen maximum growth in terms of the number of newly developed enzymes (Bush and Fisher, 2011). They are the key sources of hospital-acquired infections coupled with a high rate of mortality. Some of the examples include OXA-1, OXA-2, OXA-24/40, OXA-23, OXA-10, OXA-48, etc. (Antunes and

Table 2. Earlier studies of combination therapy with carbapenem

Isolate	Combination	% Synergy	% Antagonism	Reference
Acinetobacter baumanii	Imipenem + Ampicillin/sulbactam	88.2	Nil	Ozseven *et al*. (2012)
Acinetobacter baumanii	Imipenem + cefepime/sulbactam	70.6	Nil	Ozseven *et al*. (2012)
Acinetobacter baumanii	Meropenem + Ampicillin/ sulbactam	94.1	Nil	Ozseven *et al*. (2012)
Acinetobacter baumanii	Meropenem + cefepime/ sulbactam	8.8	Nil	Ozseven *et al*. (2012)
Acinetobacter baumanii	Meropenem + sulbactam	70	6.7	Pongpech *et al*. (2012)
Acinetobacter baumanii	Imipenem + Ampicillin/sulbactam	16	Nil	Sheng *et al*. (2011)
Acinetobacter baumanii	Imipenem + sulbactam	87.5	Nil	Song *et al*. (2007)
Pseudomonas aeruginosa	Imipenem + Gentamycin	25	Nil	Santos *et al*. (2013)
Pseudomonas aeruginosa	Imipenem + Tobramycin	75	Nil	Santos *et al*. (2013)
Pseudomonas aeruginosa	Imipenem + Rifampicin	75	Nil	Santos *et al*. (2013)
Pseudomonas aeruginosa	Imipenem + Tobramycin	15	Nil	Dundar and Otkun, (2010)
Pseudomonas aeruginosa	Imipenem + Ciprofloxacin	8	Nil	Dundar and Otkun, (2010)
Pseudomonas aeruginosa	Doripenem + Amikacin	20	Nil	He *et al*. (2012)
Pseudomonas aeruginosa	Doripenem + Colistin	3	Nil	He *et al*. (2012)
Pseudomonas aeruginosa	Doripenem+Levofloxacin	9	Nil	He *et al*.(2012)
Klebsiella pneumoniae	Meropenem + Colistin	38.5	Nil	Tascini*etal*.(2013)
Klebsiella pneumoniae	Imipenem + Colistin	38.5	Nil	Tascini*et al*. (2013)
Klebsiella pneumoniae	Imipenem + Polymyxin B	100	Nil	Elemam*et al*. (2010)
Klebsiella pneumoniae	Meropenem + Colistin	25	Nil	Stein *et al*. (2015)
Klebsiella pneumoniae	Meropenem + Tigecycline	10	Nil	Stein *et al*. (2012)
Klebsiella pneumoniae	Doripenem + Colistin	100	Nil	Lee and Burges, (2013)
Klebsiella pneumoniae	Doripenem + Polymyxin B	100	Nil	Lee and Burges, (2013)
Klebsiella pneumoniae	Meropenem + Polymyxin B	64	Nil	Pankey and Ashcraft, (2009)
Klebsiella pneumoniae	Imipenem + Tigecycline	69.2	Nil	Yim*et al*. (2011)
Klebsiella pneumoniae	Imipenem + Colistin	33.3	23.8	Souli *et al*. (2009)
Klebsiella pneumoniae	Meropenem + Polymyxin B	100	Nil	Diep *et al*. (2017)
Klebsiella pneumoniae	Meropenem + Polymyxin B	100	Nil	Kulengowski *et al*. (2017)

Fisher, 2014).As mentioned in penicillinase, class D also possesses serine at their active site (Akhter *et al.,* 2018). However, according to the study of (Willing *et al.,* 2020), serine incorporation lowers resistance to oxacillin.

ANTIBIOTIC SYNERGY WITH CARBAPENEM

The concept of "antibiotic synergy" arises when a combination of antibiotics is used to cure a particular infection instigated by drug-resistant bacteria in cases where monotherapy may not be advantageous. While using a combination, if the effect of the antibiotic combination is more than the effects of separate antibiotics, the antibiotics are said to act in synergy. Such a pair may provide better improvement and reduce mortality. While using carbapenem for combination therapy, either one or both candidates can be considered carbapenems. Combinations of other antibiotics with carbapenem have been found to be most effective for patients in the high-risk mortality class (septic shock patients). In contrast, the combinations without carbapenem exhibited worse results (Daikos*et al*., 2014). Several studies have focused on the effect of combination therapy to cure infections. Carbapenems-aminoglycosides combinations have had encouraging results against carbapenem-resistant Enterobacteriaceae (CRE) (Hirsch *et al*., 2013; Le *et al*., 2011). Combinations of colistin with meropenem showed synergism against MDR *Klebsiella pneumoniae* isolates (Ontong*et al*., 2021). Another independent study identified the combination of doripenem with amikacin to be highly effective against KPC-producing *Klebsiella pneumoniae*. The results were promising for both *in vitro* and *in vivo* infection models (Hirsch *et al*., 2013).To treat the infections caused by the PDR (pandrug-resistant) Acinetobacter baumannii, a combination of imipenem-colestimethate and imipenem-tigecycline has been found to be synergistic (Spoiala*et al*., 2010). MDR *Klebsiella pneumoniae* infections were treated with several combinations, such as colistin with aminoglycoside, fluoroquinolones, carbapenem, tetracycline, cephalosporins, piperacillin and fosfomycin (Ontong*et al*., 2021).Zusman*et al*. (2013) analysed the *in vitro* synergy between polymyxin and carbapenem combinations. This combination was successful against 77% of *Acinetobacter baumannii* isolates with only 1% antagonism, notably when meropenem or doripenem was used.

Combinations of two carbapenems are a salvage treatment option to combat XDR (extensively drugresistant) and PDRCRE. In a few studies, ertapenem and doripenem combinations have been used, where ertapenem inhibits KPCs while doripenem kills the pathogen(Bulik and Nicolau, 2011). Other carbapenem combinations effective against KPC producers are imipenem/ertapenem, imipenem/doripenem, meropenem/doripenem and ertapenem/doripenem, whereas the meropenem/ertapenem combination showed no synergy(Poirel*et al*., 2016; De Pascale *et al*., 2017). Some data show that DCC (double carbapenem combination) exhibits synergistic activity against MDR (multidrug-resistant) and XDR *K.*pneumoniae producing OXA-48 (Galani*et al*., 2018). However, the results are limited to *in vitro* conditions, and clinical evidence is awaited. Recent studies have also shown that resistance development against combination therapies is delayed compared to that against monotherapies (Tan *et al*., 2007). Table 2 depicts some of the studies where carbapenems were used for treating infections.

All these reports suggest one thing in common: DCCs proved to be of maximum efficacy, with imipenem showing maximum efficiency. However, the results cannot be directly taken into account relevant to clinical practice, as the correlation among the results *obtained in vitro* and *in vivo* depends on several factors, such as the pharmacokinetic and pharmacodynamic properties of the drug in the patient and bacterial load and antibiotic load at the infection site (Zusman*et al*., 2013).

Methods for testing synergy

The need for testing antibiotic synergy has gained excessive admiration in the last decade owing to the growing incidence of infectious organisms that are highly drug-resistant. *In vitro* drug combination studies can, to an extent, provide evidence about their *in vivo* performance, which is where synergy testing plays a key role. Synergy testing requires sophisticated techniques to measure the cumulative efficacy of the antibiotic combinations. The most common methods used in vitro to determine the synergy are the checkerboard method, MCBT (Multiple combination bactericidal test), time-kill assays and E-test (gradient diffusion test). These methods may determine if the antibiotics used in combination are antagonistic to each other (Doern, 2014). Papoutsaki*etal.*(2020) used the three E-test, chequerboard, and TKA to evaluate the invitro methods for testing tigecycline combinations against carbapenemase-producing Klebsiella pneumoniae isolates.

Checkerboard method

This method is relatively easy and can determine the activity of a combination of two antibiotics in clinically relevant concentrations. The combinations at different concentrations can be tested in a broth with a 2 ml volume or a micro broth with a 100 µl volume (Odds, 2003). The effect of different classes of antibiotics can also be determined in serial dilutions. The results of this method are expressed in terms of FIC and fractional inhibitory concentration. The FIC values are calculated from the MIC values of the individual components of

the combination and the MIC of the combination as a whole. For a combination to be synergistic, the MIC of the combination should be at least 4-fold less than the MIC of the individual components. This meansthat the FIC value should be less than 0.5 for the combination to act synergistically. FIC values in the range of 0.5-1.0 are considered to be additive; values 1-4 indicate little interaction between the drugs, while FIC values greater than 4 imply that the antibiotics in the combination are antagonistic (Saiman, 2007). This method has certain limitations. For example, it can test a combination of only two antibiotics, not more than that. The efficacy of the combination can be tested only for a fixed period, and it requires several reagents and resources to check several different combinations (Doern, 2014).

Using the broth volatilization checkerboard technique, (Netopilova*et al*., 2021) researchers evaluated in vitro antibacterial interactions against diverse S. aureus strains in both liquid and vapourphases.The synergistic effect of conventional antibiotics and lipopeptides of the pelgipeptin family was evaluated by the Checkerboard method in the study of (Costa *et al*., 2019).The results indicate that the combination of pelgipeptin B and C or chloramphenicol has a synergic effect against a multi-drug-resistant bacterial strain.

Multiple-combination bactericidal test

The MCBT is a useful technique to help clinicians decide on appropriate non antagonistic combination antibiotic therapy for patients (Smith *et al.,* 2020)

The multiple-combination bactericidal test overcomes one of the major disadvantages of the checkerboard test. It can simultaneously test two, three or even four antibiotics and determine the antimicrobial concentration required to achieve 99.9% killing. The concentration of the antibiotics to be used in the test depends on the concentration present in the serum of the patients. It allows only a fixed concentration of antibiotics to be tested. However, several combinations can be easily tested as the assay is performed in a 96-well microtiter plate. Each well can have the required antibiotic combination atappropriate concentrations. The plates are inoculated with the bacterial agents to be tested, incubated and observed for turbidity twice, at 24 h as well as 48 h. Samples from the wells with no turbidity are subcultured and examined for 99.9% killing (Aaron *et al*., 2000). The utility of this method is limited, as the results are measured only at specific time intervals (Taylor *et al*., 1983).

(Tapalsky 2018) Evaluated the sensitivity of combinations of antimicrobial drugs (AMPs) of nosocomial isolates of *Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii*-producingcarbapenemase by using MCBT.

in the study of Lim and Fitzgerald (2018). Treating resistant Pseudomonas aeruginosa lung disease in young children with cystic fibrosis.It was mentioned that MCBT is able to systematically test P. aeruginosa isolates against various combinations of antibiotics to identify the optimal sensitivity pattern. The results can be generated within 72 hours of culture, whichfurther enhances the treatment efficacy in the management of P. aeruginosa infections in individuals with CF.

Time-kill assays

The time-kill assay (TKA) determines the reduction in bacterial counts with time followed by exposure to a specific drug combination compared to the antibiotics used singly (Laishram*et al*., 2017). Similar to the MCBT test, the time-kill assay also determines the concentration of the antibiotics that is sufficient to achieve 99.9% bacterial killing. Therefore, it is considered a derivative of MCBT. However, in contrast to MCBT, where growth is checked at 24 and 48 hours, the time-kill assays employ different time points to better understand bacterial killing. This enables the detection of the rate of bacterial killing, which is crucial to predicting the infected patient's performance (Norden*et al*., 1979). There is a standard protocol for the time-kill assays that are performed in beakers of at least 10 mL volume. The antibiotic combination was mixed with a specific load of inoculum and incubated for 48 hours. In the meantime, 0.5 mL samples are withdrawn at a specific time interval to determine the viable count of bacteria in the sample. The values of time and kill are used to make a graph. Suppose there is more than a 2 log10 decrease in the growth of bacteria obtained with the antibiotic combination and with the most active antibiotic of the combination. In that case, the combination is said to exhibit synergistic behavior (NCCLS, 1999). Any difference less than this is noted as indifference. The limitation of the test is that the graph is not easy to interpret, as different bacteria grow at different rates, with the first 12 h being the most critical ones for such studies.

The results of TKA can also be depicted as the area under the killing curve (AUC). The log values of bacteria can be plotted on the Y-axis with the time on the X-axis, and the AUC can be calculated for the combination and an individual component. If both AUC values were statistically significant at $P < 0.05$, the components acted synergistically. Even though the results have high precision coupled to less experimental variation, they are not extensively used (*Laishram et al*., 2017). A key reason for this is the fact that researchers often prefer to use the drug at concentrations similar to those present in the serum. However, this is not reliable, as the drug concentration at the site of infection or in other organs may vary, and therefore, the results cannot be extrapolated. Another key point is that the drug concentration remains constant in vitro, whereas the concentration keeps changing within the body. Fac-

tors such as the properties of the drug, dose and mode of administration, inoculum size, etc. severely affect the results of TKA (Tripodi*et al*., 2007). It can be used as a reference assay to determine the bactericidal effect but is often time-consuming andlabour-intensive and can detect only limited concentrations (Laishram*et al*., 2017).

Jahan *et al*.(2021) used an Etest minimum inhibitory concentration (MIC): MIC technique and a time-kill assay to investigate the combination of fosfomycin and meropenem against 20 MBL-producing P. aeruginosa. The Etest technique and the time-kill assay yielded similar results in accordance with the isolates.

(Alrashidi*et al*., 2021) conducted a Time-Kill Assay Study on the Synergistic Bactericidal Activity of Pomegranate Rind Extract and Zn(II) against methicillin-resistant Staphylococcus aureus (MRSA), Staphylococcus epidermidis, Escherichia coli, and Pseudomonas aeruginosa, as Pomegranate Rind Extract and Zn(II) possess antimicrobial activity.

E-test

The E-test formerly known as the Epsilometer test is a method of assessing antimicrobial sensitivity by inserting an antimicrobial-impregnated strip onto an agar plate. If a bacterium or fungus is sensitive, it will not grow near an antibiotic or antifungal concentration. The results can be used to determine a minimum inhibitory concentration (MIC) for some microbial and antimicrobial combinations.

The test is also known as the gradient diffusion test. The test relies on antibiotic diffusion from a strip impregnated with the antibiotic in a continuous concentration gradient. An agar medium with the lawn growth of the test bacterium was used to perform the test. E-test strips with the specific antibiotic were placed on agar and allowed to incubate overnight. The antibiotic will slowly diffuse from the strip to kill the bacteria, leading to the formation of an elliptical zone as the antibiotic will diffuse from all sides. The point at which this clear zone touches the strip is the minimal inhibitory concentration (MIC). TheE-test can be modified in two ways to determine synergy (Doern, 2014).

The first method employs two E-test strips with different antibiotics. They were placed in agar plates with lawn bacterial growth perpendicular to each other. The strips intersect each other at the MIC of the individual antibiotic. The FIC value determines whether they are in synergy (Saiman 2007; White *et al*., 1996). In the second method, a single E-test strip with the antibiotic is placed on the lawn bacterial growth on the agar plate for 60 min. The strip is then replaced with a second strip impregnated with a different antibiotic. Control strips are also placed on the same plate without interfering with the test results (Lewis *et al*., 2002). Table 3 lists some of the studies that used these testing methods to determine synergy against *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

In (Ranu*et al.,* 2019), antibiotic combinations such as colistin + meropenem, imipenem + tigecycline, poly-

Table 3. Synergy methods used for different combination therapies

Organism	Antibiotic combination	Synergy testing method employed	References
Acinetobacter baumanii	Imipenem + Polymyxin B	Checkerboard and time-kill	Yoon *et al*., 2004
Acinetobacter baumanii	Imipenem + Polymyxin B	E test	Wareham and Bean, 2006
Acinetobacter baumanii	Meropenem + Colistin	Checkerboard	Biancofiore*et al*., 2007
Acinetobacter baumanii	Meropenem + Polymyxin B	E test and time-kill	Pankey and Ashcraft, 2009
Pseudomonas aeruginosa	Imipenem + Polymyxin B	Time-kill	Landman *et al*., 2005
Pseudomonas aeruginosa	Imipenem + Polymyxin B	Checkerboard breakpoint	Tateda*et al*., 2006
Pseudomonas aeruginosa	Meropenem + Colistin	Time-kill	Pankuch*et al*., 2008
Pseudomonas aeruginosa	Imipenem + Colistin	Time-kill	Bergen *et al*., 2011
Klebsiella pneumoniae	Imipenem + Polymyxin B	Time-kill	Bratu*et al*., 2005a
Klebsiella pneumoniae	Imipenem + Colistin	Time-kill	Souli*et al*., 2009
Klebsiella pneumoniae	Imipenem + Colistin	Time-kill	Elemam*et al*., 2010
Klebsiella pneumoniae	Doripenem + Polymyxin B	Time-kill	Urban *et al*., 2010
Klebsiella pneumoniae	Doripenem + Colistin/ Polymyxin B	Time-kill	Lee and Burgess, 2013

myxin B + azithromycin, and doripenem + sulbactam were utilized for MDR Acinetobacter infections and evaluated invitro by Epsilometric test and Broth Micro dilution, with time kill assay confirmation. (Ramadan *et al.,* 2018) characterized different carbapenemase genes carried by carbapenem-resistant (CR) A. baumannii and P. aeruginosa isolates and evaluated the in vitro effect of some colistin-based combinations by the E-test method. Papoutsaki*etal.(*2020) explored whether easier methods based on the Etest technique might offer a suitable alternative. There is a poor correlation between the synergy testing methods of tigecycline combinations, which may be associated with their different endpoints.

Comparison of methods for testing synergy

There is no gold standard method for studying synergy. However, several tests can be used to compare the results to develop a method better suited for the purpose. As each of the tests uses different results, i.e., inhibition or killing, the overall results are often discordant. The time-kill method has been shown to exhibit better synergy rates than the checkerboard method (Soulie*t al*., 2008). The results of the checker-board test and E-test are expressed in terms of MIC (minimum inhibitory concentration) values. This holds relevance in clinical diagnosis and studies. However, the time-kill methods focus on the extent of the killing of the infectious bacteria in a certain amount of time, which gives an idea about the nature of the infection. The results may vary with the type of bacteria, the bacterial load, the time frame of the infectious sample during the entire course of infection, and so on (Zusman*et al*., 2013). All these parameters are key to arriving at a single method to determine the synergy in conditions that are *in vitro* as well as *in vivo.*

For the gold standard method for testing drug-resistant variants of Escherichia coli, Enterobacter cloacae, Staphylococcus aureus, and *Pseudomonas aeruginosa,* TKA has been the gold standard method compared to the checkerboard and E-test assays. TKA methods showed an agreement value of 44-88% compared to the values 63-75% for the other methods (White *et al*., 1996). As there is not much difference between the values for all three methods, the E-test method, which is the simplest, can be used as a replacement for the checkerboard and TKA methods. A different study compared checkerboard and TKA for *Pseudomonas aeruginosa.*The results showed synergy for various antimicrobial concentrations using the TKA method, while the checkerboard method showed no difference (Cappellety and Rybak, 1996). For the determination of synergy for *Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* the TKA method was the best, followed by the checkerboard test, while the E-test detected the least synergism (Laishram*et al*., 2017). Despite this, there have been instances where more than 90% correlation have been found between the results obtained using checkerboard test and E-test (Balke*et al*., 2006).

The key points to be considered are that there is no one gold standard method for determining synergy. No two methods for testing synergy can produce comparable methods because of the different parameters. At the same time, one cannot neglect the fact that one of the synergy methods may correlate well with patient outcomes. With the limited data available, one can say that the TKA method could be a gold standard, as it provides dynamic details of how the bacteria are killed as a function of time.Checkerboard method, MCBT or E -test does not provide this information. However, the limited studies that have focused on the clinical relevance of synergy testing have not considered TKA. This means that the *in vitro* data about antibiotic synergy cannot be linked to the treatment options.

Clinical significance of combination therapy and synergy testing

Carbapenem-resistant (CR) Gram-negative bacteria (GNB) embody one of the prime health risks (Paul *et al*., 2014). Treating the infections caused by them has resulted in a higher rate of mortality and a very low recovery rate.The use of combination therapy has been a boon in such cases, as they show a significant decrease in mortality and improved recovery rate. This increased efficacy of the antibiotic combination is because of the synergistic interaction between the antibiotics. Combination therapies are vital, as they maximize the rate of bacterial killing and the extent of bacterial killing, as the different components of the combination will affect multiple bacterial pathways and kill them. They also prevent bacterial regrowth and reduce the development of resistance by the bacteria (Zavasckie*t al*., 2013).

The key success of the combination therapy is to quantify how beneficial the antibiotic combination is compared to when administered individually. Synergy testing methods have enabled testing of several antibiotic combinations with different drug concentrations in different groups of drug-resistant bacteria to determine which combination affects the bacteria the most. Such tests provide information about which drugs can be paired together and at what concentrations. It can also determine which drugs cannot be paired together. The results of the methods determine the MIC of the individual drug as well as the drugcombination as a whole to determine the mechanism behind the resistance. The *in vitro* results obtained as a result of synergy testing can be used to predict the in vivo performance with more confidence and the patient outcome and recovery (Laishram*et al*., 2017). As the studies are performed in drug concentrations mimicking the drug concentrations

in the serum during the natural infection, the results can give a fair idea of how the *in vitro* drug combination studies are effective in treating a similar infection *in vivo.*

The goal of (Li *et al.,* 2020) research was to determine how effective colistin was against CRAB clinical isolates when used alone and in combination with either meropenem or levofloxacin. Colistin's synergistic activity against CRAB isolates was proven. CRAB isolates should be treated with a mixture of colistin and meropenem. For carbapenem-resistant Gram-negative bacilli (CR-GNB) infections, the superiority of combination treatment is still debated. Antibiotic regimen activity against CR-GNB may be predicted using in vitro models. Pharmacokinetic/pharmacodynamic (PK/PD) and time–kill (TK) studies assessing the in vitro effectiveness of antibiotic combinations against CR-GNB were included in a comprehensive review and meta-analysis of (Scudeller *et al.,* 2021).

Conclusion

The handling of infections induced by drug-resistant Gram-negative bacteria is one of the major challenges faced by clinicians today. This is coupled with an increasing development of resistance against antibiotics, especially for carbapenems, the last-line antibiotics used to combat infections. In such cases where no single agent can be effective, combination therapy, where two or more antibiotics can be used, can provide better treatment efficacy and reduced mortality. Before exploring this, it is of utmost importance to determine if the antibiotics used in combination can produce a superior effect compared to when they are used alone. This synergism between the combination's individual components can be tested using several methods.

Among the methods of synergy testing that are used, no one test works under all conditions. Furthermore, the results obtained using different methods are not correlated enough to deduce the best one. This often results in a poor estimation of the treatment efficacy as well as the patient outcomes. There is a great variation in the results because of several factors, such as the type of organism tested and the type, concentration and combination of the antibiotics, which results in very little to no correlation between the results obtained using different methods. A significant drawback is that these methods are all labor intensive and timeconsuming, with the expertise needed to interpret the data, except for the E-test method. Extensive research and standardizationare required to develop a test that suits the majority of the purposes and, most importantly, allows us to compare and correlate the *in vitro* findings to the *in vivo* patient treatment efficacy and the clinical outcome. Therefore, an appropriate and reliable study of the synergistic effects of several drug combinations will drastically improve the therapeutic possibilities available for the treatment of infections caused by drug -resistant bacteria.

ACKNOWLEDGEMENTS

Thank you to the Department of Science and Technology, Government of India for providing funding tous as part of the Women Scientist Scheme No. SR/WOS-A/LS-643/2016(G),Manipal Academy of Higher Education,PalamurBiosciencesPvt.andSVSMedicalCollegefor cooperation.We Thank Dr. Murali for providing his comments on the draft version of the manuscript

Conflict of interest

The authors declare that they have no conflict of interest.

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