IN VITRO ANTIMICROBIAL SYNERGY TESTING OF *ACINETOBACTER BAUMANNII*

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that it has not previously in its entirety or in part been submitted at any university for a degree

Signature: Date: 01/09/2010

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DEDICATION

To my wife, for her love, belief in me, understanding, and support

SUMMARY

Acinetobacter baumannii has emerged as one of the most troublesome nosocomial pathogens globally. This organism causes infections that are often extremely difficult to treat because of the widespread resistance to the major antibiotic groups. Colonization or infection with multidrug-resistant *A. baumannii* is associated with the following risk factors: prolonged hospital stay, admission to an intensive care unit (ICU), mechanical ventilation, and exposure to broad spectrum antibiotics, recent surgery, invasive procedures, and severe underlying disease.

A. baumannii has been isolated as part of the skin flora, mostly in moist regions such as axillae, groin and toe webs. It has also been isolated from the oral cavity and respiratory tract of healthy adults. Debilitated hospitalized patients have a high rate of colonization, especially during nosocomial Acinetobacter outbreaks. This organism is an opportunistic pathogen as it contains few virulence factors. Clinical manifestations of *A. baumannii* include nosocomial pneumonia, nosocomial bloodstream infections, traumatic battlefield and other wound infections, urinary tract infections, and post-neurological surgery meningitis. Fulminant community-acquired pneumonia has recently been reported, indicating that this organism can be highly pathogenic.

The number of multidrug-resistant *A. baumannii* strains has been increasing worldwide in the past few years. Therefore the selection of empirical antibiotic treatment is very challenging. Antibiotic combinations are used mostly as empirical therapy in critically ill patients. One rationale for the use of combination therapy is to achieve synergy between agents.

The checkerboard and time-kill methods are two traditional methods that have been used for synergy testing. These methods are labor intensive, cumbersome, costly, and time consuming. The E-test overlay method is a modification of the E-test method to determine synergy between the different antibiotics. This method is easy to perform, flexible and time efficient.

The aim of this study was to assess the in vitro activity of different combinations of colistin, rifampicin, imipenem, and tobramycin against selected clinical strains of *A. baumannii* using the checkerboard and the E-test synergy methods. The MICs obtained with the E-test and broth microdilution method were compared. The results of the disk diffusion for imipenem and tobramycin as tested in the routine microbiology laboratory were presented for comparison.

Overall good reproducibility was obtained with all three methods of sensitivity testing. The agreement of MICs between the broth dilution and E-test methods was good with not more than two dilution differences in MIC values for all isolates, except one in which the rifampicin E-test MIC differed with three dilutions from the MIC obtained with the microdilution method. However, the categorical agreement between the methods for rifampicin was poor. Although MICs did not differ with more than two dilutions in most cases, many major errors occurred because the MICs clustered around the breakpoints.

The combinations of colistin + rifampicin, colistin + imipenem, colistin + tobramycin, rifampicin + tobramycin, and imipenem + tobramycin all showed indifferent or additive results by the E-test method. No results indicating synergy were obtained for all the above-mentioned combinations. There was one result indicating antagonistic effect for the combination of colistin + tobramycin.

The results of the checkerboard method showed results indicating synergy in four of the six isolates for which the combination of colistin and rifampicin was tested. The other two isolates showed indifferent/additive results. All the other combinations showed indifferent/additive results for all isolates except isolate 30 (col + tob) and isolate 25 (rif + tob) which showed synergism. No antagonistic results were observed by the checkerboard method.

When the results obtained with the E-test and checkerboard methods were compared, it was noted that for most antibiotic combinations an indifferent/additive result was obtained. However, for the colistin + rifampicin combination, the checkerboard method showed synergism for 4 of 6 isolates, whereas the E-test method showed indifference and an additive result in one. For the rifampicin + tobramycin, and colistin + tobramycin combinations, synergism was also shown with the checkerboard method in one isolate for each combination. The E-test method however showed an indifferent and additive result respectively.

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The E-test method was found to be a rapid, reproducible, easy-to-perform, and flexible method to determine synergistic antibiotic activity. This study was however limited by low numbers of isolates. This might explain why no synergistic results were obtained with the E-test method and few synergistic results with the checkerboard method. Genotypic analysis using pulse-field gel electrophoresis (PFGE) may be considered in future studies to determine relatedness of the

isolates which will facilitate the selection of different strains for synergy testing. Furthermore, clinical studies are needed to establish whether in vitro synergy testing is useful in the clinical setting and whether the results of synergy testing will have any bearing on the clinical outcome of patients infected with multidrug resistant *A. baumannii.*

OPSOMMING

Acinetobacter baumannii het wêreldwyd as een van die mees problematiese nosokomiale patogene verskyn. Hierdie organisme veroorsaak infeksies wat dikwels baie moeilik is om te behandel weens wydverspreide weerstandigheid teen major antibiotikagroepe. Kolonisasie of infeksie met multi-weerstandige *A. baumannii* word geassosieer met die volgende riskofaktore: verlengde hospitaalverblyf, toelating tot 'n intensiewe sorgeenheid (ICU), meganiese ventilasie, blootstelling aan breëspektrum antibiotika, onlangse chirurgie, indringende prosedures en ernstige onderliggende siekte.

A. baumannii kan deel vorm van die normale velflora, veral in die axillae, inguinale area en tussen die tone. Dit is ook al vanuit die mondholte en die respiratoriese traktus van gesonde volwassenes geïsoleer. Verswakte gehospitaliseerde pasiënte word veral gekoloniseer gedurende nosokomiale *Acinetobacter* uitbrake. Hierdie organisme is 'n opportunistiese patogeen en bevat min virulensie faktore. Kliniese manifestasies van *A. baumannii* sluit nosokomiale pneumonie, nosokomiale bloedstroom infeksies, troumatiese slagveld- en ander wondinfeksies, urienweginfeksies en meningitis wat volg op neurologiese chirurgie in. Fulminerende gemeenskapsverworwe pneumonie is onlangs beskryf en dui aan dat hierdie organisme hoogs patogenies kan wees.

Die aantal multi-weerstandige *A. baumannii* stamme het wêreldwyd toegeneem oor die laaste paar jare. Daarom is die seleksie van empiriese antibiotiese behandeling 'n uitdaging. Antibiotika kombinasies word meestal as empiriese behandeling in ernstige siek pasiënte gebruik. Die beginsel hiervan is om sinergistiese werking tussen agente te verkry.

Die "checkerboard" en "time-kill" metodes is twee tradisionele metodes van sinergisme toetsing. Hierdie metodes is werksintensief, duur en tydrowend. Die E-toets sinergisme metode is gebaseer op die E-toets metode. Hierdie metode is maklik, buigbaar en tydseffektief.

Die doel van hierdie studie was om die in vitro aktiwiteit tussen verskillende antibiotika kombinasies van colistin, rifampisien, imipenem, en tobramisien teen geselekteerde kliniese *A. baumannii* isolate te toets met die "checkerboard" en E-toets sinergisme toetsing metodes. Die minimum inhibitoriese konsentrasies (MIKs) verkry met die E-toets en "broth microdilution" metode is ook vergelyk. Die resultate van die skyfie diffusie metode (die metode wat in die roetiene

mikrobiologie laboratorium gebruik word) vir imipenem en tobramisien word ook verskaf vir vergelyking van die resultate van verskillende sensitiwiteitsmetodes.

In oorsig is goeie herhaalbaarheid van resultate verkry met al drie metodes van sensitiwiteitstoetsing. Die ooreenstemming van MIKs tussen die “broth dilution” en E-toets metodes was goed en resultate het met nie meer as twee verdunnings in MIK waardes verskil nie. Daar is een uitsondering waar die rifampisien E-toets MIK waarde met drie verdunnings van die MIK waarde verkry met die “microdilution” metode verskil. Die ooreenstemming tussen die sensitiwiteitskategorie resultate tussen die twee metodes was egter swak vir rifampisien. Alhoewel die MIKs in die meeste gevalle met nie meer as twee verdunnings in waarde verskil het nie, was daar baie major foute aangetoon omdat die MIKs rondom die breekpunte geval het.

Die kombinasies van colistin + rifampisien, colistin + imipenem, colistin + tobramisien, rifampisien + tobramisien, en imipenem + tobramisien het oorwegend slegs matige interaksie met die E-toets metode getoon. Geen sinergisme is verkry met enige van die antibiotika kombinasies met hierdie metode nie. Daar was egter een resultaat wat antagonisme getoon het vir die kombinasie van colistin + tobramycin.

Die resultate van die “checkerboard” metode het sinergisme getoon in vier van die ses isolate wat vir die kombinasie van colistin en rifampisien getoets was. Die ander twee isolate het slegs matige interaksie getoon. Al die ander kombinasies het ook slegs matige interaksie getoon, behalwe in isolaat 30 (col + tob) en isolaat 25 (rif + tob) waar die spesifieke kombinasies sinergisme getoon het. Geen antagonisme is waargeneem met die “checkerboard” metode nie.

Met vergelyking van die E-toets en “checkerboard” metodes, is dit opmerklik dat vir die meeste van die antibiotika kombinasies slegs matige interaksie verkry is. Vir die colistin + rifampisien kombinasie toon die “checkerboard” metode egter sinergisme vir 4 uit 6 isolate, terwyl die E-toets metode slegs matige interaksie toon. Vir rifampisien + tobramisien, en colistin + tobramisien kombinasies is sinergisme getoon met die “checkerboard” metode in een isolaat vir elke kombinasie. Die E-toets metode het slegs matige interaksie getoon.

Die E-toets sinergisme metode was vinnig, herhaalbaar en maklik om uit te voer. Hierdie studie word egter beperk deur lae getalle van isolate. Dit mag verklaar waarom geen sinergistiese resultate met die E-toets metode verkry is nie en die min sinergistiese resultate met die “checkerboard” metode. Genotipiese analiese met “pulse-field gel electrophoresis” mag in aanmerking geneem word in toekomstige studies om die verwantskap tussen isolate te bepaal wat die seleksie van verskillende stamme vir sinergisme toetsing sal vergemaklik. Verder, kliniese studies is nodig om te bepaal of in vitro sinergisme toetsing van waarde is en of die resultate van sinergisme toetsing ‘n rol speel in die kliniese uitkoms van pasënte geïnfekteer met multi-weerstandige *A. baumannii.*

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IN VITRO ANTIMICROBIAL SYNERGY TESTING OF *ACINETOBACTER BAUMANNII*

I. Introduction

Acinetobacter baumannii has emerged as one of the most troublesome nosocomial pathogens globally (Peleg A.Y., 2008). It has a remarkable ability to up-regulate or acquire resistance determinants. Infections due to this organism are often extremely difficult to treat because of the widespread resistance to the major antibiotic groups (Bergogne-Berezin E .1996). The emergence of multidrug resistant strains of *A. baumannii* has resulted in carbapenems becoming the mainstay treatment for Acinetobacter infections (Towner, 2009). Recently, though, there are increasing reports of carbapenem resistance accumulating worldwide. Some of these isolates are resistant to all conventional antibiotics. This resistance causes challenges in antibiotic selection for empirical therapy. Empirical therapy should thus rely on institutional-level data concerning the phenotypes and genotypes of *A. baumannii* strains endemic in a particular hospital (Towner, 2009).

There is a lack of large controlled clinical trials focusing on the treatment of *A. baumannii* infections (Maragakis L.L., 2008; Towner, 2009). This makes it difficult to evaluate the role of antimicrobial synergy of combination therapy (Maragakis L.L., 2008). Information about the best therapeutic approaches is based on in-vitro susceptibility data, small case series and retrospective analysis of observational studies (Towner, 2009).

In vitro testing has been used by researchers for some time now, for accurate prediction of clinically relevant antimicrobial synergy (White R.L., 1996). The most widely used methods for in vitro synergy are the checkerboard and the time-kill curve methods. The epsilometer (E-test) strip has also been used in vitro for performing synergy testing (White R.L., 1996). These three methods have been compared in previous studies (White R.L., 1996; Bonapace C.R., 2000). Agreement of qualitative interpretation was demonstrated among these methods, even though they use different endpoints (Bonapace C.R., 2000). There has however been conflicting results between studies testing the same antimicrobial combinations against *Acinetobacter* isolates (Maragakis L.L., 2008).

II. Literature Review: *Acinetobacter baumannii*

1. Current taxonomy

The genus *Acinetobacter* is characterized by a long history of taxonomic changes. These organisms have been moved from the family Neisseriaceae to the family Moraxellaceae (Fournier P.E., 2006). There are at least 25 different *Acinetobacter* strains which fulfill the criteria to be considered distinct species. These have been identified by DNA-DNA hybridization studies (Fournier P.E., 2006). These studies have also been used to delineate 15 genomic species (gen. sp.) which do not yet have valid names (Dijkshoorn L, 2007). These genomic species are commonly labeled by the initials of their authors e.g. Tjernberg and Using (TU) or Bouvet and Jeanjean (BJ) (Dijkshoorn L, 2007).

Acinetobacters are Gram negative coccobacilli, that are strictly aerobic and non-motile (occasionally showing twitching motility) ((Bergogne-Berezin E . 1996). The organisms exist as bacilli during rapid growth and coccobacilli in the stationary phase and have a tendency of retaining crystal violet, thus may be incorrectly identified as Gram-positive cocci.

It is difficult to differentiate *Acinetobacter* isolates according to their phenotypic characteristics (Fournier P.E., 2006; Peleg A.Y., 2008). This has led to the use of the term *A. calcoaceticus – A. baumannii* complex (Fournier P.E., 2006). The complex includes genomic species 1 (*A. calcoaceticus*), 2 (*A. baumannii*), gen. sp. 3, and 13TU, which show an extremely close relationship (Bergogne-Berezin E . 1996). *A. baumannii* seems to be the species of greatest clinical importance. Repeated isolation of other species from the *A. calcoaceticus – A. baumannii* complex might be significant, especially if clinical symptoms are also present (Bergogne-Berezin E . 1996),(Peleg A.Y., 2008). *A. calcoaceticus* is an environmental species that has been recovered from soil and water but has not been implicated in serious clinical disease ((Peleg A.Y., 2008). Figure 1 below shows the delineation of *Acinetobacter* genomic species.

TABLE 1. Delineation of *Acinetobacter* genomic species

Species	Genomic species[a]	Type or reference strain	Reference(s)
A. baumannii	2	ATCC 19606^T	51, 542
A. baylyi		DSM 14961^T	72
A. bouvetii		DSM 14964^T	72
A. calcoaceticus	1	ATCC 23055^T	51, 542
A. gerneri		DSM 14967^T	72
A. grimontii		DSM 14968^T	72
A. haemolyticus	4	ATCC 17906^T	51, 542
A. johnsonii	7	ATCC 17909^T	51, 542
A. junii	5	ATCC 17908^T	51, 542
A. lwoffii	8/9	ACTC 15309^T	51, 542
		ATCC 9957	
A. parvus		NIPH384^T	393
A. radioresistens	12	IAM 13186^T	51, 401, 542
A. schindleri		NIPH1034^T	392
A. tandoii		DSM 14970^T	72
A. tjernbergiae		DSM 14971^T	72
A. towneri		DSM 14962^T	72
A. ursingii		NIPH137^T	392
"*A. venetianus*"[b]		ATCC 31012	573
	3	ATCC 19004	51, 542
	6	ATCC 17979	51, 542
	10	ATCC 17924	51, 542
	11	ATCC 11171	51, 542
	13TU	ATCC 17903	542
	13BJ, 14TU	ATCC 17905	53, 542
	14BJ	CCUG 14816	53
	15BJ	SEIP 23.78	53
	15TU	M 151a	542
	16	ATCC 17988	53
	17	SEIP Ac87.314	53
	Between 1 and 3	10095	190
	Close to 13TU	10090	190

[a] Unless indicated otherwise, genomic species delineation is according to Bouvet and Grimont (51) and Bouvet and Jeanjean (53). BJ, Bouvet and Jeanjean; TU, Tjernberg and Ursing.

[b] "*A. venetianus*" is found in marine water but does not yet have formal species status.

Figure 1: Delineation of *Acinetobacter* genomic species.

Reproduced from: (Peleg A.Y., 2008)

2. Species of clinical importance

Acinetobacter spp. may form part of the human skin flora. Not all species of the genus *Acinetobacter* have their natural habitat in the environment. The skin carriage rate of all *Acinetobacter* species can be as high as 75% among hospitalized patients, and up to 25% among healthy individuals (Seifert H., 1997). *A. baumannii* and gen. sp. 13TU, on the other hand, were found only rarely on human skin in the study by Seifert et al., which looked at the distribution of *Acinetobacter* spp. on human skin of 40 cardiology patients and 40 healthy controls (Seifert H., 1997).

A. baumannii is the main genomic species associated with nosocomial outbreaks (Bergogne-Berezin E . 1996). Many reports of infection due to *A. baumannii* however do not include the necessary tests for specific identification to species level, but give a presumptive identification (Bergogne-Berezin E., 1996). There is a need for further investigations to define the clinical significance of Acinetobacter species other than *A. baumannii,* because these isolates are often considered as contaminants derived from the environment (Bergogne-Berezin E., 1996). However, genomic species 3 and 13TU have been implicated in nosocomial infections and *A. johnsonnii* has been reported to cause catheter related bacteremia. The main sites of infections due to *A. baumannii* are the lower respiratory tract and the urinary tract (Bergogne-Berezin E., 1996).

3. Laboratory identification

Precise species identification of Acinetobacter is not necessary in the routine clinical laboratory. The term *A. baumannii* group is sufficient for laboratory diagnosis. Exact strain identification may be required for epidemiologic purposes to identify strain relatedness. Various methods are available for molecular typing of strains for epidemiological purposes. There are also molecular methods which have been validated for the identification of Acinetobacter. Examples of these molecular methods are: amplified 16S rRNA gene restriction analysis, high resolution fingerprint analysis by amplified fragment length polymorphism, ribotyping, tRNA spacer fingerprinting, restriction analysis of the 16S – 23S rRNA intergenic spacer region and sequencing of the *rpoB* gene. All the above mentioned methods are too labor intensive to be used routinely in the clinical microbiology laboratory (Peleg A.Y., 2008).

Manual and semi-automated commercial identification systems are currently being used for species identification in the clinical microbiology laboratory. Examples are the API 20NE, Vitek 2, Phoenix, and Microscan WalkAway systems. The problem with these systems is their limited database content and the fact that they use identification substrates which have not been tailored specifically for Acinetobacter identification (Peleg A.Y., 2008). All the currently available commercial methods cannot differentiate

clinically relevant members of the *A. calcoaceticus – A. baumannii* complex. *A. baumannii*, Acinetobacter genomic species 3, and Acinetobacter genomic species 13TU are uniformly identified as *A. baumannii*. It is thus advisable to use the term *A. baumannii* group instead of *A. calcoaceticus – A. baumannii* complex when referring to these species. The distinction between *A. baumannii* group and *Acinetobacter* spp. outside the *A. baumannii* group has important infection control implications. Acinetobacter spp. outside the *A. baumannii* group rarely causes nosocomial outbreaks and therefore do not necessitate infection control measures (Peleg A.Y., 2008).

Acinetobacters are non-fastidious organisms that grow well on common laboratory media consisting of nutrient agar and tryptic soy agar (Bergogne-Berezin E . 1996). Clinical isolates, mostly *A. baumannii*, gen spp. 3, and 13TU, grow at 35 - 37 ˚C or higher, whilst some other genomic species grow only at lower temperatures. Most *Acinetobacter* strains can grow in a simple mineral medium containing a single carbon and energy source (Bergogne-Berezin E . 1996). Some *Acinetobacter* species outside the *A. calcoaceticus – A. baumannii* complex may not grow on McConkey agar. Some species may show hemolysis on sheep blood agar (e.g. *A. haemolyticus*). Members of the *A. calcoaceticus – A. baumannii* complex are never hemolytic on sheep blood agar. However, there is no single metabolic test which enables unambiguous identification of *Acinetobacter* species from other similar bacteria (Peleg A.Y., 2008).

DNA – DNA relatedness is used to classify *Acinetobacter* isolates into genomic species. The different DNA hybridization methods which have been employed are the nitrocellulose filter method, the S1 endonuclease methods, the hydroxyapatite method and a quantitative bacterial dot filter method. The latter method is the simplest, with the others being more labor intensive and not suitable for routine microbiological use (Bergogne-Berezin E . 1996). The DNA – DNA hybridization method is the gold-standard among the few validated methods for identification of *Acinetobacter* species (Peleg A.Y., 2008).

4. Epidemiology

Acinetobacter may form part of the skin flora, mostly in moist regions such as the axillae, groin and toe webs. They have also been isolated from the oral cavity and respiratory tract of healthy adults. The carriage rate in non-hospitalized patients, apart from on the skin, is generally low (Bergogne-Berezin E . 1996). Debilitated hospitalized patients have a high rate of colonization, especially during nosocomial Acinetobacter outbreaks. The predominant site of colonization in these patients is the skin, but respiratory tract or digestive systems may also be colonized. The differences between carriage rates between outpatients and hospitalized patients suggest that infecting or colonizing organisms in hospital patients may be acquired from cross-transmission or from hospital environmental sources and is usually not derived from endogenous patient sources (Bergogne-Berezin E . 1996). Colonization or infection with multidrug – resistant Acinetobacter is associated with the following risk factors: prolonged hospital stay, admission to

an intensive care unit (ICU), mechanical ventilation, and exposure to broad spectrum antibiotics, recent surgery, invasive procedures, and severity of the underlying disease (Maragakis L.L., 2008).

To investigate the environmental habitat of Acinetobacter, the distribution and frequency of *Acinetobacter* species in a variety of purchased and harvested fresh fruit and vegetables have been studied. Acinetobacter was isolated in 17% (30 of 177) samples of the produce. *A. baumannii* complex formed 56% of all isolates from cucumbers, peppers, mushrooms, lettuces, potatoes, corns, cauliflowers, radishes, mushrooms, melons, cabbages, apples, and beans. According to this study hospital food could be a natural habitat and a source for *A. baumannii* acquisition and subsequent colonization of the digestive tract of hospitalized patients (Berlau J., 1999).

A. baumannii has also been isolated from wounds of injured American and British soldiers from Afghanistan and Iraq. These strains were multidrug-resistant and mostly were part of polymicrobial infections (Paolino K., 2007). The sources for these infections were unknown, but it was suggested that prolonged environmental contamination of military field hospitals played a role as *Acinetobacter* species can survive in both moist and dry environments (Giamarellou H., 2008). Interestingly, in a study done in France, *A. baumannii* strains were isolated from body lice of homeless people. The researchers demonstrated that body lice were vectors of A*. baumannii.* This indicated that *A. baumannii* was epidemic in human body lice. *A. baumannii* association with body lice is likely due to undiagnosed transient *A. baumannii* bacteremia in people infested with body lice (La Scola B., 2004).

A review article by Villegas and Hartstein (Villegas M.V., 2003) provided examples of locations in the hospital environment where Acinetobacter has been isolated. Common sources for this organism included ventilator tubing, suction catheters, humidifiers, containers of distilled water, urine collection jugs, intravenous nutrition, multi-dose vials of medication, potable water, moist bedding articles, and inadequately sterilized reusable arterial transducers (Villegas M.V., 2003). In an outbreak of Acinetobacter infection in burns patients, wet mattresses served as environmental reservoirs of Acinetobacter (Sherertz R.J., 1985). Contaminated bedding materials may play an important role in the nosocomial spread of these organisms (Bergogne-Berezin E . 1996). Medical equipment can get contaminated through contact with both the patients and staff during handling. Therefore hospital staff may be responsible for contaminating equipment if they do not adhere to infection control measures. In respiratory ICUs, respiratory equipment can be a source of persistent outbreaks due to inadequate decontamination after use (Bergogne-Berezin E,. 1996).

Acinetobacter has an ability to persist in the hospital environment, thus are able to cause extended outbreaks (Bergogne-Berezin E., 1996). In one outbreak, the presence of airborne *Acinetobacter* species was demonstrated by settle plates (Allen K.D., 1987). The source of these organisms was probably the skin of infected or colonized patients, and/or contaminated fomites, e.g. bed linen and curtains. Airborne

Acinetobacter produces extensive environmental contamination, and was found to persist in the environment for up to 13 days after patient discharge (Allen K.D., 1987). Thus, there is a possible interchange between patients, hospital staff and inanimate items, allowing the survival of nosocomially important pathogens (Getchell-White S.I., 1989).

Acinetobacter differ from other gram negative bacteria in that they spread easily in the environment surrounding infected or colonized patients. In an in vitro study it was shown that the ability of *A. baumannii* strains to survive under dry conditions varied greatly (Wendt C., 1997). This ability correlated well with the source of the strain. Those strains which were isolated from dry sources tended to survive longer than the ones derived from wet sources (Wendt C., 1997).

5. Pathogenesis of *Acinetobacter baumannii* infections

Acinetobacters cause opportunistic infections because of limited number of virulence factors and are thus considered as low grade pathogens (Bergogne-Berezin E., 1996). Recently, there have been a number of case-reports of fulminating community – acquired pneumonia which indicated that these organisms may sometimes be highly pathogenic and cause invasive disease (Joly-Guillou, 2005). There are certain characteristics of this organism that can enhance its virulence. These include the presence of a capsular polysaccharide which makes the organism to be hydrophilic, the ability to adhere to human epithelial cells in the presence of fimbriae and/or capsular polysaccharides, the production of lipases which can damage tissue lipids and the presence of cell wall lipopolysaccharide and lipid A which are potentially toxic (Bergogne-Berezin E., 1996). The lipopolysaccharide causes resistance to complement in human serum and acts synergistically with capsular exopolysaccharide (Joly-Guillou, 2005). Little else is known about Acinetobacter's lipopolysaccharide endotoxigenic potential in humans. The capsule is a major virulence factor and is presumed to protect bacteria from host defenses (Joly-Guillou, 2005). Quorum – sensing as a widespread regulatory mechanism in gram negative bacteria has been found in clinical isolates of Acinetobacter. It might be a central mechanism for auto-induction of multiple virulence factors in Acinetobacter (Joly-Guillou, 2005).

Mixed infections involving Acinetobacter and other bacteria are more virulent than infections with *Acinetobacter* species alone (Bergogne-Berezin E., 1996). *Acinetobacter* species have the ability to obtain the necessary iron for growth in the human body. This is also an important virulence determinant (Bergogne-Berezin E . 1996).

6. Clinical manifestations of *Acinetobacter baumannii* infections

a. Nosocomial pneumonia

There is a persistent seasonal variation in the rate of Acinetobacter infection. This variation tends to increase in late summer for all major infection sites (McDonald L.C., 1999). Presently the most important role of Acinetobacter is as a cause of nosocomial pneumonia, mostly following the use of mechanical ventilation in ICU patients (Joly-Guillou, 2005; Bergogne-Berezin E., 1996). The role played by *Acinetobacter* species in ventilator associated pneumonia (VAP) appears to be increasing (Bergogne-Berezin E., 1996). An increase from 0.64% to 6.4% in the incidence of nosocomial pneumonia due to Acinetobacter between 1976–1990 has been reported in a surveillance program of the Nosocomial Infections Surveillance (NNIS) System in the USA which involved adult and pediatric patients (McDonald L.C., 1999).

Today, there are many major advances in the management of ventilated patients and there is routine use of effective procedures to disinfect respiratory equipment. These have not affected the increased incidence of VAP due to Acinetobacter (Bergogne-Berezin E., 1996). Although it is often very difficult to distinguish upper respiratory tract colonization from true pneumonia, ventilator-associated pneumonia due to *A. baumannii* does occur (Peleg A.Y., 2008). The acquisition of *A. baumannii* infection in the ICU is associated with a high APACHE II score, cardiovascular failure, respiratory failure, previous infection, previous antibiotic therapy, use of mechanical ventilation and the presence of a central venous or urinary catheter (Lortholary O., 1995).

The prognosis associated with nosocomial pneumonia is considerably worse than that due to other Gram-negative or Gram-positive bacteria, except for *Pseudomonas aeruginosa* (Bergogne-Berezin E., 1996)). Acinetobacter nosocomial pneumonia is a severe disease in ventilated patients. It is not easy to ascertain whether such critically ill patients would have survived if nosocomial pneumonia had not occurred (Bergogne-Berezin E., 1996). Fagon et al. looked at the extent to which nosocomial pneumonia increased mortality and hospital stay in ventilated patients by performing a matched retrospective cohort study in a Paris hospital (Fagon J., 1993). The authors diagnosed pneumonia by use of quantitative culture of samples from protected specimen brush and observation of intracellular organisms from bronchoalveolar lavage. They were able to match cases and controls for confounders like severity of underlying illness, age and reason for ventilation. VAP caused by *Pseudomonas* or *Acinetobacter* species was associated with considerable mortality in excess of that due to the underlying disease alone (Fagon J., 1993). The mortality attributed to Acinetobacter or Pseudomonas infection exceeded 40%, with a relative risk of death of 2.5. There was also a significantly prolonged hospital stay in the ICU by more than 10 days in patients diagnosed with pneumonia (Fagon J., 1993).

b. **Community –acquired pneumonia**

Most studies of community-acquired pneumonia due to *A. baumannii* (CAP-AB) originated from China, Taiwan, and Australia (Falagas M. E., 2007). The disease mostly occurs in patients with the following co-morbidities: chronic obstructive pulmonary disease (COPD), renal disease, diabetes mellitus, alcoholism, and heavy smoking. Community – acquired Acinetobacter infections are caused by isolates that are more susceptible than hospital-acquired strains (Falagas M. E., 2007). Clinically, patients with CAP-AB present with acute onset of dyspnea, cough and fever that tend to rapidly progress to respiratory failure and shock (Leung W., 2006). When compared to hospital-acquired pneumonia due to *A. baumannii* (HAP-AB), CAP-AB patients are likely to be smokers and have COPD. The clinical presentation tends to be more fulminating in CAP-AB and associated with bacteremia, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC) and early death. The mortality of patients with community acquired Acinetobacter pneumonia and /or bacteremia is considerable (Falagas M. E., 2007; Leung W., 2006) and can be as high as 64% (Anstey N. M., 1992). This high mortality rate can be explained by the large number of risk factors affecting patients with CAP-AB, the relatively higher average age, or inappropriate empiric treatment (Leung W., 2006).

c. Nosocomial bloodstream infections

The major and frequent manifestation of infection caused by *A. baumannii* is bacteremia, followed by respiratory tract and surgical wound infections (Cisneros J.M., 2002). During a nationwide, concurrent surveillance study done in the USA (1995 – 2002), to examine trends in the epidemiology and microbiology of nosocomial bloodstream infections, *A. baumannii* was the 10th most common etiologic agent (Wisplinghoff H., 2004). This organism was responsible for 1.3% of all monobacterial nosocomial bloodstream infections (0.6 bloodstream infection per 10 000 admissions) (Wisplinghoff H, 2004). The mean interval between admission and infection was 26 days for *Acinetobacter* species and most of the infections were in patients admitted in intensive care unit (Wisplinghoff H., 2004). *A. baumannii* bloodstream infection had a crude mortality rate of 34% - 43.4% in ICU, and 16.3% in the general wards. *Pseudomonas aeruginosa* (crude mortality - 38.7%) and Candida species (crude mortality - 39.2%) were the only organisms with crude mortality rates above *A. baumannii* (crude mortality 34-43.4%) in ICU patients (Wisplinghoff H., 2004).

In a single center study in Seville, Spain, there were 1.8 episodes of bacteremia due to *A. baumannii* per 1000 adults admitted to the hospital (Cisneros J.M., 1996). Of these patients, 25% had serious, debilitating chronic diseases (Cisneros J.M., 1996). Other risk factors for bacteremia included invasive procedures such as intravascular catheterization, urinary tract catheterization, mechanical ventilation, and prior surgery. Septic shock due to *A. baumannii* bacteremia can be as high as 25 – 30% (Cisneros J.M., 1996).

d. Traumatic battlefield and other wound infections

Injured and ischemic tissue in trauma patients facilitates colonization with *A. baumannii* (Oncul O., 2002). *A. baumannii* has been isolated from wounds of war casualties from Iraq and Afghanistan. In one study *A. calcoaceticus-baumannii* complex formed 32.5% of initial wound cultures. It did not appear to directly contribute to any substantial morbidity (viz. persistent nonunion or amputation), thus signifying that this organism is of low pathogenicity in wound infections (Johnson E. N., 2007). Gunshot wounds and external fixation tend to be associated with increased risk of Acinetobacter infection (Petersen K, 2007).

Most Acinetobacter infections in war casualties are caused by highly antibiotic-resistant strains. These infections occur in critically ill patients with severe traumatic injuries. These organisms are acquired through nosocomial transmission in field hospitals (Scott P., 2007). Murray et al. (Murray C.K., 2006) found that *Acinetobacter* species were not isolated from wounds immediately after or soon after injury from casualties who were treated at a US Military field hospital in Iraq. In a study by Petersen et al (Petersen K, 2007), which looked at trauma related infections in Iraqi war casualties, Acinetobacter followed by Pseudomonas species, and *Escherichia coli* were the most common wound isolates. Environmental contamination and transmission of organisms within healthcare facilities seem to play a significant role in acquiring Acinetobacter wound infection (Scott P., 2007).

The circumstances of combat are extremely challenging (Zapor M.J., 2005). Infection control measures such as cohorting, isolation and even proper hand washing techniques are very difficult, especially in mass casualty situations. This leads to ongoing colonization, and at the end, to wound infection (Zapor M.J., 2005). Complicated soft tissue and bone infection may follow. An increase in the rate of osteomyelitis caused by Acinetobacter was described in soldiers stationed in southwest Asia (Zapor M.J., 2005). Traumatic wounds related to natural disasters may also involve Acinetobacter. *A. baumannii* has been isolated from traumatic wounds sustained during an earthquake in Marmara, northwest of Turkey (Oncul O., 2002).

e. Urinary Tract Infections (UTI)

In many cases *A. baumannii* isolated from respiratory secretions and urinary tract specimens collected from hospitalized patients signify colonization rather than infection (Fournier P.E., 2006). Most infections due to this organism are from organ systems with a high fluid content, e.g. respiratory tract, peritoneal fluid and the urinary tract. These infections are associated with indwelling devices (Fournier P.E., 2006). *A. baumannii* is not usually implicated in uncomplicated UTI in healthy outpatients (Peleg A.Y., 2008). In a

study looking at trends in Gram-negative pathogen distribution in ICUs (1986 – 2003) (Gaynes R., 2005), *Acinetobacter* isolates comprised 1.6% of pathogens associated with UTIs in the ICU. Investigators in Spanish hospitals looked at 206 patients colonized or infected with *A. baumannii.* UTIs constituted 23%, second only to respiratory tract infections (39%) (Rodriguez-Baño J., 2004). *A. baumannii* UTIs tend to show seasonal variability (Fournier P.E., 2006). The reason for the seasonal variability is unknown, but was observed also in a study done by McDonald (McDonald L.C., 1999).

f. Meningitis

There is a steady increase in cases of nosocomial, post-neurological surgery *A. baumannii* meningitis (Peleg A.Y., 2008); (Kim M., 2009). Community-acquired meningitis due to *A. baumannii* on the other hand is very rare (Kim M., 2009). Patients with post-neurological surgery central nervous system (CNS) infection tend to be young, acquire the infection in hospital, commonly have no severe underlying diseases, and have a slow clinical course (Lu C., 1999). Mortality due to Acinetobacter meningitis has been cited to be 23% (Siegman-Igra Y., 1993). In this case series, patients were predominantly adult males and the most significant risk factor was the presence of a continuous connection between the ventricles and the external environment. The median time to develop Acinetobacter meningitis following a neurosurgical procedure was 12 days (range 1 – 40 days) (Siegman-Igra Y., 1993). These types of infections can be prevented by maintaining a closed drainage system together with timely removal of the ventricular catheters. Furthermore, the selective pressure of the antibiotics used in the neurosurgical ICU favors the growth of multi-drug resistant Acinetobacter.

Pseudomeningitis may occur, where the CSF culture is positive for Acinetobacter in the absence of clinical and laboratory features of meningitis (Kim M., 2009). Contamination of the CSF may occur during specimen collection as this organism is increasingly prevalent in the hospital environment and may colonize the skin. The specimen may also become contaminated due to contaminated specimen tubes and in the laboratory, contaminated pipettes and media. In a study by Chen (Chen H., 2005), lumbar puncture derived *Acinetobacter* isolates were more clinically insignificant than those obtained from previously placed ventricular drains. Differentiating between clinically significant and insignificant isolates enables clinicians to avoid unnecessary antibiotic treatments and helps with timely and accurate treatment of infected patients (Chen H., 2005). Most significant cases are associated with neurosurgical procedures (Chen H., 2005), (Kim M., 2009). With regard to clinical signs and symptoms in Acinetobacter meningitis, fever was the most common presentation in a study by Siegman-Igra (1993). Neck stiffness and other symptoms suggestive of meningitis were frequently absent. In the study by Chen *et al* (Chen H., 2005), the absence of fever, meningeal signs and seizures correlated with the isolation of insignificant CSF Acinetobacter isolates. Most cases of Acinetobacter meningitis (20 – 50%) were found to be polymicrobial (Siegman-Igra Y., 1993 ; Chen H., 2005).

g. Other clinical presentations

There are a limited number of case reports of Acinetobacter endocarditis in the literature. The precise species identification remains an issue in these case reports (Peleg A.Y., 2008). Cases reported in the literature involved both native and prosthetic valves (Valero C., 1999; Starakis I., 2006; Olut A.I., 2005; Gradon J.D., 1992). Risk factors for gram negative infective endocarditis are diabetes mellitus type I, endoscopy of the gastrointestinal or genital tract, patients with congenital heart diseases, dental surgery, and patients with right-sided endocarditis (Krcmery V., 2010). Any breach of the integument can lead to Acinetobacter seeding of a heart valve (Gradon J.D., 1992). A maculopapular rash involving the palms and soles has been reported in cases of Acinetobacter endocarditis. Splenomegaly seems to be a common, but not a dominant feature of Acinetobacter endocarditis (Gradon J.D., 1992). The prognosis of Acinetobacter prosthetic valve endocarditis (PVE) has been more favorable than PVE due to other pathogens. This might be due to the low virulence of *Acinetobacter* species (Olut A.I., 2005).

Acinetobacter can cause ulcerative keratitis and corneal ulcers. These infections may be related to the use of contact lenses or follow eye surgery (Kau H., 2002), (Corrigan K.M., 2001.),. There is an association between high levels of contamination of contact lenses with Acinetobacter and occurrence of adverse responses (Corrigan K.M., 2001.). Acinetobacter are not regarded as normal flora, but there is a small proportion of the general population that carries low numbers of this organism on their skin. Acinetobacter causing eye infections may have been transferred to the eye by the hands or from the hands to the contact lens (Corrigan K.M., 2001.).

There was a single case report about a Shiga toxin-producing *A. haemolyticus* strain from Uruguay (Grotiuz G., 2006). This involved a 3-month old baby who presented with bloody diarrhea of 12 hours' duration without pyrexia or other previous illnesses. Fecal samples were inoculated onto MacConkey sorbitol plates. All sorbitol negative colonies were recovered after 48h of incubation. These were then analyzed by PCR to detect the presence of shiga-toxin 1/ shiga-toxin 2(Stx 1/Stx 2) – encoding organisms. The presence of stx_2 –related sequence was then confirmed by PCR. A specially designed PCR suggested that the Shiga toxin genes of *A. haemolyticus* were carried in an infective bacteriophage. The usual enteropathogenic pathogens were not detected from the patient's stool samples (Grotiuz G., 2006).

III. Antibiotic treatment of *A. baumannii* infections

Peleg et al. (2008) described multidrug resistance to *A. baumannii* as resistance to more than two of the following five drug classes: ceftazidime or cefepime (antipseudomonal cephalosporins); imipenem or meropenem (carbapenems); ampicillin-sulbactam, ciprofloxacin or levofloxacin (fluoroquinolones), and gentamicin, tobramycin, and amikacin (aminoglycosides). The number of multidrug-resistant *A. baumannii* has been increasing worldwide in the past few years (Li J., 2006). Therefore the selection of empirical antibiotic treatment is very challenging (Towner, 2009). This should rely on institutional-level data relating to the phenotypes and genotypes whenever possible. Reports in the literature that provide knowledge about the best therapeutic approaches with regards to Acinetobacter include in- vitro susceptibility data, small case series and retrospective analysis of observational studies (Towner, 2009).

1. Polymyxins

Due to limited treatment options, physicians have returned to the use of polymyxin B or polymyxin E (colistin) for the most drug-resistant Acinetobacter infections (Maragakis L.L., 2008). These antibiotics are cationic polypeptides that interact with the lipopolysaccharide layer of Gram-negative bacteria and are bactericidal against *A. baumannii* (Towner, 2009). Colistin exists commercially in two forms: colistin sulfate for oral and topical use, and colistimethate sodium for parenteral use. Both these forms can be delivered by inhalation or nebulization (Towner, 2009). Nebulized forms are used in patients with nosocomial pneumonia.

Colistin is useful for treating infections due to carbapenem-resistant isolates (Towner, 2009). A favorable treatment outcome with colistin in up to 76.9% of cases has been reported for all nosocomial infections due to multi-resistant *P aeruginosa* or *A. baumannii* (Kallel H., 2006)-. In that study by Kallel (2006), favorable clinical response was seen in 73.8% of cases treated for only VAP using colistin. In another study (Garnacho-Montero J., 2008) there was an equivalent clinical response in patients with ventilator associated pneumonia (VAP) treated with colistin or with imipenem according to the antibiotic susceptibility results of *A. baumannii.* In a Brazilian study (Levin A.S., 2003), a good outcome was reported in 58% of patients when colistin was used to treat nosocomial infections due to *P. aeruginosa* and *A. baumannii* in non–cystic fibrosis patients. Colistin was also found to be effective in a neutropenic rat thigh infection model against *A. baumannii* (Pantopoulou A., 2007).

There are a number of studies that looked at the in vitro interaction of colistin with rifampicin against nosocomial strains of *A. baumannii* susceptible only to colistin (Giamarellos-Bourboulis E.J., 2001; Yoon J., 2004; Pantopoulou A., 2007; Song J.Y., 2008). In one of these studies more than 50% of isolates showed

synergy depending on the exposure time with these two antibiotics (Giamarellos-Bourboulis E.J., 2001). The efficacies of colistin + rifampicin and imipenem + rifampicin combinations were also compared in a neutropenic mouse pneumonia model (Song J. Y., 2009). In this study colistin showed good in vitro activity against carbapenem resistant *A. baumannii* isolates and it was bactericidal at concentrations 4x MIC and 8x MIC. The combinations of imipenem + rifampicin and colistin + rifampicin were found to be synergistic and bactericidal at concentrations of 1x MIC. It was recommended that rifampicin be added to either imipenem or colistin for the treatment of carbapenem – resistant *A. baumannii* infections (Song J. Y., 2009).

The existing knowledge about the pharmacokinetics and pharmacodynamics of colistin is limited. The current dosing regimens are based on experience obtained as far back as 30 years ago (Li J., 2006). It is very important to administer colistin in dosages that provide maximal activity with minimal potential for the development of resistance (Li J., 2006). Fortunately, resistance to colistin has remained relatively low. Recently, heteroresistance and resistance to colistin has been reported in Australia and Korea, respectively (Li J., 2006; Ko K.S., 2007)). In the Australian study, resistant subpopulations of *A. baumannii* (Li J., 2006) were assumed to be responsible for the significant regrowth in the time–kill studies. The heteroresistance observed was unlikely to be related to previous exposure to colistin as this drug was never used before in the patients from whom the isolates were obtained. These subpopulations cannot be detected by the most commonly used commercial automated systems and the disk diffusion susceptibility test.

Heteroresistance denotes the existence of a subpopulation, within a culture population of a susceptible isolate that is able to grow in a substantially high colistin concentration (Li J., 2006). From the study by Li et al (2006), it was demonstrated that hetero-colistin-resistant *A. baumannii* cannot be differentiated from colistin-susceptible *A. baumannii* by broth microdilution MIC measurement, commercial automated systems and disk diffusion. A population analysis profile method was used to detect the hetero-colistin-resistant subpopulation of *A. baumannii.* The clinical significance of a heteroresistant subpopulation is unclear. It could relate to the in vivo emergence of colistin resistance after the use of the drug (Matthaiou D.K., 2008). Monotherapy with colistin for the treatment of infections due to hetero-colistin-resistant *A. baumannii* may be problematic, hence this drug should be used judiciously and appropriately (Li J., 2006).

Colistin and polymyxin B resistance is rare world-wide. High colistin resistance rates in Acinetobacter species were reported in a Korean study (Ko K.S., 2007). Another Korean study (Park Y.K., 2009), suggested that most colistin-resistant *Acinetobacter* species isolates emerged independently. There was no clonal spreading of an individual bacterial clone. The mechanism of colistin resistance in *Acinetobacter* species has not been fully revealed (Park Y.K., 2009). Gram-negative organisms become resistant to polymyxins through adaptive mechanisms after exposure to these agents. Resistance to colistin can also emerge through mutational mechanisms (Matthaiou D.K., 2008). The former mechanism of resistance is unstable and regresses after the withdrawal of the antibiotics. The latter mechanism, which involves

mutational mechanisms, is stable and inheritable (Matthaiou D.K., 2008). In the study by Matthaiou et al (Matthaiou D.K., 2008), the use of colistin was found to be an independent and a strong factor associated with the isolation of colistin-resistant organisms. Other significant factors included the duration of colistin administration, inappropriate colistin dosing and duration of ICU stay (Matthaiou D.K., 2008).

2. Carbapenems

The carbapenems (e.g. imipenem and meropenem) have been used as the mainstay treatment for Acinetobacter infection up until the past few years (Towner, 2009). According to Jones et al. (Jones R.N., 2006), imipenem is the more potent agent, compared to meropenem for the treatment of multiresistant Acinetobacter infection. However, in Greece it was observed that the discordance between imipenem and meropenem activity favors meropenem among *A. baumannii* isolates (Ikonomidis A., 2006). This is in contrast to the surveillance results of North America and Europe which established that imipenem is more potent than meropenem (Rhomberg P.R., 2003 ; Jones R.N., 2006). Overexpression of efflux pumps affects meropenem to a greater extent. Resistance to imipenem in *A. baumannii* is due to the presence of carbapenemases, such as OXA–58 and VIM–1, which hydrolyses imipenem more efficiently than meropenem. These carbapenemases are more prevalent in Greece (Ikonomidis A., 2006). Therefore, susceptibility to imipenem does not predict susceptibility to meropenem or vice versa (Maragakis L.L., 2008).

3. Sulbactam

Sulbactam is a β-lactamase inhibitor with an intrinsic activity against many *Acinetobacter* strains. This intrinsic activity may be due to the ability of sulbactam to bind with penicillin-binding proteins (PBP) of imipenem-resistant and –susceptible isolates. Sulbactam as monotherapy is not advised for severe Acinetobacter infections. Commercially, sulbactam is available in combination with a β-lactam agent (e.g. ampicillin). This combination does not appear to contribute to activity or synergy (Maragakis L.L., 2008).

There are few case reports which describe the success of sulbactam, alone or in combination with ampicillin for the treatment of Acinetobacter (Levin A.S., 2003; Jiménez-Mejías M.E., 1997; Smolyakov R., 2003). In one study (Jiménez-Mejías M.E., 1997) the authors reported on clinical features and the outcomes of eight cases of nosocomial *A. baumannii* meningitis treated with ampicillin-sulbactam. The outcome was good in six of the eight cases. A Brazilian study looked at the clinical efficacy of the ampicillin-sulbactam combination for the treatment of *A. baumannii*. Improvement or cure rate was 67.5%

in that retrospective study where most patients had severe infections (Levin A.S., 2003). Ampicillin – sulbactam significantly decreased the risk of death in one study (p= 0.02) (Smolyakov R., 2003). In a study done in France (Wolff M., 1999)) involving a mouse pneumonia model caused by two different isolates of *A. baumannii*, sulbactam with the following antibiotics: imipenem; ticarcillin; ticarcillin-clavulanic acid; and rifampicin were tested in triple combinations: ticarcillin-sulbactam-clavulanic acid; β-lactams- sulbactam - rifampicin, which resulted in enhanced survival. The results from that study suggested that the use of non - classical combinations of β-lactams, β-lactamase inhibitors, and rifampicin should be considered during the treatment of nosocomial pneumonia due to *A. baumannii* (Wolff M., 1999).

Rifampicin monotherapy leads to rapid development of resistance in vitro and in vivo. In an experimental pneumonia murine model, the development of rifampicin resistance was prevented by the use of rifampicin in combination with imipenem or sulbactam (Pachon-Ibanez M.E., 2006). The deduction from the reported data is that more experience with the application of sulbactam in the treatment of Acinetobacter infections is needed either as monotherapy or combination therapy (Giamarellou H., 2008).

4. Tigecycline

Tigecycline, a new class of tetracycline – related antibiotics, the glycylcyclines, was approved by the FDA in June 2005 (Towner, 2009; Giamarellou H., 2008; Karageorgopoulos D. E., 2008). This antibiotic is able to evade the major mechanisms of resistance in tetracyclines, viz. the tet (A–E) and tet (K) efflux pumps and the tet (M) and tet (O) determinants that provide ribosomal protection (Peleg A.Y., 2007) 2). In preliminary studies, tigecycline was found to have activity against several Gram-positive and Gram-negative bacteria, including Acinetobacter (Giamarellou H., 2008)). Tigecycline antibiotic has a large volume of distribution, thus is able to achieve high levels in many tissue sites including the lungs. Further advantages are that there is no need for dosage adjustment due to age, severe renal impairment or hemodialysis (Giamarellou H., 2008). The FDA has approved the antibiotic only for complicated intra-abdominal and complicated skin infections and community – acquired pneumonia (Karageorgopoulos D. E., 2008).

Presently there is limited clinical experience with tigecycline (Towner, 2009 ; Giamarellou H., 2008)). There are few clinical reports about the use of tigecycline in patients infected with *A. baumannii* (Towner, 2009). One such report looked at the use of tigecycline in 34 patients with infections involving multidrug-resistant *A. baumannii* (Gordon N.C., 2009). Sixty eight percent of patients showed a positive clinical outcome. A poor correlation between microbiological clearance and clinical outcome was noted, mostly in patients with

respiratory tract infections. This was indicated by cultures which remained positive whilst the patient responded clinically.

There is controversy surrounding the use of tigecycline for bloodstream infections (Gordon N.C., 2009); (Towner, 2009)). This is due to the suboptimal concentrations of tigecycline in the blood (Towner, 2009) at the current recommended dose (Gordon N.C., 2009). The use of tigecycline for *A. baumannii* bacteremia is not recommended if another alternative is available (Peleg A.Y., 2007). Tigecycline is bacteriostatic against *A. baumannii* (Peleg A.Y., 2007). The development of bloodstream infection in two patients receiving therapeutic doses of tigecycline has been described (Peleg A.Y., 2007). These patients were receiving tigecycline for other indications, when they were diagnosed with *A. baumannii* bacteremia at Pittsburgh Medical Center, USA. One patient, a 76 year old woman, was on tigecycline after culturing vancomycin-resistant *Enterococcus faecium*. *A. baumannii* was cultured nine days later, from two blood cultures and a tracheal aspirate. The second patient was a 60 year old man who had a ventricular-assisted device inserted for ischemic cardiomyopathy. Post-operative wound sepsis developed 14 months later and cultures grew *A. baumannii* and *Enterobacter cloacae*. The latter organism was regarded as the significant one, and ertapenem was started. The patient did not respond clinically and intravenous tigecycline was commenced. Wound purulence decreased, showing clinical response. *A. baumannii* was subsequently grown 16 days later from two blood cultures following a new onset of fever. No susceptibilities were available for tigecycline. The patient did well on meropenem and amikacin, for which the isolate was susceptible. This report raised the question about the use of tigecycline to treat *A. baumannii* bacteremia as both patients developed *A. baumannii* infections whilst on tigecycline. Multidrug efflux pumps may be responsible for the tigecycline non-susceptibility in Acinetobacter (Peleg A.Y., 2007). Over 50% of patients treated for *A. baumannii* bacteremia had a positive outcome in one study (Gordon N.C., 2009). The explanation was the eradication of the underlying source of infection. Also, there could have been synergy between tigecycline and other antibiotics which were used (Gordon N.C., 2009).

A group in Italy looked at the in vitro activity of tigecycline in combination with various antibiotics against multidrug - resistant *A. baumannii* (Principe L., 2009). They demonstrated the in vitro synergy of tigecycline in combination with colistin, levofloxacin, amikacin and imipenem. This synergy was only observed among tigecycline non-susceptible strains (Principe L., 2009). According to the authors, more studies are needed to clarify the molecular mechanisms involved in synergy between tigecycline and other antibiotics (Principe L., 2009).

High tigecycline resistance in multidrug–resistant multiple clones of *A. baumannii* has been reported when E-test and disk diffusion methods were used (Navon-Venezia S., 2007). In that study, 60% of the isolates were resistant to tigecycline, 12% were intermediate and 22% were susceptible (Navon-Venezia S., 2007). This high tigecycline resistance could be due to the methods used, disk diffusion method and E-test (Thamlikitkul V., 2007)). In a study done in Thailand, there was a discrepancy in susceptibility results of

tigecycline against *Acinetobacter* species when different methods were used. The E- test method tends to give 4-fold higher MICs than those determined by the broth microdilution method. According to the authors, the E-test method might not be accurate in testing of tigecycline against *Acinetobacter* spp. (Thamlikitkul V., 2007).

IV. Combination therapy

Antibiotic combinations are used mostly as empirical therapy in critically ill patients with possible polymicrobial infections. One rationale for the use of combination therapy is to achieve synergy which increases the activity of either antibiotic. Synergy implies that significantly greater activity is provided by the two antibiotics combined than that provided by the sum of each antibiotic alone. Another rationale is to administer lower doses of either antibiotic to decrease their toxicity. Combination therapy has also been used to prevent the development of resistance to either antibiotic.

There are a number of ways that antibiotics may interact (Moellering, 1979). Some of the mechanisms involved in synergistic effects include sequential blockade of a given metabolic pathway (e.g. in the use of trimethoprim and sulfonamides); one drug causing changes in the bacterial surface, allowing better penetration of the second prescribed drug (e.g. in enterococci: penicillin and aminoglycoside combinations; vancomycin with an aminoglycoside) and inhibition of an enzyme responsible for antibiotic inactivation (e.g. clavulanic acid and amoxicillin combination).

Antibiotic combinations which have been found to be synergistic in vitro have been used clinically in the treatment of patients with neutropenic sepsis and in the treatment of enterococcal endocarditis (Eliopoulos G.M., 1982). Combination therapy is also used in the empirical treatment of patients with sepsis to provide initial broad antibiotic cover against the most common Gram positive and Gram negative organisms and to treat polymicrobial infections e.g. brain abscesses, intra-abdominal, pelvic, and necrotizing lung infections (Klastersky J., 1982). Another clinical rationale for the use of antibiotic combinations is to prevent the development of resistance e.g. in tuberculosis treatment. Combining antibiotics prevents the emergence of resistant strains, which might have occurred rapidly if a single antibiotic was used.

The following combinations have been shown to provide enhanced activity against strains of *A. baumannii*: colistin plus rifampicin; polymyxin B plus rifampicin plus imipenem; rifampicin plus imipenem, tobramycin or colistin; rifampicin plus sulbactam/ampicillin, colistin plus minocycline; imipenem plus sulbactam, colistin plus tigecycline; (Yoon J., 2004; Montero A.; 2004; Tripodi M., 2007; Song J.Y., 2008) In most of these cases, the mechanism of positive interaction is unknown. In one study, it was suggested that the probable role of polymixin B was its rapid permeabilization of the outer membrane, allowing enhanced penetration

and activity of imipenem and rifampicin (Yoon J., 2004). Data regarding the best combinations for synergy, as mentioned above, is mostly derived from in vitro and in vivo animal studies (Karageorgopoulos D. E., 2008). The good results obtained from these studies do not necessarily correlate with clinical findings (Karageorgopoulos D. E., 2008). Clinical trials are too few to recommend the use of specific combinations for the treatment of multidrug-resistant *A. baumannii* (Towner, 2009; Karageorgopoulos D. E., 2008).

Timurkaynak et al (Timurkaynak F., 2006) used the checkerboard method to determine whether combinations of colistin, rifampicin, meropenem, azithromycin and doxycycline act synergistically against multidrug-resistant strains of *Pseudomonas aeruginosa* and *A. baumannii*. Five strains of *A. baumannii* were selected based on differences in colistin MICs. The combination of colistin and rifampicin was fully synergistic against four *A. baumannii* strains. When time-kill studies were used by Giamarellos-Bourboulis et al to assess the interaction of colistin and rifampicin on multidrug-resistant *A. baumannii*, the activity of colistin was increased in the presence of rifampicin (Giamarellos-Bourboulis E.J., 2001).

Song et al (Song J.Y., 2008) retrospectively evaluated the safety and effectiveness of a combination of colistin and rifampicin in 10 patients with ventilator-associated pneumonia caused by carbapenem-resistant (only susceptible to colistin) *A. baumannii*. The mean duration of colistin/rifampicin therapy was 8.1 ± 1.8 days. With regard to clinical outcome, 70% (7 patients) of the patients benefitted from the combination of colistin + rifampicin. Six patients showed microbiological eradication of follow-up cultures taken after seven days of colistin/rifampicin. When Saballs et al (Saballs M., 2006) treated 10 patients with different infections caused by carbapenem-resistant *A. baumannii* with 6 – 21 days of imipenem/rifampicin combination, seven patients were clinically cured. The cure rates between the study by Song et al and Saballs et al were similar, but high-level rifampicin resistance (MIC = 256 mg/l) developed in seven patients during treatment in the latter study. The MICs of rifampicin were not changed in the former study. The differences could be due to differences in antibiotic combinations or infectious diseases (Song J.Y., 2008).

In a mouse pneumonia model by Montero et al, the combination of imipenem + tobramycin was the most active combination against moderately carbapenem-resistant (MIC 8 mg/l) A*. baumannii.* In infections caused by highly carbapenem-resistant (MIC 512 mg/l) strain rifampicin + imipenem and rifampicin + tobramycin were the most active combinations. According to the investigators, imipenem can still be used against *A. baumannii* with moderate levels of imipenem resistance, preferably in combination with aminoglycosides. For *A. baumannii* strains with high resistance to imipenem, a combination of rifampicin with imipenem, tobramycin or colistin may be useful, only if resistance to rifampicin is moderate (Montero A., 2004).

Yoon et al (Yoon J., 2004) looked at in vitro double and triple synergistic activities of polymyxin B, imipenem, and rifampicin against multidrug-resistant *A. baumannii* by checkerboard method. The combination of polymyxin B + imipenem, polymyxin B + rifampicin, and polymyxin B, imipenem, and rifampicin demonstrated synergy. Polymyxin B + imipenem and polymyxin B + rifampicin were bactericidal for seven of eight isolates within 24 hour.

In the in vitro comparative study by Tripodi et al (Tripodi M., 2007), the activity of colistin, rifampicin, imipenem and sulbactam/ampicillin alone and in combination against multidrug-resistant A. baumannii was evaluated by time-kill studies. Bactericidal effect was shown for colistin but not for imipenem, rifampicin or sulbactam/ampicillin used as single agents. The combination of rifampicin + imipenem or sulbactam/ampicillin was synergistic for all isolates. colistin + rifampicin were only synergistic in the study for isolates showing higher minimum inhibitory concentrations for rifampicin.

V. In vitro techniques for measuring antibiotic synergism

Synergistic antibiotics are often used to treat serious infections (Berenbaum M.C., 1978) particularly in immunosuppressed or granulocytopenic patients (Norden C.W., 1979). Two traditional methods that have been used for synergy testing are the checkerboard and time kill (killing curve) methods. The time-kill method measures the rate of bacterial killing by antibiotics (Norden C.W., 1979).The checkerboard method examines the ability of antibiotic combinations to inhibit growth of an organism. The checkerboard method is one of the most frequently used techniques to assess drug interactions. The term checkerboard arises from the pattern that is generated by the multiple dilutions of the two antibiotics being tested.

Historically, the checkerboard method was performed in an array of tubes using broth macrodilution. This was cumbersome, time consuming and expensive. Presently, testing is done using microtiter trays. The results are calculated mathematically and expressed in terms of a fractional inhibitory concentration (FIC) index. This is equal to the sum of the FICs for each drug. The FIC for a drug is defined as the minimal inhibitory concentration (MIC) of the drug in combination divided by the MIC of the drug used alone. If the FIC index is ≤ 0.5, the antibiotic combination is interpreted as being synergistic; FIC index >0.5 and ≤1.0 as additive (Timurkaynak F., 2006), between 1 and 4 as indifferent and > 4 as antagonistic (Schwalbe R., 2007).

The Epsilometer (E-test) is a relatively new agar diffusion method for antimicrobial susceptibility testing (Arroyo L. A., 2009; Bonapace C.R., 2000). It is a preformed and predefined gradient of antibiotic concentrations immobilized in a dry format onto the surface of a plastic strip. The continuous concentration gradient is calibrated across a corresponding MIC range covering 15 twofold dilutions. When applied to the

surface of an inoculated agar plate, the antibiotic on the E-test strip is instantaneously transferred to the agar in the form of a stable and continuous gradient directly beneath and in the immediate area of the strip. A number of investigations have documented E-test performance to be equivalent to reference MIC methods with a high degree of reproducibility in most laboratory settings (Schwalbe R., 2007).

The E-test overlay method is a modification of this method to determine synergy between different antibiotics. The E-test strip of the first antibiotic to be tested is placed on an agar plate inoculated with the test organism. The plate is then incubated at room temperature for 1 hour. The E-test strip is then lifted off the agar surface and replaced with the strip of the second antibiotic. This is placed exactly on top of the gradient of the first antibiotic. The plate is incubated for 24 hours at 37 °C. The fractional inhibitory concentrations (FIC) are calculated to evaluate the effect of the antibiotic combination. Interpretation of results is as for the checkerboard method. The E-test overlay method is easy to perform, flexible and time efficient (Manno G., 2003; Haddad F.A., 2005).

Haddad et al (Haddad F.A., 2005) evaluated antibiotic combinations against multidrug-resistant A. baumannii using the E-test overlay method. This method was found to be less labor-intensive. It also has a high correlation with the checkerboard method. Manno et al (Manno G., 2003) assessed synergy of various combinations of antibiotics against 131 isolates of *Burkholderia cepacia* complex using the E-test overlay method. The agreement between the E-test and checkerboard methods was 90%. In a Turkish study by Kocazeybek et al (Kocazeybek B.S., 2002), various antibiotic combinations were investigated using the E-test overlay method in multiresistant *P. aeruginosa*. The agreement rate between the E-test and checkerboard method was 100%. The E-test method was evaluated as a good alternative for combination investigation due to its ease of application and evaluation and also for its good agreement in that study with the checkerboard method (Kocazeybek B.S., 2002).

White et al (White R.L., 1996) and Bonapace et al (Bonapace C.R., 2000) compared antibiotic synergy by E-test, time kill, and checkerboard methods. There was poor agreement between the E-test and other methods in these studies. This can be attributed to improper methodology inconsistent with the manufacturer's recommendations on use of the E-test method for synergy testing (Haddad F.A., 2005). In these two studies the E-test strips were placed on the Muller-Hinton agar in a cross formation, with a 90^0 angle at the intersection between the scales at their respective MICs for the organism.

The E-test overlay method has also been compared to checkerboard and time-kill methods for synergy testing of antifungal agents against *Candida* species (Lewis R.E., 2002). The agreement between the E-test and checkerboard methods was poor. Overall, agreement between E-test and time-kill studies was good. Of the three methods, E-test was the simplest to use and yielded reproducible results for testing antifungal combinations (Lewis R.E., 2002).

VI. Aim of the study

The aim of this study is to assess the in vitro activity of different combinations of colistin, rifampicin, imipenem and tobramycin against selected clinical strains of *A. baumannii* from patients in a tertiary hospital using, the checkerboard and E-test synergy methods. .

Objectives

- To compare E-test and broth dilution MIC testing methods
- To determine if the following antibiotic combinations exhibit in vitro synergy against *Acinetobacter baumannii* isolates with different antibiotic susceptibility profiles, including multi-drug resistant (MDR) *A. baumannii:*

    Colistin (col) + rifampicin (rif)

    colistin + imipenem (imi)

    colistin + tobramycin (tob)

    rifampicin + tobramycin

    rifampicin + imipenem

    imipenem + tobramycin

- To evaluate the E test synergy method using the checkerboard method as a reference standard.

VII. Material and Methods

Study design:

This is a descriptive study that was conducted at the NHLS Medical Microbiology laboratory which is situated at Tygerberg Hospital, Cape Town. The laboratory is a P2 facility with adequate infrastructure. Safety precautions were adhered to according to the laboratory safety manual. Ethical approval was obtained from the Committee of Human Research of Stellenbosch University (ethics number: N08/02/037).

Bacterial strains:

Acinetobacter baumannii isolates were collected from all routine clinical specimens over a six month period from January 2008 – June 2008.

Personal information of patients from which isolates were obtained remained anonymous by coding isolate samples to ensure confidentiality. A total of 50 isolates, identified by the automated Vitek 2 analyzer (Biomerieux), were collected. Strains were selected randomly, based on specific antibiotic sensitivity patterns representing strains which were very resistant (only colistin and tobramycin sensitive) and those that were fully sensitive to all tested antibiotics. Each isolate was stored in cryobroth at -80°C for subsequent synergy testing. Stored isolates were subcultured twice on two successive days on blood agar prior to in vitro testing. Only one isolate from each patient per admission period was included in this study.

Selection of isolates for further MIC determinations and synergy testing:

All isolates were identified by the automated Vitek 2 system. Susceptibility testing was done by disk diffusion method for imipenem and tobramycin due to limitations of the Vitek susceptibility method for Acinetobacter testing. Colistin susceptibility was determined by the E-test method as only identification of the selected isolates was done by Vitek 2.system at that time. Rifampicin does not form part of the panel for routine testing of *A. baumannii*. The isolates were then grouped according to their antibiotic profiles. Ten isolates were purposely selected based on the antibiogram patterns of tobramycin, colistin and imipenem. Due to time constraints and the labor intensiveness of the procedure, only selected isolates were tested with the checkerboard method. Three isolates were sensitive to tobramycin and resistant to imipenem (isolates 1, 23, and 25); three were sensitive to imipenem and resistant to tobramycin (isolates 14, 16, and 17); two isolates were both resistant to imipenem and tobramycin (isolates 3 and 6) and two isolates (9 and 30) were sensitive to both imipenem and tobramycin. All ten isolates were sensitive to colistin.

Determination of minimum inhibitory concentrations (MICs):

MICs of the ten selected isolates were determined in triplicate by the E-test method according to the manufacturer's instructions (AB Biodisk, Solna, Sweden) and the broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI Document M7-A7, 2006). The concentrations of the E-test strips (AB Biodisk, Solna, Sweden) were 0.016 – 256 µg/ml for colistin, tobramycin 0.064 – 1024 µg/ml, and for rifampicin and imipenem was 0.002 – 32 µg/ml. The final concentrations tested by broth microdilution were 0.064 – 32 µg/ml, for colistin (Sigma-Aldrich, USA), rifampicin (Sigma-Aldrich, USA) and imipenem (Abtek biological ltd, Liverpool, UK), and 0.125 – 64 µg/ml for tobramycin (Sigma-Aldrich, USA). The lowest concentration of antibiotic that showed no growth was recorded as the MIC. The susceptibility of colistin, imipenem, and tobramycin were interpreted according to CLSI guidelines (CLSI Document M100-S20, 2010). The susceptibility breakpoint for rifampicin was established as ≤ 2 µg/ml, based on a previous study by Hogg et al (1998).

Comparison of the E-test and broth microdilution susceptibility methods

The MIC results were interpreted as susceptible, intermediately-resistant or resistant according to the CLSI criteria. Discrepancies in the results were characterized as very major, major or minor errors

very major error	broth dilution result resistant and E-test or disc diffusion result susceptible (false-susceptible)
major error	broth dilution result susceptible and E-test or disc diffusion result resistant (false-resistant)
minor error	broth dilution result resistant or susceptible and E-test or disc diffusion result intermediately resistant or vice versa

Synergy testing:

The following antibiotic combinations were tested with the E-test (all ten isolates in triplicate) and checkerboard synergy methods (three isolates tested in duplicate): colistin + rifampicin; colistin + imipenem; colistin + tobramycin; rifampicin + tobramycin; rifampicin + imipenem and imipenem + tobramycin.

The antibiotic combinations were chosen based on published data and the easy availability of these antibiotics in our hospital. The median MIC value obtained for each antibiotic with the broth microdilution method was used to determine the MIC dilutions in the checkerboard method.

E-test synergy method:

Mueller-Hinton agar plates were inoculated with swabs saturated with suspensions of the test organism equivalent to a 0.5 McFarland standard. The E-test strip of antibiotic A was placed on the inoculated agar surface for one hour at room temperature. After one hour, the strip was removed and the E-test strip of antibiotic B was placed on top of the imprint of the first strip which was then incubated at 35°C for 24h as described previously (Manno G., 2003). E-test strips were handled by using an E-test vacuum pen (Nema C88 – AB BioMerieux-Sweden). The MIC was interpreted as the value at which the inhibition zone intersected the scale on the E-test strip.

Calculation of fractional inhibitory concentration index (FIC Index):

FIC (FIC index) = FIC drug A + FIC drug B = $MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$

FIC of antibiotic A = MIC of antibiotic A in combination with antibiotic B /MIC of antibiotic A alone

FIC of antibiotic B = MIC of antibiotic B in combination with antibiotic A /MIC of antibiotic B alone

The results were interpreted as follows:
FIC index ≤0.5, synergistic;
FIC index >0.5 and ≤1.0, additive
FIC index >1.0 and ≤4, indifferent

FIC index > 4, antagonistic

Checkerboard synergy method:

The checkerboard method was performed according to the method described by Schwalbe (2007).

Preparation of checkerboard microdilution panels

The number of combination panels that had to be prepared was determined: one for each isolate, and one panel for the dilution of the second antibiotic.

Antibiotic powders used were colistin sulfate salt (Sigma-Aldrich, USA), tobramycin (Sigma-Aldrich, USA), imipenem (Abtek biological ltd, Liverpool, UK), and Rifampicin (Sigma-Aldrich, USA). The latter antibiotic was stored in a foil, both in powder and solution forms because of its photosensitivity. Sufficient quantities of antibiotic solutions were prepared. The concentration of antibiotic prepared prior to dilution was four times higher than the highest concentration to be tested. For example, if the initial concentration for antibiotic A was 512 µg/ml and for antibiotic B was 64 µg/ml, then it was necessary to start with a concentration of 2 048 µg/ml for antibiotic A and 256 µg/ml for antibiotic B. The median MICs determined with the broth dilution method (appendix A) were used to configure the concentrations to be used in the checkerboard panels (see figure 2, below).

For each panel, the following quantities of antibiotic solutions were required:

a. antibiotic A : 16 wells x 0.05ml = 0.8 ml

b. antibiotic B : 24 wells x 0.05ml = 1.2ml

<u>For one isolate:</u>

Two panels were prepared; one for the combination drug panel (panel 1) and the other (panel 2) was used for making antibiotic dilutions for the second antibiotic (antibiotic B)

For panel 1(see figure 1, below):

a. 50 µl of sterile Cation-supplemented Muller-Hinton broth (CSMHB) was dispensed into every well except those of column 1 and column 12

b. 50 µl of sterile CSMHB was added to G12

c. 50 µl of antibiotic solution A was added to wells A12 – G12 and wells A11 – H11.

d. serial dilutions were made from column 11 to column 2 and 50 µl discarded from column 2

e. 50 µl were taken from B1 – B11 from panel 2 and dispensed in the corresponding row of panel 1.

f. step e was repeated for the next higher concentration

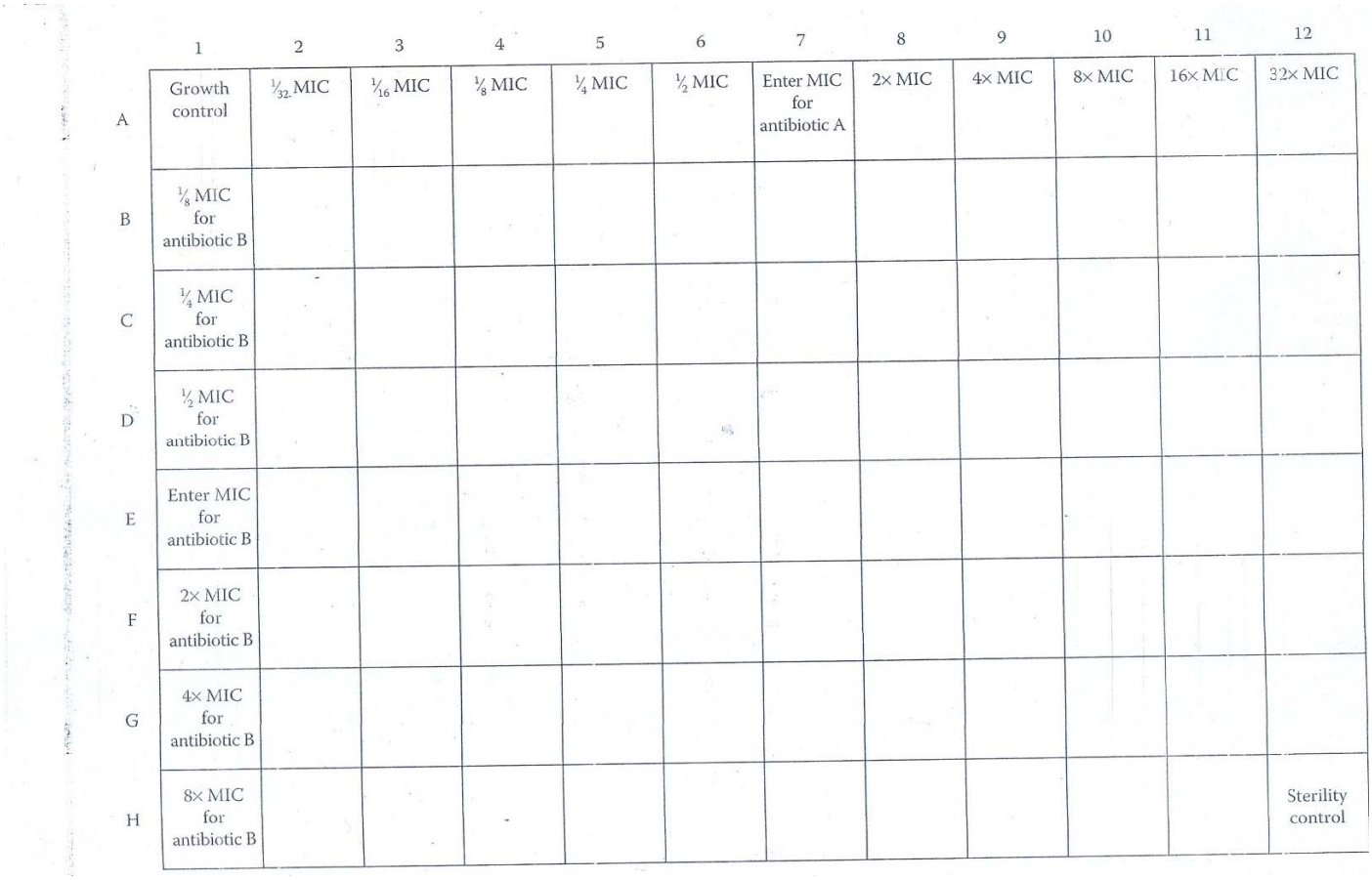
For panel 2:

a. 100 µl of CSMHB was dispensed into every well except those of row A and row H

b. 100 µl of antibiotic solution B were added to H1 – H11 and G1 – G12

c. serial dilutions were made from each well in row B (the final volume in each well is 100 µl (0.1ml)

the prepared panels were stored at -80^{0}C overnight. The trays were covered with lids and placed in sealed plastic bags



	1	2	3	4	5	6	7	8	9	10	11	12
A	Growth control	1/32 MIC	1/16 MIC	1/8 MIC	1/4 MIC	1/2 MIC	Enter MIC for antibiotic A	2× MIC	4× MIC	8× MIC	16× MIC	32× MIC
B	1/8 MIC for antibiotic B											
C	1/4 MIC for antibiotic B											
D	1/2 MIC for antibiotic B											
E	Enter MIC for antibiotic B											
F	2× MIC for antibiotic B											
G	4× MIC for antibiotic B											
H	8× MIC for antibiotic B											Sterility control

Figure 2: an example of a worksheet template for broth microdilution checkerboard panel (Reproduced from Schwalbe et al., 2007)

Preparation of inoculum:

Frozen stored isolates were subcultured twice on two successive days onto blood agar media to obtain fresh cultures for synergy testing. Three to five colonies of the selected isolate were touched with a sterile swab and then transferred to 5 ml of sterile CSMHB to make a 0.5 McFarland standard (1.5 x 10^8 CFU/ml)

A total of at least 5ml of diluted inoculum was required for one panel, including sufficient excess volume for the reservoir

0.1ml of the adjusted inoculum suspension was added to 9.9 ml of sterile CSMHB (1:100 dilution) to achieve approximately 10^6 CFU/ml

Inoculation and incubation of checkerboard panel:

50 µl of the diluted inoculum was added to every well except H12, using a multichannel pipette

50 µl of the diluted inoculum was added to growth control well A1

A purity blood agar plate was inoculated by subculturing 0.001 ml of inoculum from the reservoir onto a blood agar plate using a 1-µl loop

An inoculum verification plate was prepared as follows:

a. 50 µl of diluted inoculum was added to 0.45ml of sterile 0.85% saline

(dilution 10^{-1})

b. 0.1ml was transferred from dilution 10^{-1} to 0.9 ml of sterile 0.85% saline and vortexed well (dilution 10^{-2})

c. 0.1 ml was transferred from dilution 10^{-2} to 0.9ml of sterile 0.85% saline and vortexed well (dilution 10^{-3})

d. 0.1ml of dilution10^{-3} was plated onto a blood agar plate and spread for quantitation by streaking in several directions with a sterile loop

The trays were placed in plastic bags and incubated at 35^0C for 20h

The purity and inoculum verification plates were incubated at 35^0C for 20h.

Reading MIC panels

For each panel, the growth control well is examined for heavy turbidity to determine organism viability. The purity plates are also examined to check that cultures do not show mixed growth. Growth or no growth was recorded with the help of a viewing device for all the wells as shown on the worksheet (see Appendix C for an example of a worksheet)

The MICs for the individual antibiotics in the checkerboard method was read as follows:

MIC of antibiotic A: row A – lowest concentration showing inhibition of growth

MIC for antibiotic B: column 1 - lowest concentration showing inhibition of growth

These MICs were used in the formula to calculate the FIC indices. Several combination concentrations were tested along the checkerboard and multiple FIC indices are therefore calculated. The FIC index was worked out from rows in the middle with most reaction. Synergism or antagonism is reported even if exhibited only once within the checkerboard.

Definition of terms

- Synergy – the result with the combination is significantly better than the sum of their independent activities when measured separately; the MIC of the combination is ≥ 2 dilutions LOWER than MIC of the most active drug alone
- Additive - An additive combination of two drugs produce the same effect as the combination (the equally effective concentrations), when the agents are used alone
- Indifferent – the result with two drugs is equal to the best individual result; the MIC of the combination is within ± 1 dilution compared to the most active drug alone
- Antagonism – the result of the combination is significantly less than the best individual result; the MIC of the combination is ≥ 2 dilutions higher than the MIC of the most active drug alone.

Quality control:

The following quality control strains were used for MIC testing only, as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2006). There are no CLSI criteria for interpretation with synergy methods for these quality control strains. The QC strains were used just for the E-test and broth dilution MICs, and not for synergy testing. See Appendix C for an example of a worksheet template for broth microdilution checkerboard panel

a. *Escherichia coli* ATCC 25922
b. *Pseudomonas aeruginosa* ATCC 27853

VIII. Results:

Determination of MICs and comparison of susceptibility methods:

The MICs obtained with the E-test and broth microdilution methods are shown in appendix A (tables 1-8). Both methods were done in triplicate for the 10 selected isolates. With both methods, all isolates were sensitive to colistin. The MICs for the control microorganisms were within the expected ranges. The number of colonies on the inoculum verification plates was counted, and was between 75 and 150.

All isolates were resistant to rifampicin by E-test method (Table 3, Appendix A) (MICs, 4 – 8 µg/ml; resistance breakpoint ≤2 µg/ml) (Hogg G. M., 1998). However, by broth microdilution seven isolates tested susceptible (2-3 double dilutions lower than E-test MICs). These constituted major errors (broth microdilution susceptible, E-test false resistant).

With imipenem, minor errors were found in only two isolates, testing intermediate resistant with the E-test method and resistant with the broth microdilution method. Excellent categorical agreement was obtained with the other isolates.

With tobramycin, discrepancies were found in two isolates, one testing susceptible with the E-test method, but resistant with broth microdilution method (very major error) and one testing intermediate with E-test, but susceptible with broth microdilution method (minor error).

Table 9 (Appendix A) shows the MIC median values and their interpretative categories for colistin, rifampicin, imipenem, and tobramycin by both the E-test and broth microdilution methods. The results of the disk diffusion for imipenem and tobramycin as tested in the routine microbiology laboratory are also presented for comparison. With imipenem, the disk diffusion method gave one very major error. With tobramycin, the disk diffusion method showed discrepant results for the same two isolates as found with the E-test method, as well as further discrepant results in two other isolates (1 very major and 3 major errors).

Overall good reproducibility was obtained with all three methods. The agreement of MICs between the broth dilution and E-test methods was good with not more than 2 dilution differences in MIC values for all isolates. The E-test MICs matched the broth microdilution MICs or differed by 1 double dilution in 73% of tests. However, the categorical agreement between the methods for rifampicin was poor. Although MICs did not differ with more than two dilutions in most cases, many major errors occurred because the MICs clustered around the breakpoints.

MICs of antibiotic combinations with E-test combination testing:

The MICs obtained for the respective antibiotic combinations with the E-test combination testing method is shown in appendix B, Tables 1-10. For all ten isolates, combination testing was done in triplicate and showed good reproducibility. The median MIC values obtained for the combinations were used for the calculation of the FIC index.

Results of the E-test combination testing method:

The following tables (Tables 1-6) show the median MICs obtained with the E-test method for the respective antibiotic combinations. The FIC index was calculated and interpreted according to the formula described in the methods chapter. For isolates with MICs above the upper threshold concentration tested, the FIC index could not be calculated as the exact MIC was not determined. The combinations of colistin + rifampicin, colistin + imipenem, colistin + tobramycin, rifampicin + tobramycin, rifampicin + imipenem, and imipenem + tobramycin all showed indifferent or additive results. No synergistic results were obtained by the E-test method for all the above-mentioned combinations. There was one antagonistic result for the combination of colistin + tobramycin (isolate 16).

Table 1: Antibiotic combination MICs and FIC index – E-test method

Col + rif

Isolate	MIC col (µg/ml)	MIC col + rif	MIC rif (µg/ml)	MIC rif + col	FIC index	interpretation
1	0.5	0.5	4.0	0.032	1.008	indifferent
3	1.0	1.0	4.0	0.125	1.031	indifferent
6	1.0	1.0	8.0	0.125	1.016	indifferent
9	0.5	1.0	8.0	0.064	2.008	indifferent
14	1.0	1.0	4.0	0.064	1.016	indifferent
16	0.5	0.5	4.0	0.064	1.016	Indifferent
17	0.5	0.5	4.0	0.064	1.016	indifferent
23	1.0	0.5	4.0	0.064	0.516	additive
25	1.0	0.5	4.0	0.064	0.516	additive
30	1.0	1.0	4.0	0.125	1.031	indifferent

FIC, fractional inhibitory concentration; Col, colistin; Rif, rifampicin; Imi, imipenem; Tob, tobramycin

Table 2: Antibiotic combination MICs and FIC index – E-test method

Col + imi

Isolate	MIC col (µg/ml)	MIC col +imi	MIC imi (µg/ml)	MIC imi + col	FIC index	interpretation
1	0.5	1.0	8.0	0.125	2.016	indifferent
3	1.0	1.0	8.0	0.125	1.016	indifferent
6	1.0	1.0	16.0	0.125	1.009	indifferent
9	0.5	1.0	0.25	0.125	2.5	indifferent
14	1.0	1.0	0.25	0.125	1.5	Indifferent
16	0.5	1.0	0.25	0.25	3.0	indifferent
17	0.5	1.0	0.25	0.25	3.0	indifferent
23	1.0	0.5	32	0.25	0.509	additive
25	1.0	1.0	>32	0.125	ND	ND
30	1.0	1.0	>32	0.125	ND	ND

ND = not determined

Table 3: Antibiotic combination MICs and FIC index – E-test method

Col + tob

Isolate	MIC col (µg/ml)	MIC col + tob	MIC tob (µg/ml)	MIC tob + col	FIC index	interpretation
1	0.5	1.0	4.0	1.0	2.25	indifferent
3	1.0	1.0	128	1.0	1.008	indifferent
6	1.0	2.0	2.0	1.0	2.5	indifferent
9	0.5	0.5	1.0	1.0	2.0	indifferent
14	1.0	1.0	8.0	1.0	1.125	indifferent
16	0.5	2.0	4.0	4.0	5.0	antagonistic
17	0.5	0.5	16	1.0	1.063	indifferent
23	1.0	1.0	2.0	0.5	1.25	indifferent
25	1.0	0.5	1.0	1.0	1.5	indifferent
30	1.0	0.5	2.0	1.0	1.0	additive

Table 4: Antibiotic combination MICs and FIC index – E-test method

Rif + tob

Isolate	MIC rif (µg/ml)	MIC rif + tob	MIC tob (µg/ml)	MIC tob + rif	FIC index	interpretation
1	4.0	0.5	4.0	4.0	1.125	indifferent
3	4.0	4.0	128	32	1.25	indifferent
6	8.0	0.5	2.0	4.0	2.063	indifferent
9	8.0	0.125	1.0	1.0	1.016	indifferent
14	4.0	2.0	8.0	8.0	1.5	indifferent
16	4.0	0.5	4.0	4.0	1.125	indifferent
17	4.0	2.0	16	16	1.5	indifferent
23	4.0	0.5	2.0	1.0	0.625	additive
25	4.0	0.125	1.0	1.0	1.031	indifferent
30	4.0	0.125	2.0	2.0	1.031	indifferent

Table 5: Antibiotic combination MICs and FIC index – E-test method

Rif + imi

Isolate	MIC rif (µg/ml)	MIC rif + imi	MIC imi (µg/ml)	MIC imi + rif	FIC index	interpretation
1	4.0	4.0	8.0	4.0	1.5	indifferent
3	4.0	4.0	8.0	4.0	1.5	indifferent
6	8.0	8.0	16.0	4.0	1.25	indifferent
9	8.0	0.25	0.25	0.25	1.031	indifferent
14	4.0	0.25	0.25	0.25	1.063	indifferent
16	4.0	0.5	0.25	0.25	1.125	indifferent
17	4.0	0.5	0.25	0.25	1.125	indifferent
23	4.0	8.0	32	4.0	2.125	indifferent
25	4.0	4.0	>32	8.0	ND	ND
30	4.0	4.0	>32	4.0	ND	ND

ND= not determined

Table 6: Antibiotic combination MICs and FIC index – E-test method

Imi + tob

Isolate	MIC imi (µg/ml)	MIC imi+ tob	MIC tob (µg/ml)	MIC tob + imi	FIC index	interpretation
1	8.0	0.5	4.0	4.0	1.06	indifferent
3	8.0	8.0	128	64	1.5	indifferent
6	16.0	0.25	2.0	2.0	1.016	indifferent
9	0.25	0.125	1.0	1.0	1.5	indifferent
14	0.25	0.25	8.0	4.0	1.5	indifferent
16	0.25	0.25	4.0	2.0	1.5	indifferent
17	0.25	0.25	16	4.0	1.25	indifferent
23	32	0.25	2.0	1.0	0.508	additive
25	>32	0.125	1.0	1.0	ND	ND
30	>32	0.064	2.0	2.0	ND	ND

ND= not determined

Results of the checkerboard method:

The following tables (Tables 7-12) show the MICs and FIC indices obtained with the checkerboard method for the respective antibiotic combinations. This method was done on selected isolates. The selection was based on broth dilution MICs (median values) of the respective antibiotics. These median MICs were also used to configure the antibiotic concentrations in the checkerboard microdilution panels (see Appendix C for an example of a worksheet). The MICs for the individual antibiotics as determined in the checkerboard (row A for antibiotic A and column one for antibiotic B) were used in the formulae to calculate the FIC indices. For col + rif combination, rifampicin susceptible isolates and one resistant isolate (isolate 3) was selected. Only those isolates susceptible to imipenem were selected for the checkerboard method in the col + imi combination. Isolate 9 was done in duplicate to assess reproducibility. For the col + tob combination, only the tobramycin susceptible isolates were selected. For rif + tob combination, three isolates susceptible to both antibiotics, two resistant to both antibiotics and three isolates resistant to rif, but susceptible to tob were chosen. For the rif + imi, and imi + tob combinations, four isolates susceptible to imi were tested Some isolates were tested in duplicate to check for reproducibility of this method. The FIC index was calculated and interpreted according to the formula described in the methods chapter. Synergistic results were observed in four of the six isolates for which the combination of colistin and rifampicin was tested. The other two isolates showed indifferent/additive results. All the other combinations showed indifferent/additive results for all isolates except isolate 30 (col + tob) and isolate 25 (rif + tob) which showed synergism. No antagonistic results were observed by the checkerboard method.

Table 7: Antibiotic combination MICs and FIC index - checkerboard method

Col + rif

Isolate	MIC col (µg/ml)	MIC col + rif	MIC rif (µg/ml)	MIC rif + col	FIC index	interpretation
1	0.125	0.125	1.0	0.125	1.125	indifferent
3	0.5	0.064	16	1.0	0.191	synergistic
6	0.5	0.064	2	0.125	0.253	synergistic
16	0.25	0.125	2.0	1.0	1.0	additive
23	0.125	0.032	1.0	0.125	0.381	synergistic
30	0.25	0.032	1.0	0.064	0.192	synergistic

Table 8: Antibiotic combination MICs and FIC index - checkerboard method (isolate 9 done in duplicate)

Col + imi

Isolate	MIC col (µg/ml)	MIC col+imi	MIC imi (µg/ml)	MIC imi+col	FIC index	interpretation
9a	0.125	0.064	0.125	0.064	1.024	indifferent
9b	0.125	0.064	0.125	0.032	0.768	additive
16	0.125	0.064	0.125	0.064	1.024	indifferent
17	0.5	0.5	0.125	0.032	1.256	indifferent

Table 9: Antibiotic combination MICs and FIC index - checkerboard method

Col + tob

Isolate	MIC col (µg/ml)	MIC col+tob	MIC tob (µg/ml)	MIC tob+col	FIC index	interpretation
9	0.25	0.125	0.25	0.125	1.0	additive
14	0.125	0.125	1.0	0.5	1.5	indifferent
16	0.125	0.064	8.0	4.0	1.012	Indifferent
23	0.5	0.125	1.0	0.5	0.75	additive
30	1.0	0.25	2.0	0.064	0.282	synergistic

Table 10: Antibiotic combination MICs and FIC index- checkerboard method (isolates 9 & 23 done in duplicate to assess reproducibility)

Rif + tob

Isolate	MIC rif (µg/ml)	MIC rif+tob	MIC tob (µg/ml)	MIC tob+rif	FIC index	interpretation
1	4.0	0.25	4.0	2.0	0.563	additive
9a	2.0	0.5	0.25	0.125	0.75	additive
9b	2.0	1.0	0.25	0.125	1.0	additive
14	0.5	0.25	2.0	0.5	0.75	additive
16	1.0	1.0	4.0	0.5	1.125	indifferent
17	2.0	2.0	16	4.0	1.25	indifferent
23a	1.0	1.0	1.0	0.125	1.125	Indifferent
23b	1.0	1.0	1.0	0.25	1.25	Indifferent
25	0.5	0.064	1.0	0.064	0.192	synergistic
30	1.0	1.0	2.0	0.125	1.063	indifferent

Table 11: Antibiotic combination MICs and FIC index - checkerboard method (isolate 16 done in duplicate to assess reproducibility)

Rif + imi

Isolate	MIC rif (µg/ml)	MIC rif+imi	MIC imi (µg/ml)	MIC imi+rif	FIC index	interpretation
9	4.0	1.0	0.064	0.032	0.75	additive
14	0.5	0.5	0.064	0.032	1.5	indifferent
16a	1.0	0.5	0.125	0.064	1.012	indifferent
16b	2.0	0.25	0.125	0.064	0.637	additive
17	4.0	2.0	0.25	0.064	0.756	additive

Table 12: Antibiotic combination MICs and FIC index - checkerboard method (isolate 16 done in duplicate to assess reproducibility)

Imi + tob

Isolate	MIC imi (µg/ml)	MIC imi+tob	MIC tob (µg/ml)	MIC tob+imi	FIC index	interpretation
9	0.25	0.032	0.25	0.25	1.128	indifferent
14	0.032	0.064	4.0	0.5	2.125	indifferent
16a	0.064	0.016	4.0	2.0	0.75	additive
16b	0.064	0.032	4.0	2.0	1.0	additive
17	0.064	0.032	16	8.0	1.031	indifferent

Comparison of the results obtained with the E-test and checkerboard methods:

Tables 13-18 give the results obtained for isolates tested with both combination testing methods for the respective antibiotic combinations. The median MIC values were used for the calculation of the FIC index in the combination testing methods. It is noted that for most antibiotic combinations an indifferent/additive result was obtained. However, for the col + rif combination, the checkerboard method showed synergism for 4 of 6 isolates, whereas the E-test method showed indifference and an additive result in one. For the col + tob combination, synergism was shown in one isolate with the checkerboard method, whereas the E-test method showed an additive result. In another isolate tested with this combination, antagonism was found with the E-test method, but the checkerboard reaction showed indifference. For rif + tob, synergism was also shown with the checkerboard method in one isolate; however the E-test result was indifferent.

Table 13: Comparison of E-test and checkerboard methods

Col + rif

Isolate number	Interpretation – E-test	Interpretation-checkerboard
1	indifferent	indifferent
3	indifferent	synergistic
6	Indifferent	synergistic
16	Indifferent	indifferent
23	additive	synergistic
30	indifferent	synergistic

Table 14: Comparison of E-test and checkerboard methods

Col + imi

Isolate number	Interpretation – E-test	Interpretation-checkerboard
9	indifferent	9a indifferent 9b additive
16	indifferent	indifferent
17	indifferent	indifferent

Table 15: Comparison of E-test and checkerboard methods

Col + tob

Isolate number	Interpretation – E-test	Interpretation-checkerboard
9	indifferent	additive
14	indifferent	indifferent
16	antagonistic	indifferent
23	indifferent	additive
30	additive	synergistic

Table 16: Comparison of E-test and checkerboard methods

Rif + tob

Isolate number	Interpretation – E-test	Interpretation-checkerboard
1	indifferent	additive
9	indifferent	additive
14	Indifferent	additive
16	Indifferent	indifferent
17	Indifferent	indifferent
23	additive	indifferent
25	Indifferent	synergistic
30	indifferent	indifferent

Table 17: Comparison of E-test and checkerboard methods

Rif + imi

Isolate number	Interpretation – E-test	Interpretation-checkerboard
9	indifferent	additive
14	indifferent	indifferent
16	indifferent	16a indifferent 16b additive
17	indifferent	additive

Table 18: Comparison of E-test and checkerboard methods

Imi + tob

Isolate number	Interpretation – E-test	Interpretation-checkerboard
9	indifferent	indifferent
14	indifferent	indifferent
16	indifferent	indifferent
17	indifferent	indifferent

VIII. Discussion

Acinetobacter baumannii is a serious growing problem worldwide due to its multidrug resistance. Few antibiotics may be active against this species. Presently there are no particularly promising antibiotics on the horizon for the near future (Timurkaynak F., 2006). This has resulted in the use of old drugs, like colistin, until new antibiotics can be developed (Li J., 2006). Colistin belongs to the polymyxin family. Information about the pharmacokinetic, pharmacodynamic and toxic profile of colistin is limited. This antibiotic was discovered over 50 years ago, thus it was never exposed to drug development processes needed for compliance with contemporary regulatory requirements (Li J., 2006). In line with the literature, it was established in this study that compared with rifampicin, imipenem and tobramycin; colistin had the best in vitro activity against all the tested *A. baumannii* isolates which showed 100% susceptibility to colistin.

In the present study MIC determination by E-test was compared to the broth microdilution method. Overall good reproducibility was obtained with both methods. The agreement of MICs between the broth dilution and E-test methods was good with not more than 2 dilution differences in MIC values for most isolates. The E-test MICs matched the broth microdilution MICs or differed by 1 double dilution in 73% of tests. However, the categorical agreement between the methods for rifampicin was poor. Although MICs did not differ with more than two dilutions in most cases, many major errors occurred because the MICs clustered around the breakpoints.

The results of the disk diffusion for imipenem and tobramycin as tested in the routine microbiology laboratory were also presented. There was generally good correlation between the broth microdilution, E-test and disk diffusion methods for imipenem. Tobramycin MICs showed good correlation between E-test and broth dilution methods, but some errors were noted with the disk diffusion method. Swensen et al (Swensen J.M., 2004) compared the broth dilution and the disk diffusion methods and found that categorical agreement for tobramycin and imipenem were comparable for these two methods.

Published information regarding the treatment of multidrug resistant *A. baumannii* is limited (Manikal V.M., 2000). Because of the nephrotoxicity and neurotoxicity which are reported to be associated with colistin, the combination of colistin with other antibiotics was tested. The antibiotic combinations might allow the use of lower concentrations of the antibiotics, thus minimizing toxicity. Colistin in combination is suggested to cause rapid permeabilization of the outer cell membrane, thus allowing increased penetration by and activity of the other antibiotic in combination (Yoon J., 2004). In our study, synergy was demonstrated between colistin and rifampicin for four of the six tested isolates by the checkerboard method. All these isolates were resistant to rifampicin individually. The E-test method showed indifferent results for all these isolates, except one, which showed additive results.

There are few studies that have reported on the activity of antibiotic combination against *A. baumannii*. Most have produced variable results, probably due to differences in the strains selected and/or the methods used to evaluate synergy (Haddad F.A., 2005). Rifampicin has been shown to be bactericidal in vitro against *A. baumannii* (Thornberry C., 1983). The combination of colistin and rifampicin acted synergistically against *A. baumannii* in a number of studies (Giamarellos-Bourboulis E.J., 2001; Hogg G.M., 1998, Timurkaynak F., 2006; Tripodi M., 2007). Hogg et al used the checkerboard method to assess in vitro activity of colistin + rifampicin against 13 strains of *A. baumannii* (Hogg G.M., 1998). The combination of colistin and rifampicin was synergistic against 11 isolates (FICs, 0.07 – 0.063) and indifferent against two isolates. No antagonism was observed in that study. Time-kill method was used by Giamarellos-Bourboulis et al (Giamarellos-Bourboulis E.J., 2001) to investigate the interaction of colistin and rifampicin on 39 multidrug-resistant (MDR) *A. baumannii* isolates. Synergy was observed in six isolates (15.4%) at 6h of growth and in 20 isolates (51.3%) at 24h of growth. No synergy was found at 2 and 4h of growth (Giamarellos-Bourboulis E.J., 2001). Timurkaynak et al assessed the in vitro activity of colistin, azithromycin, doxycycline and rifampicin against five MDR strains of *A. baumannii* and five MDR strains of *Pseudomonas aeruginosa*. They also used the checkerboard method to determine whether the combinations of colistin with the abovementioned antibiotics act synergistically against these strains. The combination of colistin and rifampicin was synergistic against four *A. baumannii* and two *P. aeruginosa* strains (Timurkaynak F., 2006). Tripodi et al used time kill studies to evaluate nine MDR *A. baumannii* isolates using colistin, rifampicin, imipenem and sulbactam/ampicillin (Tripodi M., 2007). Synergy was observed with combinations of rifampicin + imipenem or sulbactam/ampicillin for all isolates and with colistin + rifampicin for isolates showing higher (4μg/ml) minimum inhibitory concentrations for rifampicin Co-administration of rifampicin with colistin enhances colistin activity (Pantopoulou A., 2007). The best in vitro results were observed when rifampicin was combined with colistin. Even though colistin is associated with nephrotoxicity, in patients without underlying renal disease, 7–11 days of colistin/rifampicin combination would be safe without serious adverse events (Song J.Y., 2008).

The combination of colistin and imipenem showed indifferent results by both the E-test and checkerboard methods, except for one isolate which was additive by checkerboard method. All the tested isolates were individually susceptible to both antibiotics. The results in our study differed to those of Manikal et al (Manikal V.M., 2000) and Haddad et al (Haddad F.A., 2005) which demonstrated additive and synergistic results by E-test and checkerboard methods, respectively, rather than indifferent results. Souli et al (Souli M., 2009) also concluded in their study that colistin and imipenem combination improved bactericidal activity against *Klebsiella pneumoniae* isolates susceptible either to both agents or to colistin. In this study both the checkerboard and E-test methods gave indifferent results, no synergy or antagonism was found for the colistin/imipenem combination. This is probably due to the low number of isolates tested that no synergy was observed.

The combinations of rifampicin and imipenem showed indifferent results by E-test, but additive results by checkerboard. The selected isolates tested with both combination methods were all susceptible to

imipenem individually. Saballs et al (Saballs M., 2006) treated 10 patients with different clinical presentations, who were infected with carbapenem resistant *A. baumannii*. Seven patients were clinically cured by the rifampicin/imipenem combination. In that study in vitro development of high level resistance (MIC = 256 mg/l) to rifampicin was observed in seven (70%) of the treated patients during treatment. The authors concluded that the rifampicin/imipenem combination should not be used against carbapenem resistant *A. baumannii* isolates (Saballs M., 2006). In a mouse pneumonia mouse model study by Montero et al, it was shown that a combination of rifampicin with imipenem, tobramycin or colistin may be useful (Montero) against carbapenem resistant *A baumannii* isolates. The combination of imipenem and tobramycin was the most active therapy against pneumonia caused by moderately carbapenem-resistant (imipenem MIC 8mg/l) strains. Our in vitro study showed indifferent results for this combination by both E-test and checkerboard method, except for one isolate which was additive by the latter method.

When results obtained with the E-test and checkerboard methods were compared, there was generally good agreement between the methods, except for the combinations of colistin/rifampicin and rifampicin/tobramycin. The checkerboard method showed more synergy results than the E-test method which showed more indifferent results for the colistin/rifampicin combination. For the rifampicin/tobramycin combination, the checkerboard method showed additive results compared to indifferent E-test results. Whereas more synergism or additivity was observed using the checkerboard methods in our study (Tables 13-18), the E-test method revealed indifference more frequently. There was no antagonism detected by the checkerboard method and one was detected by E-test method. In another study by White et al, that compared E-test, checkerboard, and time kill methods for detecting synergy, concordance between the E test and the checkerboard or time-kill curves method was present in 75% of cases. When the same methods were compared against *A. baumannii*, synergy was not detected by the E-test method (Bonapace C.R., 2000). This finding is similar to our present study, as the E-test method failed to detect synergy. The difference between our study and the one by Bonapace et al is that E-test strips were crossed at a 90^0 angle so that the scales met at the MIC of each drug alone, and the fractional inhibitory concentration index was calculated on the basis of the resultant zone of inhibition. The study by Manno et al (Manno G., 2003), which used the same E-test overlay method as ours, against *Burkholderia cepacia* complex, agreement between the E test and checkerboard was observed in 18 of 20 instances.

Data regarding the best combinations for synergy, as mentioned above, is mostly derived from in vitro and in vivo animal studies (Karageorgopoulos D.E., 2008). The good results obtained from these studies do not necessarily correlate with clinical findings (Karageorgopoulos D.E., 2008). It is also worthwhile to note that when interpreting the results of combination studies, one must take into account the important fact that what is called synergism may not be clinically relevant because such an interaction may occur in vitro only with antibiotic concentrations that are higher than those that can be achieved in patients (Moellering R.C. 1978). Clinical trials are too few to recommend the use of specific combinations for the treatment of multidrug-resistant *A. baumannii* (Towner 2009, Karageorgopoulos D.E., 2008).

From this study there was reasonable correlation between the checkerboard and E-test methods but synergy was detected for the rifampicin/colistin combination by checkerboard method, and not by E-test. The checkerboard method is indeed laborious, time consuming, expensive, and not suitable for the routine microbiology laboratory. More studies need to be performed to assess correlation between the E-test and checkerboard methods with a higher number of isolates. This study was limited by low numbers of selected isolates. The checkerboard method is indeed not an easy method to perform and expertise is needed to perform this method. Genotypic analysis using pulsed-field gel electrophoresis (PFGE) may be considered in future studies to determine relatedness of the isolates which will facilitate the selection of different strains for synergy testing. Furthermore, clinical studies are needed to establish whether in vitro synergy testing is useful in the clinical setting and whether the results of synergy testing will have any bearing on the clinical outcome of patients infected with multidrug resistant *A. baumannii*.

IX. Conclusions

The combination of colistin and rifampicin was synergistic against most *A. baumannii* isolates which were selected for this study. Synergy was detected by the checkerboard method; however the E-test method failed to detected synergism. Most combinations were indifferent and some additive by E-test method. There was good correlation between E-test and broth microdilution methods for the determination of colistin, imipenem, tobramycin and rifampicin MICs against A*. baumannii.* More studies to compare the checkerboard and E-test methods are needed as the latter method is much easier to perform and may be suitable for routine laboratory testing. In addition, clinical studies to investigate the usefulness of in vitro synergy testing in the clinical setting are recommended.

X. References

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APPENDIX A

Table 1: MIC determined by E-test method – colistin (a, b & c = MICs done in triplicate)

Isolate number	E-test MIC's µg/ml				interpretation
	a	b	c	Median	
1	0.5	0.5	0.5	0.5	S
3	0.5	1.0	1.0	1.0	S
6	0.5	1.0	1.0	1.0	S
9	0.5	0.5	0.5	0.5	S
14	0.5	1.0	0.5	0.5	S
16	0.5	0.5	0.5	0.5	S
17	0.5	0.5	0.5	0.5	S
23	1.0	1.0	1.0	1.0	S
25	0.5	1.0	1.0	1.0	S
30	1.0	1.0	1.0	1.0	S

Table 2: MIC by broth microdilution – colistin (a, b & c = MICs done in triplicate)

Isolate number	Broth microdilution MIC's µg/ml				interpretation
	a	b	c	Median	
1	1.0	2.0	2.0	2.0	S
3	2.0	2.0	2.0	2.0	S
6	2.0	2.0	2.0	2.0	S
9	1.0	2.0	1.0	1.0	S
14	2.0	2.0	1.0	2.0	S
16	1.0	1.0	2.0	1.0	S
17	2.0	1.0	2.0	2.0	S
23	1.0	1.0	1.0	1.0	S
25	1.0	1.0	1.0	1.0	S
30	0.25	1.0	0.25	0.25	S

Table 3: MIC by E-test method – rifampicin

Isolate number	E-test MIC's µg/ml				interpretation
	a	b	c	Median	
1	4.0	4.0	8.0	4.0	R
3	8.0	4.0	4.0	4.0	R
6	8.0	8.0	8.0	8.0	R
9	8.0	4.0	8.0	8.0	R
14	2.0	4.0	4.0	4.0	R
16	4.0	4.0	4.0	4.0	R
17	4.0	4.0	4.0	4.0	R
23	4.0	4.0	4.0	4.0	R
25	4.0	4.0	4.0	4.0	R
30	4.0	4.0	4.0	4.0	R

Table 4: MIC by broth microdilution – rifampicin

Isolate number	Broth microdilution MIC's µg/ml				interpretation
	a	b	c	Median	
1	2.0	2.0	4.0	2.0	S
3	4.0	8.0	8.0	8.0	R
6	2.0	2.0	8.0	2.0	S
9	4.0	4.0	8.0	4.0	R
14	2.0	2.0	8.0	2.0	S
16	2.0	2.0	4.0	2.0	S
17	4.0	4.0	4.0	4.0	R
23	1.0	4.0	1.0	1.0	S
25	1.0	2.0	0.5	1.0	S
30	0.5	0.5	0.5	0.5	S

Table 5: MIC by E-test method – imipenem

Isolate number	E-test MIC's µg/ml				interpretation
	a	b	c	Median	
1	8.0	8.0	8.0	8.0	I
3	16.0	8.0	8.0	8.0	I
6	16.0	16.0	16.0	16.0	R
9	0.25	0.25	0.25	0.25	S
14	0.25	0.25	0.25	0.25	S
16	0.25	0.25	0.25	0.25	S
17	0.25	0.25	0.25	0.25	S
23	32.	32	32	32	R
25	>32	>32	>32	>32	R
30	32	>32	>32	>32	R

Table 6: MIC by broth microdilution – imipenem

Isolate number	Broth microdilution MIC's µg/ml				interpretation
	a	b	c	Median	
1	32	32	32	32	R
3	32	32	32	32	R
6	32	32	32	32	R
9	0.125	0.125	4.0	0.125	S
14	0.125	0.25	16	0.25	S
16	0.125	0.125	0.25	0.125	S
17	0.125	0.125	0.25	0.125	S
23	>32	>32	>32	>32	R
25	>32	>32	>32	>32	R
30	>32	>32	>32	>32	R

Table 7: MIC by E-test method – tobramycin

Isolate number	E-test MIC's µg/ml				interpretation
	a	b	c	Median	
1	2.0	4.0	4.0	4.0	S
3	256	128	128	128	R
6	2.0	2.0	2.0	2.0	S
9	2.0	1.0	1.0	1.0	S
14	8.0	8.0	8.0	8.0	I
16	4.0	4.0	8.0	4.0	S
17	16	16	16	16	R
23	2.0	2.0	2.0	2.0	S
25	1.0	1.0	1.0	1.0	S
30	1.0	2.0	2.0	2.0	S

Table 8: MIC by broth microdilution – tobramycin

Isolate number	Broth microdilution MIC's µg/ml				interpretation
	a	b	c	Median	
1	8.0	16	64	16	R
3	>64	>64	>64	>64	R
6	2.0	2.0	8.0	2.0	S
9	2.0	0.5	1.0	1.0	S
14	4.0	4.0	32	4.0	S
16	2.0	2.0	16	2.0	S
17	16	32	32	32	R
23	1.0	0.5	2.0	1.0	S
25	0.5	0.5	1.0	0.5	S
30	0.5	0.5	1.0	0.5	S

Table 9 MICs of colistin, rifampicin, imipenem, and tobramycin by E-test and broth microdilution methods together with disk diffusion results for imipenem and tobramycin

Isolate no.	MIC (median values)µg/ml									
	Col		rif		Imi			tob		
	E-test	Broth microdi-lution	E-test	Broth microdi-lution	E-test	Broth microdi-lution	Disk diffusion	E-test	Broth microdi-lution	Disk diffusion
1	0.5(S)	2.0(S)	4.0(R)	2.0(S)	8.0(I)	32(R)	R	4.0(S)	16(R)	S
3	1.0(S)	2.0(S)	4.0(R)	8.0(R)	8.0(I)	32(R)	R	128(R)	>64(R)	R
6	1.0(S)	2.0(S)	8.0(R)	2.0(S)	16(R)	32(R)	R	2.0(S)	2.0(S)	R
9	0.5(S)	1.0(S)	8.0(R)	4.0(R)	0.25(S)	0.125(S)	S	1.0(S)	1.0(S)	S
14	0.5(S)	2.0(S)	4.0(R)	2.0(S)	0.25(S)	0.25(S)	S	8.0(I)	4.0(S)	R
16	0.5(S)	1.0(S)	4.0(R)	2.0(S)	0.25(S)	0.125(S)	S	4.0(S)	2.0(S)	R
17	0.5(S)	2.0(S)	4.0(R)	4.0(R)	0.25(S)	0.125(S)	S	16(R)	32(R)	R
23	1.0(S)	1.0(S)	4.0(R)	1.0(S)	32(R)	>32(R)	R	2.0(S)	1.0(S)	S
25	1.0(S)	1.0(S)	4.0(R)	1.0(S)	>32(R)	>32(R)	R	1.0(S)	0.5(S)	S
30	1.0(S)	0.25(S)	4.0(R)	0.5(S)	>32(R)	>32(R)	S	2.0(S)	0.5(S)	S

S, susceptible, I, intermediate, R, resistant

APPENDIX B

Table 1: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC's (µg/ml) – a	b	c	median
1	Col + Rif	0.5	0.5	0.5	0.5
	Rif + col	0.016	0.032	0.064	0.032
	Col + imi	1.0	1.0	0.5	1.0
	Imi + col	0.125	0.064	0.125	0.125
	Col + tob	1.0	0.5	1.0	1.0
	Tob + col	0.5	1.0	1.0	1.0
	Rif + tob	0.5	0.5	0.25	0.5
	Tob + rif	2.0	4.0	4.0	4.0
	Rif + imi	4.0	4.0	2.0	4.0
	Imi + rif	4.0	4.0	4.0	4.0
	Imi + tob	0.25	0.5	0.5	0.5
	Tob + imi	4.0	4.0	2.0	4.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 2: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC– (µg/ml)-a	b	c	median
3	Col + Rif	1.0	1.0	1.0	1.0
	Rif + col	0.125	0.25	0.064	0.125
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.125	0.125	0.125	0.125
	Col + tob	1.0	1.0	1.0	1.0
	Tob + col	2.0	1.0	1.0	1.0
	Rif + tob	4.0	4.0	4.0	4.0
	Tob + rif	32	32	64	32
	Rif + imi	4.0	4.0	4.0	4.0
	Imi + rif	4.0	4.0	4.0	4.0
	Imi + tob	8.0	4.0	16	8.0
	Tob + imi	128	32	64	64

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 3: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)-a	b	c	median
6	Col + Rif	2.0	1.0	1.0	1.0
	Rif + col	0.064	0.125	0.125	0.125
	Col + imi	2.0	1.0	1.0	1.0
	Imi + col	0.125	0.125	0.125	0.125
	Col + tob	2.0	1.0	2.0	2.0
	Tob + col	1.0	1.0	2.0	1.0
	Rif + tob	0.5	0.5	0.25	0.5
	Tob + rif	4.0	4.0	4.0	4.0
	Rif + imi	8.0	8.0	8.0	8.0
	Imi + rif	8.0	4.0	4.0	4.0
	Imi + tob	0.25	0.25	0.25	0.25
	Tob + imi	4.0	2.0	2.0	2.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 4: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)-a	b	c	median
9	Col + Rif	1.0	1.0	0.5	1.0
	Rif + col	0.064	0.064	0.064	0.064
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.25	0.125	0.064	0.125
	Col + tob	1.0	0.5	0.5	0.5
	Tob + col	0.5	1.0	1.0	1.0
	Rif + tob	0.125	0.25	0.125	0.125
	Tob + rif	2.0	1.0	1.0	1.0
	Rif + imi	0.125	0.5	0.25	0.25
	Imi + rif	0.25	0.25	0.25	0.25
	Imi + tob	0.125	0.125	0.125	0.125
	Tob + imi	1.0	1.0	0.5	1.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 5: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)- a	b	c	median
14	Col + Rif	1.0	1.0	1.0	1.0
	Rif + col	0.064	0.064	0.25	0.064
	Col + imi	2.0	1.0	1.0	1.0
	Imi + col	0.064	0.125	0.125	0.125
	Col + tob	2.0	0.5	1.0	1.0
	Tob + col	1.0	1.0	1.0	1.0
	Rif + tob	2.0	2.0	0.5	2.0
	Tob + rif	8.0	8.0	8.0	8.0
	Rif + imi	0.125	0.25	0.25	0.25
	Imi + rif	0.25	0.25	0.25	0.25
	Imi + tob	0.25	0.25	0.25	0.25
	Tob + imi	4.0	4.0	2.0	4.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 6: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml) a	b	c	median
16	Col + Rif	0.064	0.5	0.5	0.5
	Rif + col	0.064	0.064	0.064	0.064
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.25	0.25	0.25	0.25
	Col + tob	2.0	2.0	1.0	2.0
	Tob + col	4.0	4.0	0.5	4.0
	Rif + tob	0.5	0.5	0.25	0.5
	Tob + rif	4.0	4.0	8.0	4.0
	Rif + imi	0.5	0.5	0.25	0.5
	Imi + rif	0.25	0.25	0.25	0.25
	Imi + tob	0.25	0.25	0.125	0.25
	Tob + imi	2.0	2.0	4.0	2.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 7: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)- a	b	c	median
17	Col + Rif	0.5	0.5	1.0	0.5
	Rif + col	0.064	0.125	0.064	0.064
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.25	0.25	0.25	0.25
	Col + tob	0.5	0.5	0.5	0.5
	Tob + col	1.0	1.0	0.5	1.0
	Rif + tob	2.0	2.0	2.0	2.0
	Tob + rif	16	16	16	16
	Rif + imi	0.5	0.5	0.5	0.5
	Imi + rif	0.25	0.25	0.25	0.25
	Imi + tob	0.25	0.25	0.25	0.25
	Tob + imi	4.0	4.0	4.0	4.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 8: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)- a	b	c	median
23	Col + Rif	0.5	0.5	0.5	0.5
	Rif + col	0.064	0.064	0.064	0.064
	Col + imi	1.0	0.5	0.5	0.5
	Imi + col	0.125	0.25	0.25	0.25
	Col + tob	1.0	1.0	1.0	1.0
	Tob + col	0.5	0.5	0.5	0.5
	Rif + tob	0.25	0.5	0.5	0.5
	Tob + rif	1.0	1.0	1.0	1.0
	Rif + imi	4.0	8.0	8.0	8.0
	Imi + rif	4.0	4.0	4.0	4.0
	Imi + tob	0.5	0.25	0.25	0.25
	Tob + imi	1.0	1.0	1.0	1.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 9: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)- a	b	c	median
25	Col + Rif	0.5	0.5	0.5	0.5
	Rif + col	0.064	0.064	0.064	0.064
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.125	0.125	0.0125	0.125
	Col + tob	0.5	0.5	0.5	0.5
	Tob + col	1.0	1.0	1.0	1.0
	Rif + tob	0.125	0.125	0.064	0.125
	Tob + rif	1.0	1.0	1.0	1.0
	Rif + imi	4.0	4.0	4.0	4.0
	Imi + rif	8.0	8.0	4.0	8.0
	Imi + tob	0.125	0.125	0.125	0.125
	Tob + imi	1.0	1.0	0.5	1.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

Table 10: Antibiotic combination MIC- Etest method

Isolate	Antibiotic combination	MIC – (µg/ml)- a	b	c	median
30	Col + Rif	1.0	1.0	1.0	1.0
	Rif + col	0.125	0.125	0.125	0.125
	Col + imi	1.0	1.0	1.0	1.0
	Imi + col	0.125	0.125	0.125	0.125
	Col + tob	0.5	0.5	0.5	0.5
	Tob + col	1.0	1.0	1.0	1.0
	Rif + tob	0.125	0.125	0.125	0.125
	Tob + rif	2.0	2.0	2.0	2.0
	Rif + imi	4.0	4.0	4.0	4.0
	Imi + rif	4.0	4.0	4.0	4.0
	Imi + tob	0.064	1.064	0.064	0.064
	Tob + imi	2.0	2.0	2.0	2.0

col, colistin; rif, rifampicin; imi, imipenem; tob, tobramycin

APPENDIX C

	1	2	3	4	5	6	7	8	9	10	11	12
A	GC	A 0.5	A 1	A 2	A 4	A 8	A 16	A 32	A 64	A 128	A 256	A 512
B	B1	A 0.5 + B1	A 1 + B 1	A 2 + B 1	A 4 + B 1	A 8 + B 1	A 16 + B 1	A 32 + B 1	A 64 + B 1	A 128 + B 1	A 256 + B 1	A 512 + B 1
C	B2	A 0.5 + B2	A 1 + B 2	A 2 + B 2	A 4 + B 2	A 8 + B 2	A 16 + B 2	A 32 + B 2	A 64 + B 2	A 128 + B 2	A 256 + B 2	A 512 + B 2
D	B4	A 0.5 + B4	A 1 + B 4	A 2 + B 4	A 4 + B 4	A 8 + B 4	A 16 + B 4	A 32 + B 4	A 64 + B 4	A 128 + B 4	A 256 + B 4	A 512 + B 4
E	B8	A 0.5 + B8	A 1 + B 8	A 2 + B 8	A 4 + B 8	A 8 + B 8	A 16 + B 8	A 32 + B 8	A 64 + B 8	A 128 + B 8	A 256 + B 8	A 512 + B 8
F	B16	A 0.5 + B16	A 1 + B 16	A 2 + B 16	A 4 + B 16	A 8 + B 16	A 16 + B 16	A 32 + B 16	A 64 + B 16	A 128 + B 16	A 256 + B 16	A 512 + B 16
G	B32	A 0.5 + B32	A 1 + B 32	A 2 + B 32	A 4 + B 32	A 8 + B 32	A 16 + B 32	A 32 + B 32	A 64 + B 32	A 128 + B 32	A 256 + B 32	A 512 + B 32
H	B64	A 0.5 + B64	A 1 + B 64	A 2 + B 64	A 4 + B 64	A 8 + B 64	A 16 + B 64	A 32 + B 64	A 64 + B 64	A 128 + B 64	A 256 + B 64	BC

Example of a broth microdilution checkerboard panel (Reproduced from Schwalbe et al., 2007)