THE IN VITRO ANTIMICROBIAL ACTIVITY OF AMIKACIN AND CEFTAZIDIME AGAINST MULTIPLE RESISTANT GRAM-NEGATIVE BACILLI IN NOSOCOMIAL INFECTIONS

BY

MARIUS JOHANNES JOOSTE

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INTERNAL EXAMINER : MR. A.W. VAN RIJSWIJK

EXTERNAL EXAMINER : DR. R. ROBSON

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I hereby declare that the content of this thesis is my own work and all views expressed are my own and not necessarily those of the Cape Technikon.

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M.J. JOOSTE

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"It is characteristic of science and progress that they continually open new fields to our vision..."

Louis Pasteur

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ABBREVIATIONS

G.N.B.	- gram-negative bacilli
M.I.C.	- minimum inhibition concentration
C.F.U.	- colony-forming units
Amp.	- Ampicillin
Am.	- Amikacin
Ma.	- Cephamandole
Fox.	- Cefoxitin
Tet.	- Tetracyline
Sxt.	- Trimethoprim-sulfamethoxazole
Gnt.	- Gentamicin
Tob.	- Tobramycin
Ctz.	- Ceftazidime
U.T.I.	- Urinary tract infection
P.B.P.	- Penicillin-binding proteins
E. coli	- Escherichia coli
R	- Resistant
S	- Sensitive
I	- Intermediate
F.I.C.	- Fractional Inhibitory concentration

CHAPTER 1

INTRODUCTION

1.1 NOSOCOMIAL INFECTION

Nosocomial or hospital-aquired infection, can be defined as an infection not present when the patient enters a hospital. It usually manifests itself seventy-two hours after admission and sometimes it is not apparent until after the patient has been discharged. When the incubation period is unknown, any infection developing after admission to a hospital, may be classified as a nosocomial infection. the problem of hospital acquired infection is a global one and every hospital is affected. It is estimated that five to ten percent of patients develop evidence of a hospital-acquired infection (Haley et al. 1985).

Patients in intensive care units and post-operative surgery wards are often at risk of acquiring hospital infection. Data from a project by Haley et al. (1981) identified a number of patient characteristics which were associated with an increased risk of acquiring a nosocomial infection. These nosocomial infections involve various anatomical sites and are primarily the urinary tract, post-operative wounds, the respiratory

tract and the blood stream (Wenzel et al. 1976). Unfortunately the hospital environment is the source of infection. The use of antibiotics play a major role in the spread of infection in hospitals. Antibiotics excert selective pressure on the patients normal bacterial flora and thus leads to colonization of potential pathogens. This colonization may also be prolonged with the use of antibiotics and prolonged hospital stay (Price and Sleigh 1970). Patients most likely to be colonized are those in intensive care units as these patients often have impaired host defences and have invasive monitoring devices in place. Factors promoting colonization have been described by Haverkom and Michel (1979). They are:

* Duration of stay in the hospital;
* Prolonged antibiotic therapy;
* Location in the hospital;
* Disruption of normal bacterial flora in the bowel or pharynx;
* Invasive devices.

Because of this everpresent problem the first International Conference on Nosocomial Infections encouraged the development of infection surveillance programs (Garner et al. 1971). Today every major hospital worldwide has an infection control program. A review by Wenzel (1987) illustrates the important role of the infection control team and how the problem

of hospital-acquired infection is investigated and managed.

1.2 HISTORICAL BACKGROUND

In order to appreciate the problem of hospital-acquired infections today, one must go back in history. Hospitals in the eighteenth century were "frightful places where many patients died of nosocomial infections". Patients were often grouped together and conditions were attrocious. Highly communicable diseases were probably responsible for killing many patients. Decontamination of hands or instruments were unknown and post-operative infection was inevitable (LaForce 1987).

The role of the hands in the transmission of infection was demonstrated by Ignaz Semmelweis in 1846 who published the first experimental observation on the causes of puerperal fever. Through his studies and observations he found that sepsis was transmitted by physicians from cadavers to pregnant women in the delivery rooms. He introduced hand washing with a chlorinated lime solution before physicians were allowed to enter the wards. Through this measure the mortality rate dropped dramatically (Semmelweis 1981).

One of the first studies on hospital epidemiology was probably done by James Simpson in 1860. He concluded

that the mortality rate on amputees were much higher for those that remained in hospital, compared to those that were sent home to recuperate (LaForce 1987). It was only until late in the nineteenth century that Louis Pasteur founded the science of bacteriology and soon after that advances were made into the problems of infection. The greatest contribution to the science of medicine was the discovery of antibiotics. In 1928 Sir Alexander Fleming made a discovery that would eventually save millions of lives. In his laboratory he discovered the mould that led to the development of Penicillin. Later other antibiotics such as the sulfonamides were developed and in 1940 the first aminoclycoside was discovered (Singer and Underwood (1962). One can assume that many thought that the problems of bacterial infections were something of the past, however resistance to the antibiotics emerged as will be discussed in section 2.1.

1.3 NOSOCOMIAL URINARY TRACT INFECTIONS

Urinary tract infections are one of the most common hospital-associated infections (Krieger et al. 1983, Haley et al. 1981). At Tygerberg hospital similar trends have been noted and of the gram-negative bacilli isolated, Escherichia coli is the most common pathogen in urinary tract infections. The sensitivity patterns of the Gram-negative bacilli most frequently isolated from urinary tract infections over a four

year period, are shown in tables 1.1 to 1.3. The organisms responsible for these infections are usually those that colonize the patient's gastro-intestinal tract. These G.N.B. are also usually resistant to the commonly used antibiotics (Rose and Schreier 1968). The main sources of urinary tract infections are catheters (Garibaldi et al. 1982), contaminated antiseptic solutions, urine measuring containers and urinometers (Rutala et al. 1981).

1.4 NOSOCOMIAL RESPIRATORY INFECTIONS

Nosocomial pneumoniae is one of the infections associated with a high mortality rate (Stamm et al. 1977 and Gross et al. 1980). Deaths that result from *nosocomial pneumonia can be as high as 50%* (Graybill et al. 1973, Stevens et al. 1974). These high mortality rates are usually associated with gram-negative bacilli and the deathrate associated with Pseudomonas aeruginosa infections are as high as 80% (Pennington et al. 1973, Bryan and Reynolds 1984, Tillotson and Lerner 1968, Stevens et al. 1974). The use of potent new antibiotics often does not eradicate the infecting gram-negative bacilli from the respiratory tract (Schentag et al. 1985) and the incidence of relapse and superinfection are high (Tillotson and Finland 1969, Reitberg et al. 1984).

The major factors contributing to nosocomial pneumonia are:

* Intubation (Garibaldi et al. 1981)
* Intensive care units (Johanson et al. 1972)
* Immunosuppression (Fanta and Pennington 1983)
* Antibiotic therapy (Louria and Kaminski 1962, Johanson et al. 1972)
* Chronic pulmonary disease (Simon et al. 1980)
* Surgery (Garibaldi et al. 1981, Eickhoff 1980)

The gram-negative bacilli most frequently associated with hospital-acquired pneumonia are Pseudomonas aeruginosa and Klebsiella pneumoniae (Maki 1978). The G.N.B. most frequently isolated at Tygerberg hospital and their sensitivity patterns are shown in table 1.4 and 1.5.

1.5 POST-OPERATIVE WOUND INFECTIONS

Post-operative infectious complications are frequently the cause of mortality in the surgical patient. In the United States of America surgical wound infections account for the second most encountered hospital-acquired infection (Dixon 1978). The majority of these wound infections are usually uncomplicated and only involve the skin and subcutaneous tissues. Unfortunately wound infections sometimes become serious and involve the fascia and

muscle. Although gram-positive cocci such as Staphylococcus aureus are the most frequent organisms found in wound infections, resistant G.N.B. are often isolated as well. The antibiotic susceptibility of these gram-negative bacteria isolated at Tygerberg hospital are shown in table 1.6 to 1.10.

The reservoirs or sources for these organisms that are incriminated in post-operative surgery are:

* Personel (Walter and Kundsin 1969, Hambraeus and Laurell 1980, Dineen and Drusin 1973)
* The patient (Altemeier et al. 1968)
* Environment (Eickhoff 1962, Bassett et al. 1970).

The modes of transmission of the causitive organisms have been discussed in detail by Mayhall (1987).

1.6 INTRAVASCULAR INFECTIONS

Relatively infrequent to the other hospital-acquired infections are those infections involving the blood-stream. These bloodstream infections are often device-related. Bacteria gain access to the blood-stream via the site at which the specific device penetrates the skin and the majority of infections result from skin bacterial flora colonizing the catheter entry site (Wenzel 1981). Antimicrobial therapy also plays a role in the colonizing of the

skin with specific strains (Maki 1981). Hospital personnel's failure to perform appropriate hand washing also play a major role, especially in the intensive care unit (Preston et al. 1981, Albert and Condie 1981). Contaminated disinfecting solutions onto the skin of patients have also been responsible for a few outbreaks (Berkelman et al. 1981).

Although Staphylococcus aureus and Staphylococcus epidermidis are frequently the causes of device-related infection, G.N.B. are also responsible for such infections. The antibiotic sensitivity patterns of G.N.B. isolated from blood cultures at Tygerberg hospital are shown in table 1.11 and 1.12.

1.7 MICROBIOLOGY

The microbiology laboratory has an important role to play in the control of hospital infections (Ristuccia and Cunha 1987). During an outbreak the resources of the microbiology laboratory are indispensable, especially for correct identification of the infecting organisms and their susceptibility patterns. The microbiology laboratory together with the infection control team must be constantly on the alert for the possibility of pseudo-infections. These Pseudo-epidemics can be defined as increased recovery of common/uncommon organisms by smear or culture from a body fluid/tissue that does not correlate clinically

with disease usually associated with the organism (Ristuccia and Cunha 1987).

During the 1950's and 1960's, Staphylococcus aureus was the dominant organism in hospital-acquired infections and the use of antibiotics were shown to encourage the epidemic transmission of these penicillin-resistant organisms in hospitals throughout the world (Berntsen and McDermott 1960). Infections by G.N.B. have since become the major threat to hospitalized patients (Rogers 1959, Finland 1970). Organisms such as E. coli, Klebsiella pneumoniae, Serratia marcescens, Enterobacter spp., Proteus spp., Pseudomonas aeruginosa and Acinetobacter spp. increasingly contributed to the problem of hospital-acquired infection. These normal bacterial flora have become increasingly resistant to a variety of antibiotics and this problem has been encountered worldwide (Salzman and Klemm 1967). In tables 1.1 to 1.12 these resistant patterns are clearly indicated.

In this study the organisms were isolated from patients with nosocomial infections, and selected according to their resistance to the more commonly used beta-lactam and aminoglycoside antibiotics. The test organisms will be tested against the following antibiotics:

* Amoxicillin

* Amikacin
* Cephamandole
* Cefoxitin
* Ceftazidime
* Tetracycline
* Trimethoprim-sulfamethoxazole
* Gentamicin
* Tobramycin

On these resistant organisms, the minimum inhibition concentrations of amikacin and ceftazidime will be determined. Organisms with resistance to these two antimicrobials will be selected for in vitro testing of their combined activity.

TABLE 1.1

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
ESCHERICHIA COLI ISOLATED FROM U.T.I.

Antibiotic	1984	1985	1986	1987
Amikacin	94	98	97	93
Amoxicillin	34	33	30	32
Ceftazidime	-	-	-	-
Cefoxitin	96	93	95	96
Cefamandole	51	49	44	44
Cotrimoxazole	41	42	48	47
Gentamicin	94	97	91	94
Tobramycin	94	96	90	91
% of total isolates	47.6	50.6	53.1	55.2

TABLE 1.2

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
KLEBSIELLA PNEUMONIAE ISOLATED FROM U.T.I.

Antibiotic	1984	1985	1986	1987
Amikacin	95	98	98	94
Amoxicillin	1	2	3	3
Ceftazidime	-	-	-	-
Cefoxitin	87	84	91	93
Cefamandole	27	26	27	34
Cotrimoxazole	17	19	24	35
Gentamicin	61	53	51	61
Tobramycin	63	54	51	60
% of total isolates	20.8	21.6	19.6	17.9

TABLE 1.3

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
SERRATIA MARCESCENS ISOLATED FROM U.T.I.

Antibiotic	1984	1985	1986	1987
Amikacin	42	68	62	50
Amoxicillin	-	16	2	3
Carbenicillin	38	50	-	-
Ceftazidime	-	-	-	-
Cefamandole	-	12	4	1
Cefoxitin	4	12	6	12
Cotrimoxazole	9	12	20	22
Gentamicin	27	68	43	32
Netilmycin	25	72	43	35
Tobramycin	28	59	25	30
% of total isolates	0.6	0.7	0.9	1.1

TABLE 1.4

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
KLEBSIELLA PNEUMONIA ISOLATED FROM LUNG INFECTIONS

Antibiotic	1984	1985	1986	1987
Amikacin	99	99	99	89
Amoxicillin	2	5	3	6
Ceftazidime	-	-	-	-
Cefamandole	70	53	54	62
Cefoxitin	89	91	93	93
Cotrimaxazole	65	53	56	61
Gentamicin	77	63	63	76
Tobramycin	78	63	64	70
% of total isolates	23.1	22.2	19.1	16.0

TABLE 1.5

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
PSEUDOMONAS AERUGINOSA ISOLATED FROM LUNG INFECTIONS

Antibiotic	1984	1985	1986	1987
Amikacin	97	97	98	95
Amoxicillin	NT	NT	NT	NT
Carbenicillin	73	73	NT	NT
Ceftazidime	-	-	-	99
Netilmicin	93	92	87	92
Gentamicin	92	90	87	94
Tobramycin	95	90	86	94
% of total isolates	10.9	11.5	12.5	12.3

N.T. - Not tested.

TABLE 1.6

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
ESCHERICHIA COLI ISOLATED FROM POST-OPERATIVE WOUNDS

Antibiotic	1984	1985	1986	1987
Amikacin	98	99	99	93
Amoxicillin	43	44	38	41
Carbenicillin	-	-	-	-
Ceftazidime	-	-	-	-
Cefamandole	83	89	89	83
Cefoxitin	93	96	96	95
Chloramphenicol	73	63	63	35
Cotrimoxazole	74	74	69	64
Gentamicin	98	97	98	96
Netilmycin	98	98	98	94
Pipercillin	50	NT	25	44
Tetracycline	61	59	60	54
Tobramycin	97	97	97	92
% of isolates	11.0	38.6	36.7	35.9

N.T. - Not tested.

TABLE 1.7

PERCENTAGE ANTIBIOTIC SENSITIVITY OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM POST-OPERATIVE WOUNDS

Antibiotic	1984	1985	1986	1987
Amikacin	99	97	99	91
Amoxicillin	2	3	2	6
Carbenicillin	-	-	-	-
Ceftazidime	-	-	-	-
Cefamandole	59	51	52	60
Cefoxitin	90	91	92	89
Chloramphenicol	40	50	46	46
Cotrimoxazole	55	46	50	54
Gentamicin	68	57	57	65
Netilmycin	66	68	74	72
Pipercillin	14	14	31	9
Tetracycline	66	76	74	72
Tobramycin	69	57	56	61
% of isolates	9.6	31.8	31.3	26.9

TABLE 1.8

PERCENTAGE ANTIBIOTIC SENSITIVITY OF *ENTEROBACTER CLOACAE* ISOLATED FROM POST-OPERATIVE WOUNDS

Antibiotic	1984	1985	1986	1987
Amikacin	99	100	99	90
Amoxicillin	18	50	55	43
Carbenicillin	-	-	-	-
Ceftazidime	-	-	-	-
Cefamandole	61	85	78	69
Cefoxitin	10	8	8	3
Chloramphenicol	-	-	-	-
Cotrimoxazole	85	88	87	85
Gentamicin	94	97	94	85
Netilmycin	96	98	97	88
Pipercillin	-	-	60	50
Tetracycline	87	90	90	87
Tobramycin	96	96	91	82
% of isolates	3.6	12.5	13.8	15.0

TABLE 1.9

PERCENTAGE ANTIBIOTIC SENSITIVITY OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM POST-OPERATIVE WOUNDS

Antibiotic	1984	1985	1986	1987
Amikacin	96	97	95	91
Amoxicillin	-	-	-	-
Carbenicillin	83	84	-	0
Ceftazidime	50	92	87	91
Cefamandole	-	-	-	-
Cefoxitin	-	-	-	-
Chloramphenicol	-	-	-	-
Cotrimoxazole	-	-	-	-
Gentamicin	89	80	82	90
Netilmycin	91	83	77	84
Pipercillin	94	94	82	87
Tetracycline	1	3	-	-
Tobramycin	91	80	76	84
% of isolates	8.2	11.8	13.1	12.3

TABLE 1.10

PERCENTAGE ANTIBIOTIC SENSITIVITY OF *ACINETOBACTER ANITRATUS* ISOLATED FROM POST-OPERATIVE WOUNDS

Antibiotic	1984	1985	1986	1987
Amikacin	-	96	89	83
Amoxicillin	-	0	4	3
Ceftazidime	-	-	-	-
Cefamandole	-	0	3	1
Cefoxitin	-	1	7	2
Chloramphenicol	-	0	50	16
Cotrimoxazole	-	21	27	16
Gentamicin	-	46	41	26
Netilmycin	-	53	62	71
Pipercillin	-	-	-	-
Tetracycline	-	23	29	22
Tobramycin	-	81	70	44
% of total isolates	-	7.5	6.3	12.5

TABLE 1.11

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
ESCHERICHIA COLI ISOLATED FROM BLOOD CULTURES

Antibiotic	1984	1985	1986	1987
Amikacin	-	-	97	70
Amoxicillin	-	38	30	16
Ceftazidime	-	-	100	100
Cefamandole	-	81	64	43
Cefoxitin	-	94	97	94
Cotrimoxazole	-	65	56	31
Gentamicin	-	97	93	84
Netilmycin	-	97	96	82
Tetracycline	-	64	55	38
Tobramycin	-	95	91	71
% of isolates	-	39,9	40,5	42,1

TABLE 1.12

PERCENTAGE ANTIBIOTIC SENSITIVITY OF
KLEBSIELLA PNEUMONIAE ISOLATED FROM BLOOD CULTURES

Antibiotic	1984	1985	1986	1987
Amikacin	-	-	97	70
Amoxicillin	-	38	30	16
Ceftazidime	-	-	100	100
Cefamandole	-	81	64	43
Cefoxitin	-	94	97	94
Cotrimoxazole	-	65	56	31
Gentamicin	-	97	93	84
Netilmycin	-	97	96	82
Tetracycline	-	64	55	38
Tobramycin	-	95	91	71
% of isolates	-	39,9	40,5	42,1

CHAPTER 2

LITERATURE STUDY

2.1 EVOLUTION AND EMERGENCE OF RESISTANCE TO ANTIMICROBIALS

One of the major factors contributing to the development of bacterial resistance is the repeated exposure of bacteria to antimicrobial agents. Because of the development of resistance, the efficacy of various antibiotics against a number of infections has been lost. In 1940 the sulfonamides were first used extensively as antibacterial agents against Shigella infections in Japan. By 1950 up to 90% of these isolates were resistant to the sulfonamides (Mitsuhashi 1977). Fortunately there were other drugs such as streptomycin, chloramphenicol and tetracycline, but resistance to these antibiotics also developed. In 1955 a strain resistant to these agents was isolated (Mitsuhashi 1969). Later Mitsuhashi (1977) found that the transfer of resistance required cell to cell contact and was independent of chromosomal transmissibility. The term R-factor was adopted to describe this transferable extra chromosomal plasmid.

In France Chabbert and Baudens (1966) reported an outbreak of salmonellosis and resistance to ampicillin, kanamycin, streptomycin, chloramphenicol, tetra-

cycline, neomycin and sulfonamide was encountered. In Mexico outbreaks of Shigella dysenteriae infections showed that the organisms developed resistance to ampicillin, streptomycin, sulfonamides, tetracycline and chloramphenicol (Olarte et al. 1976). In the United States of America Salmonella spp. with R-factors was reported in a childrens hospital (Smith 1966). An outbreak of gastro-enteritis caused by Salmonella heidelberg in a hospital in Puerto Rico, resistance to kanamycin, neomycin, Streptomycin and ampicillin was mediated by R-factors (Rice et al. 1976). In Mexico an outbreak of Salmonella typhi, carrying a R-factor also caused concern because these organisms were resistant to chloramphenicol, streptomycin, tetracycline and sulfinamides (Gangorasa 1972).

During 1973 a large outbreak of nosocomial infections due to Serratia marcescens occurred at the Vanderbilt University Medical Complex, Nashville, Tenessee. A major characteristic was the high-level of resistance to gentamicin and carbenisilin (Schaberg et al. 1981). This high-level resistance was shown to operate at three levels of organizations:

* Dissemination of individual strains;
* Dissemination of a plasmid among different strains.
* Movement of a discrete genetic element or transposon between plasmids.

In South Africa Woods et al. (1972) demonstrated that plasmid-mediated aminoglycoside resistance occurred among coliform bacilli. Studies done by Botha et al. (1981) also demonstrated plasmid-mediated enzymes from gram-negative bacteria. It has since been found that some species of bacteria are more resistant to particular antibiotics in certain parts of the world. In a study done by O'Brien et al. (1978) the prevalence of bacterial resistance to antibiotics differed substantially in separate regions of the world, namely France and U.S.A.

The emergence of multiple resistance in normal human flora such as E. coli, Klebsiella pneumoniae, Enterobacter spp., Serratia spp., and Pseudomonas aeruginosa, is of great concern (Gill and Hook 1965).

2.2 MECHANISMS OF RESISTANCE TO ANTIMICROBIALS

The ability of organisms to develop resistance to antimicrobials began long before the discovery of antibiotics. Bacterial enzymes evolved to protect the bacteria against other toxic substances normally found in nature. With the introduction of the first antibiotics the immediate response of bacteria was to develop resistance. This evolutionary process continues in bacteria today and thus far no antibiotic has been developed that is effective against all known organisms (Murray and Moellering 1978).

Resistance to antimicrobial agents has been established to be chromosomally or extra chromosomally (R-plasmid) mediated, however, there are also other mechanisms by which bacteria develop resistance. These other mechanisms are likely to involve target site and permeability changes (Murray and Moellering 1978). One or more of these mechanisms can be found in a resistant organism.

2.2.1 TARGET SITE MODIFICATION

The point at which the antibiotic exerts its effect on the bacteria is called the target site. The antibiotic attaches to this target site or bacterial ribosome and thus leads to the prevention of protein synthesis, for example aminoglycosides attach to the 30's subunit of the ribosome. Eliopoulos et al. (1984) demonstrated that mutants with an altered ribosomal structure lack susceptibility to streptomycin. This alteration of the ribosomal binding site was also demonstrated by Moellering (1983).

The ultimate targets of the beta-lactam antibiotics are the penicillin-binding proteins (P.B.P.'s) on the inner cell membrane. To reach these P.B.P.'s the beta-lactam antibiotic must pass through the outer membrane and avoid hydrolysis by the beta-lactamases (Malouin and Bryan 1986). Murray and Moellering (1978) suggested that alteration of the target site complex

envisaged an increased concentration of competing substances, synthesis of a resistant target site, and synthesis of alternate target sites.

2.2.2 ENZYMATIC INACTIVATION

Enzymes that mediate bacterial drug resistance are produced by most bacteria. Enzymes are produced in high concentrations in the periplasmic space of the bacteria, they are:

* Beta-lactamases (Matthew 1979);
* Aminoglycoside-modifying enzymes (Davies 1977);
* Chloramphenicol acetyl transferases (Okamoto et al. 1967).

The above mentioned enzymes can mediate bacterial drug resistance by three broad mechanisms (Livermore 1986):

(i) Some enzymes reduce or destroy antibiotic activity by altering the drugs chemically.

(ii) Alterations in target enzymes can cause resistance.

(iii) Acquisition of a new antibiotic-resistant enzyme may result in the bypassing of one that is antibiotic sensitive.

2.2.2.1 Beta-Lactamases

The beta-lactamases are enzymes produced by some stains of G.N.B. as well as Staphylococcus aureus. The enzymes produced by the G.N.B. remain within the cell, in the periplasmic space, whereas those released by Staphylococcus aureus reach high concentrations outside the cell wall (Medeiros 1984). These enzymes are the organisms natural defence against beta-lactam antibiotics. The gram-negative enzymes may inactivate the antibiotic within the cell by binding to the antibiotic or hydrolyses of the beta-lactam ring. In the latter case the enzyme attaches to the antibiotic, and forms an intermediate complex, which when broken down, has no antibacterial properties (Matthew 1979).

According to Murray and Moelling (1978) the beta-lactamases have been classified basically into two broad types:

* The cephalosporinases which are active against cephalosporins. They are often inducible and mostly chromosomally mediated.

* The penicillinases which have activity against penicillin, ampicillin and cephalosporins. They are produced constitutively and are mostly plasmid-mediated. Within the penicillinases two major subtypes can be identified viz. the T.E.M. peni-

cillinases and the "O" type penicillinases.

Constitutive enzymes are plasmid mediated and are produced irrespective of the presence of a substrate (Sanders 1986). On the other hand, inducible enzymes are produced when the organism is exposed to an enzyme inducer. These enzymes are classified by Richmond and Sykes as type 1 enzymes. They are distinctive for their susceptibility to inhibition by cloxacilin but not clavulanic acid (Sykes and Matthew 1979). Type 1 beta-lactamases can be induced by one of two mechanisms (Sanders and Sanders 1986):

* Exposure of wild type organism to an enzyme inducer;
* Spontaneous mutation.

The working mechanisms of beta-lactamase induction or derepression has been described by Sanders (1986). Regardless of the mechanism involved, the organisms are resistant to most of the beta-lactam antibiotics, once induction of the beta-lactamases has occurred (Sanders and Sanders 1985). These mechanisms of resistance have been demonstrated by Bryan and co-workers (1984) and this indicates that resistance to beta-lactam antibiotics is related to the capability of chromosomal beta-lactamases to hydrolyze a beta-lactam substrate.

The gram-negative bacteria known to possess beta-lactamases are: (Sanders 1984, Quintiliani 1984)

* Inducible : Enterobacter spp., Proteus spp., Citrobacter spp., Serratia spp., Acinetobacter sp. and Pseudomonas aeruginosa.

* Constitutive : E. coli, Haemophilus spp.

The problems with inducible beta-lactamases are that they are not evident at initial laboratory sensitivity testing whereas the constitutive beta-lactamases will be discernable by laboratory testing (Quintiliani 1984).

Another possible mechanism of bacterial resistance have been described by Then and Angehrn (1982) who suggest "trapping" of non-hydrolyzable cephalosporins by cephalosporinases produced by Enterobacter cloacae and Pseudomonas aeruginosa. According to the authors the enzymes bind to the antibiotic molecules and prevent them from binding to the penicillin binding proteins. The antibiotic was "trapped" in an inactive form.

2.2.2.2 Aminoglycoside modifying enzymes

One of the best known mechanisms of resistance to the

aminoglycosides results from several types of amino-glycoside - modifying enzymes, most of which are plasmid-mediated. These enzymed account for most of the resistance amongst gram-negative bacteria (Price et al. 1981, Phillips et al. 1986, Woods et al. 1972, Botha et al. 1981). A review done by Mayer (1986) illustrates the aminoglycoside resistance patterns worldwide and these are mostly related to inactivating enzymes mediated by plasmids. These aminoglycoside-modifying enzymes have been described by Phillips and Shannon (1984) and are devided into three classes:

* Acetyltransferases (AAC)
* Adenylyltransferases (AAD)
* Phosphatransferases (APH)

These enzymes act on the amino or hydroxyl group on the aminocyclitol ring of the aminoglycoside. The amino or hydroxyl groups are numbered, depending upon the ring they are in and their location within the ring. The enzymes are then numbered accordingly, for example acetyltransfarases 2 (AAC (2))(Murray and Moellering 1978).

2.2.2.3 Chloramphenicol acetyltransferase

Chloramphenicol was made available for clinical use in 1948. Resistance to the antibiotic soon developed but the recognition of plasmid-mediated transmission of

antibiotic resistance was only made in 1955 (Watanabe 1963). In 1964 it was observed that strains of E. coli carrying transmissible elements were able to inactivate chloramphenicol (Miyamura 1964). It soon became clear that resistance to chloramphenicol was plasmid-mediated due to an enzyme chloramphenicol acetyltransferase (CAT). The antibiotic was inactivated by acetylation of the hydroxyl group (Shaw 1967, Okamoto et al. 1967).

2.2.3 DECREASED PERMEABILITY

The presence of an added layer, a permeability barrier on gram-negative bacteria has long been suspected but its molecular architecture and properties have only been elucidated recently (Nikaido and Vaara 1985). According to Nikaido (1986) this barrier corresponds to the outer membrane, a structure located outside the cytoplasmic membrane and peptidoglycon layer. In this outer membrane porins were discovered and studies show that every gram-negative species produce these proteinaceous porins or channels through which nutrient molecules or antibiotics can travel (Nikaido 1986, Kaneko et al. 1984). This mechanisms of resistance can be naturally occurring or acquired.

2.2.3.1 Naturally occurring

The ability of the added layer to retard the entry of different antimicrobial agents varies from species to species. Gram-negative bacilli are absolutely resistant to penicillin G whereas gram-positive bacteria do not possess this permeability layer (Suginaka *et al.* 1975). An example of a naturally occurring permeability barrier is the resistance of enterococci to gentamicin.

The enterococci are only inhibited *in vitro* by high concentrations of gentamicin. When gentamicin is combined with a penicillin, the enterococci are inhibited at much lower concentrations. This is explained by the fact that the penicillins interfere with the bacterial cell wall synthesis and allows easier penetration of the gentamicin (Glew *et al.* 1975).

2.2.3.2 Acquired decreased permeability changes

Bacterial resistance can develop from "closing" of some proteinaceous porins and these permeability changes may result from protein deletions. Such a permeability barrier has been described by Burns *et al.* (1985) for chloramphenicol resistance in *Haemophilus influenzae*. A study by Gutmann *et al.* (1985) revealed that a possible single-step mutation involving outer membrane proteins, can provide permeability

changes resulting in cross-resistance to noladixic acid, trimethoprim, and chloramphenicol by using any of these antibiotics. Studies by Bush et al. (1985) suggest that the major cause of resistance to beta-lactam antibiotics in strains of Enterobacter cloacae was a lack of penetration across the outer membrane. Similar finding were observed by Werner et al. (1985).

2.3 ANTIBACTERIAL CHEMOTHERAPEUTIC AGENTS

In hospitalised patients with severe life-threatening infections the selection of appropriate antimicrobial chemotherapy depends on many factors. From the initial clinical diagnosis of the patient, chemotherapy is selected on the most likely pathogens or pathogens to be found in a given disease state. A knowledge of the susceptibility patterns of specific bacterial strains is always helpful. Patients with leukopenia, serious organ failures, malignancy, burns, septic shock are immuno-compromised and initial antimicrobial therapy should be able to drastically reduce the bacterial load and give a broad coverage as the infecting organisms are usually not known at that stage. The aminoglycosides combined with a beta-lactam antibiotic is usually the drugs of choice (Baltch and Smith 1985, Klastersky et al. 1980, Gaya 1986, Holloway 1986).

2.4 AMINOGLYCOSIDES

The aminoglycosides (aminoglycosidic aminocyclitols) must surely be the most important class of antimicrobial agents available today. They continue to be the drugs of choice in the treatment of gram-negative bacillary infections.

The first aminoglycoside to be discovered was streptomycin (Waksman et al. 1944). They isolated streptomycin from streptomyces griseus and after development streptomycin was used in clinical medicine. Since then various aminoglycosides have been developed viz. neomycin, kanamycin, gentamicin, tobramycin, sisomicin, amikacin and netilmicin.

The aminoglycosides differ amongst themselves by the amino sugars attached to the aminocyclitol nucleus. Their chemical similarities give them similar properties, such as absorption, distribution, excretion and toxicity, but their antibacterial spectrum and clinical applications differ. Generally the clinical use of the aminoglycosides are:

(i) Severe infections due to gram-negative bacteria (Moellering 1986).

(ii) Combined with a beta-lactam antibiotic for treating serious infections caused by Pseudo-

monas aeruginosa (Lau et al. 1977, Love et al. 1979).

(iii) Infections following colo-rectal or gynaecological surgery or prophylaxis in colo-rectal surgery (Clarke et al. 1979).

(iv) Enterococcal bloodstream infections in combination with penicillin G. (Gutschik et al. 1977).

(v) Systemic staphylococcal infections in combination with a penicillin (Steigbigel et al. 1975).

The advantages and disadvantages of the aminoglycosides are well known (Siegenthaler et al. 1986, Waldvogel 1984). They are chemically stable drugs with broad spectrum antibacterial activity. Their synergistic effect with beta-lactam antibiotic are well known. The main advantages are:

* Chemical stability;
* Broad antibacterial spectrum;
* Rapid bactericidal action;
* Experience over many years;
* Race allergic side effects;
* Synergism with beta-lactam antibiotics.

The main disadvantages are:

* The lack of absorbsion after oral administration;
* Poor distribution within the host especially into the serebrospinal fluid;
* Nephrotoxicity and ototoxicity are of major importance when using aminoglycosides and this is due to the narrow therapeutic range between suboptimal serum concentrations and toxic levels. The toxicities to the kidney and the inner ears have been compared in many studies (Fong et al. 1981, Kahlmeter et al. 1978, Lerner et al. 1977).

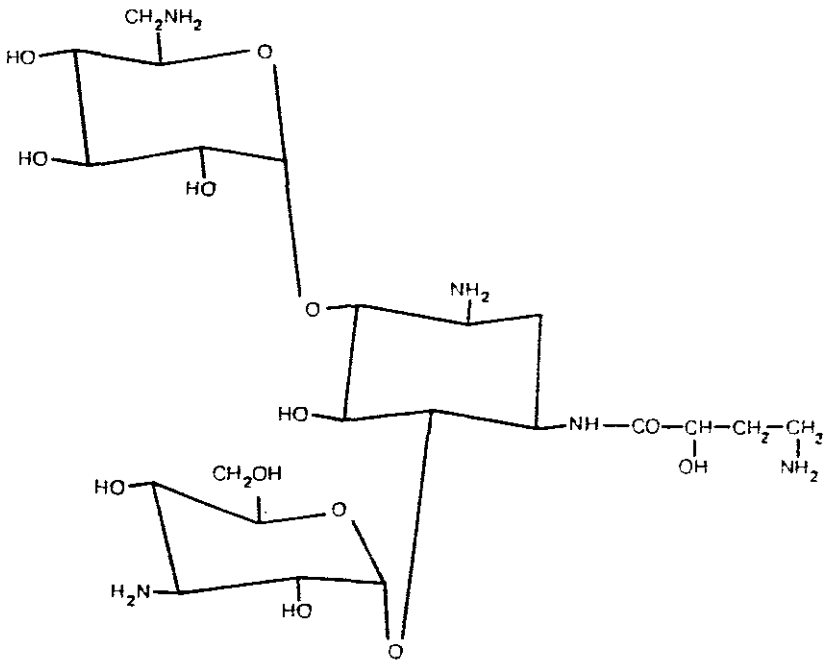
2.4.1 AMIKACIN

Resistance to the other aminoglycosides led to the development of amikacin. It is a semisynthetic derivative of kanamycin A, developed by chemically modifying the basic kanamycin structure to protect it from the inactivating mechanisms of resistant organisms. The molecular formula of the compound in its free base form is C22H43N5 013 (Ristuccia and Cunha 1985). The structure of amikacin is shown in fig. 2.1.

2.4.1.1 Mechanism of action

The bactericidal activity of amikacin is similar to that of gentamicin and tobramycin (Finland et al. 1976). Like the other aminoglycosides it acts direct-

Fig 2.1 STRUCTURE OF AMIKACIN



ly on the 30s and 50s bacterial ribosomal sub-units to inhibit protein synthesis. The drug is transported across the cell membrane and with the marked accumulation of the drug within the cell, the inhibition of the protein synthesis takes place (Ristuccia and Cunha 1985). The rate of transport of the drug across the cell membrane can be altered by the presence of divalent cations, the pH of the environment and the amount of oxygen present. In trials done by Young and Hewitt (1973) it was observed that the antimicrobial activity of the aminoglycosides was significantly reduced in an anaerobic or acidic environment.

2.4.1.2 Spectrum of activity

Amikacin is active against a wide variety of gram-negative bacteria (Knothe 1976). Studies have shown that amikacin is especially effective against isolates of Pseudomonas aeruginosa and Enterobacteriaceae resistant to gentamicin and tobramycin (Tally et al. 1975, Meyer et al. 1975, Young and Hindler 1986). This has been one of the major advantages of amikacin as proven clinically by successful treatment of patients infected with resistant organisms where other aminolgycoside therapy had failed (Sharp et al. 1974).

The structural difference of amikacin to gentamicin and tobramycin makes it less vulnerable to inactivation by aminoglycoside inactivating enzymes. This

makes amikacin useful against gentamicin and tobramycin resistant bacteria, which can be explained by the fact that gentamicin and tobramycin are vulnerable to inactivation by eight of the aminoglycoside-modifying enzymes, but amikacin is only effected by two of these enzymes.

Studies have shown that emergence of resistant strains to a particular aminoglycoside occurred following an increase in the use of the drug (Betts et al. 1984), however, increase in the usage of amikacin has not led to the increase of resistance against the drug (Price and Siskin 1984, Saavedra et al. 1984). Studies by Price et al. 1981) found that intensive use of amikacin led to a decrease in resistance to gentamicin and tobramycin among gram-negative bacteria. These findings were similar to those by Levine et al. (1985) and Larson et al. (1984). Resistant G.N.B. isolated at Tygerberg hospital have already shown resistance to amikacin (unpublished data). In a trial done by Michea-Hamzehpour et al. (1986) on mice it was observed that bacterial resistance developed soon after monotherapy was administered but that this did not occur with amikacin monotherapy. As gentamicin and tobramicin resistance occurs in certain G.N.B. isolated at Tygerberg hospital, amikacin is the aminoglycoside of choice, therefore it was chosen for this project.

2.5 BETA-LACTAM ANTIBIOTICS

The cephalosporins remain the largest single class in the beta-lactam group of antibiotics. These agents have been developed generally with activity against gram-negative bacteria (Neu 1982, Schumacher 1983). The cephalosporins may be classified by those chemical structure, clinical pharmacology, beta-lactamase resistance or their antibacterial spectrum. The accepted generation scheme are based on general features of antimicrobial activity and these groups are (Mandell 1985):

First generation

Cephalexin

Cephradine

Cefaclor

Cefadroxil

Cephalothin

Cephaloridine

Cephazolin

Second generation

Cefuroxime

Cefamandole

Cefoxitin

Third generation

Cefotaxime

Latamoxef

Cefsulodin

Cefoperazone

Ceftriaxone

Ceftazidime

The second and third generation cephalosporins were developed to withstand the various beta-lactamases produced by gram-negative bacilli. These drugs exhibit activity against Enterobacteriaceae and Pseudomonas aeruginosa (Fass 1983, Farber and Moellering 1982). These third generation cephalosporins are not intrinsically nephotoxic and none exhibit ototoxicity. They have a fairly wide toxic/therapeutic ratio and it was reasoned that these antibiotics could replace the more toxic aminoglycosides (Harper 1981). Unfortunately the emergence of resistance to these agents have resulted after their use alone in serious infections (Moellering 1982, Bittner et al. 1983, King et al. 1983).

2.5.1 CEFTAZIDIME

This antibiotic was discovered in a research program by Glaxo and was designed to find a substance for the treatment of serious infections due to a wide range of

gram-negative bacteria including Pseudomonas aeruginosa. This compound had to be resistant to inactivating bacterial enzymes. It had to be bactericidal, show good penetration of the bacterial cells, and had to have good pharmokinetic properties. Ceftazidime satisfied all of these requirements (Muggleton 1981). The chemical structure of ceftazidime is (6R, 7R) -7- (Z) -2 (2-Aminothiazol -4-YL) -2- (2-Carboxyprop -2- Yloxymino) Acetamido -3- (Pyridimium -1- Ylmethyl) Ceph -3- EM -4- Carboxylate) and is derived from cephalosporin C. The structure of ceftazidime is shown in fig. 2.2.

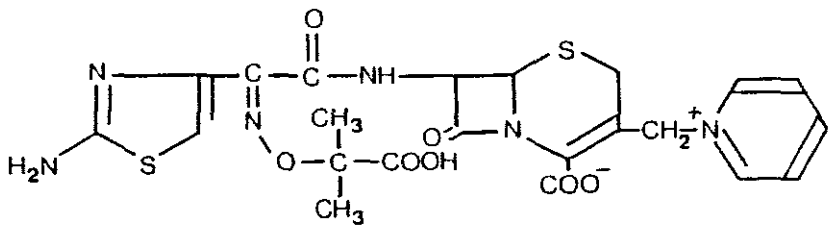
2.5.1.1 Mechanism of action

Beta-lactam antibiotics bind to specific targets located in the cytoplasmic membrane of bacteria to exert their inhibitory effect. These target proteins are termed penicillin-binding proteins (P.B.P's). The beta-lactams, such as ceftazidime, inhibit the P.B.S.'s as substrate analogs of the acyl -D- alanyl -D- alanine component of peptidoglycan (Tipper 1985). Ceftazidime has efficient penetration of the bacterial cell and has resistance to bacterial enzyme degradation (Muggleton 1981).

2.5.1.2 Spectrum of activity

In-vitro studies show that ceftazidime has broad-

Fig 2.2 STRUCTURE OF CEFTAZIDIME



spectrum antibacterial activity against gram-negative bacilli including Pseudomonas aeruginosa (Brumfitt and Hamilton-Miller 1981, Wilkinson and Gentry 1981, Verbist and Verhaegen 1981, Harper 1981). Favourable results were obtained by clinical studies done by Mandell et al. (1983) who suggested the use of ceftazidime as single-drug therapy in the treatment of hospitalised patient with pneumonia. In clinical studies done by Van Dalen et al. (1983) patients in an intensive care unit were treated with ceftazidime and these results show that this drug is an effective and relatively safe agent for the treatment of serious infections caused by gram-negative bacilli including Pseudomonas aeruginosa. In patients with septicaemia, ceftazidime proved to be effective and excellent cure rates were recorded (Wardle 1985 and Gozzard et al. 1982). Ceftazidime has also proved to be safe and effective when used as first-line monotherapy to treat febrile episodes in neutropenic and immunocompromised patients (Parapia 1985).

A very important factor to take into consideration when selecting an antibiotic with broad-spectrum activity is its effect on colonisation resistance. Ceftazidime has very little activity against anaerobic bacteria and this preservation of flora prevents the establishment of resistant pathogens.

So far the in-vitro activity and clinical efficiency

of ceftazidime are impressive but unfortunately there have been reports of adverse affects viz. resistant strains developed, following ceftazidime treatment (Scully and Neu 1984). In a study by Cone et al. (1985) three patients developed significant superinfection during ceftazidime therapy alone. Four out of twenty-one patients receiving ceftazidime had a positive direct coomb's test, but none demonstrated any significant hemolysis.

2.6 MONOTHERAPY VERSUS COMBINATION THERAPY

It seems, judging from the multiple resistance found in gram-negative bacilli in nosocomial infection, that monotherapy does have its disadvantages. No antibiotic has complete coverage of all infecting organisms and superinfection has been reported (Gribble et al. 1983). At present monotherapy in the critically ill patient is difficult to justify as therapeutic failures do occur because of the emergence of resistance (Wardle et al. 1981, King et al. 1983, Eron et al. 1983, Maslow et al. 1983, and Bouza et al. 1983). Other workers have stated that monotherapy may be inadequate as compared to combination therapy (Bodey et al. 1977 and Rahal 1978).

Combination therapy dates back to the beginning of the antimicrobial era. When streptomycin and kanamycin were deficient in their activity against streptococci,

the addition of a penicillin was necessary. According to Young (1984) the rationale for combination antibiotic therapy includes the following:

* No single agent has true broad-spectrum coverage whereas combination therapy offers a comprehensive spectrum of activity.

* To decrease the rate of emergence of resistance.

* To enhance antimicrobial activity by means of additive or synergistic interactions.

* To permit dosage reduction of the individual components of a regimen in order to avoid toxicity.

The main disadvantages of combination therapy have been discussed by Cohen (1975) and some of the most important ones are:

* Drugs selected for their broad spectrum activity will frequently be suboptimal for the specific organism in the particular case.

* A multiplicity of drugs may be physically difficult to administer.

* Adverse drug reactions increase with the number of drugs administered.

* A combination of antibiotics may minimize the likelyhood that the original infecting organism will become resistant, but probably increase the likelyhood of the patient's gastro intestinal tract becoming repopulated with organisms resistant to both antibiotics.

* Microbiologic antagonism of the good effects of one antibiotic by another.

2.7 SYNERGISM, ANTAGONISM AND INDIFFERENCE

The word synergism is derived from the Greek word synergos and means "to work together". It is defined in Websters dictionary as "cooperative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently". In relation to antibiotics one could define synergism as the ability of two drugs acting together to increase the rate of bactericidal action, as compared when either drug is used alone. There are unfortunately no universally accepted definitions of the terms "synergism, antagonism and indifference". According to Jawetz (1967) "antagonisms may be defined to those instances where a combination of antimicrobial agents results in a total effect smaller than that produced by the more effective single member of the combination when acting alone. This can be demonstrated in vitro by a decrease either in the inhibitory activity or in

the bactericidal rate of a drug combination below that of its components.

In the category of "indifference" the combined effect of the two antibiotics is equal to that of the combination or is equal to the arithmetic sum of the effects of the two individual drugs.

Combination of antimicrobial agents may act synergistically by a number of mechanisms (Lacey 1958). Unfortunately there is no single *in vitro* test method to detect all these synergistic interactions. There are different methods employed to detect *in vitro* synergism and the one most often used is the agar dilution method or checkerboard titration. The definition of synergism in interpreting the checkerboard method involves a fourfold or greater decrease of each drug used in combination as compared with the M.I.C. of each drug alone. This decrease can be demonstrated on a graph by the method as described by Sabath (1967). A variant of this is the interaction index (Berenbaum 1978). An important fact to take into consideration in interpreting these results is that synergism may not be clinically relevant because such an interaction occurs only with concentrations of antibiotics higher than those that can be achieved in the patient. Recently, Norden *et al*. (1979) compared various *in vitro* methods for the measurement of synergy between two antibiotics and these were the standard

checkerboard method, calculation of an interaction index, the construction of isoboles, and killing curves. He found that the discrepancies between the checkerboard on the one hand and the interaction index and isobole methods on the other are an artifact due to the conventional design of the checkerboard method and the need to allow for error. If this artifact is allowed for, all these methods give concordant results. The killing-curve method as generally used does not enable one to determine synergy and should not be used for this purpose. The purpose of this study is not only a comparitive *in vitro* study of amikacin and ceftazdime, but also to determine whether the combination of amikacin and ceftazidime would prove synergistic *in vitro* against selected resistant gram-negative bacilli isolated from patients at Tygerberg hospital. Although similar *in vitro* studies have been done elsewhere (Gombert and Aulicino 1986) one must keep in mind that a combination of two antibiotics may react differently against a specific microbial strain and that resistance patterns differ worldwide. It is thus incorrect to state that a combination is synergistic without naming a specific microbial strain.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Fortum ceftazidime pentahydrate was supplied by Glaxo Pharmaceuticals S.A.

Amikacin base was supplied by Bristol/Mead Johnson S.A.

All sensitivity discs used in the study were manufactured by Oxoid and supplied by C.A. Milsch.

Oxoid Mueller-Hinton Broth and Mueller-Hinton sensitivity agar were supplied by C.A. Milsch.

MacFarland 0,5 standard prepared by adding 0,5ml of 0,048M $BaCL_2$ to 99,5ml 0,36N H_2SO_4.

Petri dishes supplied by Protea Laboratory Services.

Falcon sterile disposable pipettes supplied by Laboratory and Scientific.

3.2 INTRODUCTION

Susceptibility tests are done in the laboratory to determine the *in vitro* sensitivity of organisms to assist in determining appropriate antibiotic therapy. Susceptibility testing are done on potential pathogens, especially those organisms that are known to develop resistance when exposed to antimicrobial agents. The discs sensitivity method and the agar dilution method for minimum inhibitory concentrations will be the methods used for susceptibility testing in this study.

3.3 ORGANISM SELECTION

Gram negative bacteria isolated from sputa, wounds, blood and urine from patients at Tygerberg Hospital, were investigated. Organisms presenting resistance to the different cephalosporins and aminoglycosides were selected for this trial. These organisms were:

Klebsiella pneumoniae,
Escherichia coli,
Serratia marcescens,
Enterobacter spp.,
Pseudomonas aeruginosa, and
Acinetobacter anitratus.

These gram negative bacteria were isolated and identified according to the methods described by Cowan and Steel (1974). The test organism were collected over a period of eighteen months and kept on nutrient agar slopes at $4^{o}C$. Disc sensitivity tests were done on these organisms against a variety of antibiotics, including ceftazidime and amikacin. Zone sizes were measured and compared to a reference chart. Minimum inhibition concentrations of amikacin and ceftazidime were determined against these organisms. Organisms resistant to amikacin or ceftazidime were selected for synergy testing.

3.4 ANTIMICROBIAL DISC SUSCEPTIBILITY TESTING

The disc diffusion susceptibility test has been the method of choice for determining the sensitivity of organisms to antibiotics. In these techniques paper discs impregnated with specific antibiotics are placed onto the surface of special agar medium which had been inoculated with the test organism. The antibiotic diffuses radially from the disc into the medium and growth of the test organism is inhibited in a zone around the antibiotic disc if the organism is susceptible to the antibiotic. Different methods have been developed over the years, and currently the methods in use are:

* The Kirby-Bauer and Ericsson methods,

* The Comparitive and Stokes methods.

The latter methods are favoured by British laboratories. These methods compare the zone of inhibition in respect of a test organism with that of a antibiotic susceptible control organism. These methods do not require the rigid standardization of the Kirby-Bauer method.

The Kirby-Bauer method on the other hand specifies the medium to be used, the inoculum density and antibiotic disc content.

The reliability of the disc diffusion methods depend on several factors influencing performance. These include pH, depth of medium, growth rate of the organism, incubation time and temperature.

The standardized disc diffusion method developed by Bauer et al. (1966) is the method currently recommended by the National Committee for Clinical Laboratory Standards (1975) and is also the method recommended by the South African Institute for Medical Research.

3.4.1 KIRBY-BAUER METHOD

3.4.1.1 Medium

Mueller-Hinton agar prepared and sterilised according

to the manufacturer's instructions. The pH of the medium was adjusted to be within the range 7.2 - 7.4. The medium was dispensed into sterile petri-dishes to give a depth of between four and six millimetre (25 ml. per plate). Plates were stored at 2 - 8°C and used within seven days. Before use, these plates were dried with the lids ajar in an incubator for about fifteen to twenty minutes.

3.4.1.2 Inoculation of test plates

At least four to five isolated colonies of the same morphological type on a MacConkey agar plate were selected. Each colony was touched with a sterile wire stab and transferred to a tube with 4ml of Mueller-Hinton broth.

The inoculated test tubes were incubated in a water-bath at 37°C until a turbidity was achieved comparable to a MacFarland 0.5 standard. If the turbidity exceeded that of the MacFarland standard, the test broth was diluted with sterile broth until it compared with the turbidity of the turbidity standard.

Within fifteen minutes after adjusting the turbidity of the test broth's, a sterile cotton swab on a wooden applicator was dipped into the adjusted test broth. Excess fluid was removed from the swab by rotating the swab several times against the inside wall of the test

tube.

The entire surface of the Mueller-Hinton sensitivity agar plates were streaked with the swabs. It is important that the surface of the sensitivity agar plates must be dry.

Within fifteen minutes the sensitivity discs were applied with a sterile needle to the inoculated plates. The plates were inverted and incubated at $35^{o}C$.

After eighteen hours of incubation the plates were examined. The zones of inhibition around the antibiotic discs were measured to the nearest millimeter.

To control the accuracy and precision of the test procedure standard control strains such as E. coli (NCTC 10418) and Ps. aeruginosa (NCTC 10662)were included.

The antibiotic disc concentrations used in this trial are shown in table 3.1.

The zone sizes were interpreted by reference to a interpretive standards chart as shown in table 3.2.

3.5 MINIMUM INHIBITION CONCENTRATION (M.I.C.)

Minimum inhibition concentrations can be performed in broth or agar dilutions. The agar dilution method was selected in preference to the broth method because of its better reproducibility and its economy in time and material (Ericsson and Sherris 1971). The broth dilution has the advantage that the minimum bactericidal concentrations (M.B.C.) can be determined by transferring broth to nutrient agar for counting surviving organisms. In this study the agar dilution method as described by the International Collaborative Study Group was used (Ericsson and Sherris 1971). The M.I.C. can be termed as the lowest concentration of an antibiotic at which no visible bacterial growth occurs for a given bacterial strain.

TABLE 3.1

ANTIBIOTIC CONTENT OF DISCS

ANTIBIOTIC	DISC CONTENT µg
Amikacin	30
Ampicillin	10
Ceftazidime	30
Cefamandole	30
Cefoxitin	30
Gentamicin	10
Tetracycline	30
Trimethoprim-sulfamethoxazole	1.25/23.75
Tobramycin	10

ZONE DIAMETER INTERPRETIVE STANDARDS CHART

Antimicrobial Agent	Disc Content	Zone Diameter, nearest whole mm			Approximate M.I.C. Correlates	
		Resistant	Intermediate	Susceptible	Resistant	Sensitive
Amikacin	30 µg	14 or less	15-16	17 or more	32mg/L or more	16mg/L or less
Ampicillin - when testing gram-negative enteric organisms and enterococci	10 µg	11 " "	12-13	14 " "	32mg/L " "	8mg/L " "
Cefamandole	30 µg	14 " "	15-17	18 " "	32mg/L " "	8mg/L " "
Gentamicin	10 µg	12 " "	13-14	15 " "	8mg/L " "	4mg/L " "
Tetracycline	30 µg	14 " "	15-18	19 " "	12mg/L " "	4mg/L " "
Trimethoprim-sulfamethoxazole	1.25/23.75 µg	10 " "	11-15	16 " "	8/152mg/L " "	2/38mg/L " "
Tobramycin	10 µg	12 " "	13-14	15 " "	8mg/L " "	4mg/L " "
Ceftazidime	30 µg	14 " "	15-17	18 " "	32mg/L " "	16mg/L " "
Ceftoxitin	30 µg	14 " "	15-19	20 " "	64mg/L " "	16mg/L " "

3.5.1 ANTIBIOTIC STOCK AND WORKING SOLUTIONS

Ceftazidime stock solution was prepared by weighing out ceftazidime pentahydrate. The potency of the powder viz. 116mg base equivalent to 100mg active ceftazidime has to be allowed for in the preparation of the stock solution. The powder was dissolved in 0.2% sodium carbonate to give a stock solution of 5120mg/L.

Similarly amikacin base powder was weighed and diluted with sterile water to give a stock solution of 5120mg/L.

These stock solution were distributed in 10ml aliquots and stored in a deep freeze. The concentrations are stable for one month at $-20^{o}C$.

Working solutions were prepared by two fold dilutions of the stock solutions with sterile water to a concentration of 2.5mg/L.

3.5.2 PREPARATION AND INOCULATION OF AGAR PLATES

Test plates were prepared by dispensing 19ml of Mueller-Hinton sensitivity agar as used in 3.4.1.1 into sterile screw-top glass bottles. On the day of use these stock agars were melted in a steamer at $100^{o}C$ and allowed to cool to more or less $50^{o}C$

in a 50°C water bath.

From the working antibiotic solutions one ml was added to the cooled agar and mixed gently. The agar was poured immediately into a sterile Petri dish and allowed to harden. These agar dilutions gave a final concentration of 256mg/L to 0,0625mg/L of each antibiotic used in the trial. A control plate without antibiotic was also made. The plates were dried at 37° for one hour.

The test organisms were inoculated in Mueller-Hinton broth and incubated in a waterbath at 37°C to obtain an inoculum comparable to a MacFarland 0.5 turbidity standard. The number of colony forming units per ml achieved in this manner are approximately 1 x 10^8C.F.U./ml. To obtain an inoculum of 10^5 - 10^6C.F.U./ml the standardized broth was diluted 1:50 with sterile broth.

To inoculate the agar plates a Denley multipoint inoculator was used and worked on the same principles as the one described by Steers et al. (1959).

The multipoint inoculator is a electrically driven machine that permits the inoculation of twenty different organisms onto an agar plate. A replicating pin head consisting of twenty pins and a marker transfer a fixed amount of inoculum onto the agar plates.

The seeded plates were incubated at 37°C for eighteen hours. The plates were examined for growth. The position of the inocula were identified with a template (Ericsson and Sherris 1971).

3.6 TESTS OF COMBINED ANTIBACTERIAL ACTION

To demonstrate whether the interaction between two different antimicrobials are synergistic, indifferent or antagonistic, the following methods can be used viz. diffusion method or the dilution method. These interactions all involve the static action of the drugs concerned and are demonstrable by both methods.

3.6.1 DIFFUSION METHODS

One of the easiest ways of demonstrating synergy between two antimicrobials, is by placing paper strips impregnated with antibiotics at right angles to each other on an agar plate inoculated with the test organism. This method has the advantage of demonstrating the activity of each antimicrobial separately as well as the combined action of both antimicrobials (Garrod and Waterworth, 1962). An example of this is demonstrated in fig. 3.1.

The same effect can be demonstrated by using antibiotic discs. The distance of the discs are critical and if they are placed too far apart the effect will

not be seen.

These tests are simply discriptive and the results obtained from these methods are questionable for application in clinical situations (Sabath et al. 1967).

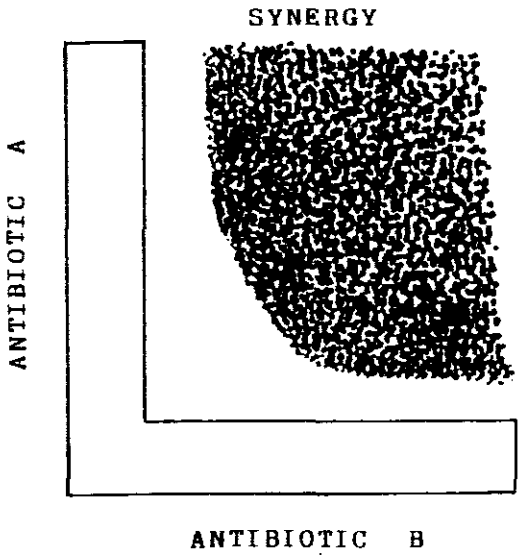
3.6.2 AGAR DILUTION METHOD

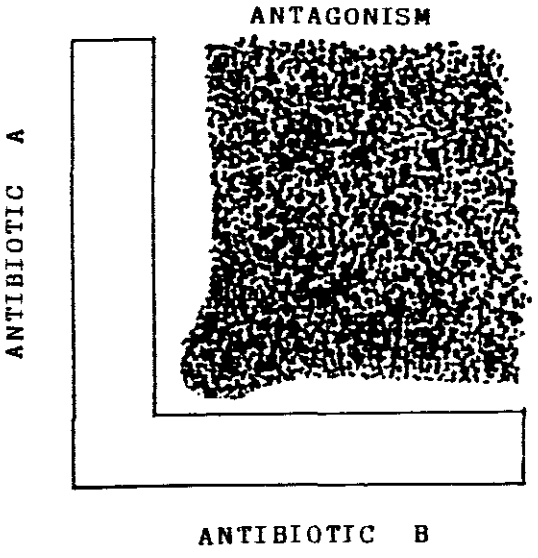
The interaction of two drugs can be more precisely determined by performing a checkerboard titration according to the principles described by Sabath et al. (1967). In this method a suitable range of dilutions of both drugs are added to agar so that every concentration of each is present alone, and in every possible combination with the other, and the whole test inoculated with a standard inoculum.

3.6.2.1 Antibiotic stock and working solutions

Amikacin and ceftazidime stock solutions were prepared as described in section 3.5.1. This gave stock solutions of 5120 mg/L. Working solutions were prepared from each antibiotic by double diluting with Meuller-Hinton broth to a concentration of 2.5 mg/L. From each antibiotic working concentration 0,5 ml was added to 0,5 ml of each possible concentration of the other antibiotic, for example 2,0 mg/L of amikacin was mixed with every concentration of ceftazidime.

Figure 3.1: Illustrations of synergism by use of the difussion method





3.6.2.2 Preparation and inoculation of test plates

Meuller-Hinton agar base was prepared as described in section 3.5.2. The 19 ml agar stock in the glass bottles were melted and allowed to cool to $\pm 50^{o}C$. From every different combination of amikacin and ceftazidime together as well as alone, one ml of each was added to the cooled agar. This was gently mixed and poured into a sterile Petri dish. This gave agar plates with antibiotic concentrations ranging from 0,0625 mg/L to 128 mg/L.

The test organisms were inoculated and adjusted as described in section 3.5.2. The inoculation of the test plates was done with a Denley multipoint replicator as described in section 3.5.2.

The seeded plates were incubated at $37^{o}C$ for eighteen hours. The test plates were again read against a template and plotted on a checkerboard chart as shown in figure 3.2.

3.7 FRACTIONAL INHIBITORY CONCENTRATION INDEX

The third way of expressing results from the checkerboard titrition was to calculate the interaction index (Berenbaum 1978). In this procedure the fractional inhibitory concentrations of each antibiotic was determined, ie. AC/AB where AC is the concentrations

of the antibiotic A in combination and AB the M.I.C. when A is used alone. Similarly the index of antibiotic B is determined the same way. When the sum of the two fractions is less than one, the combination is regarded as synergistic. When the sum is greater than one, the combination is antagonistic, for example AC/AB + BC/BB = less than one. The reaction is thus regarded as synergistic.

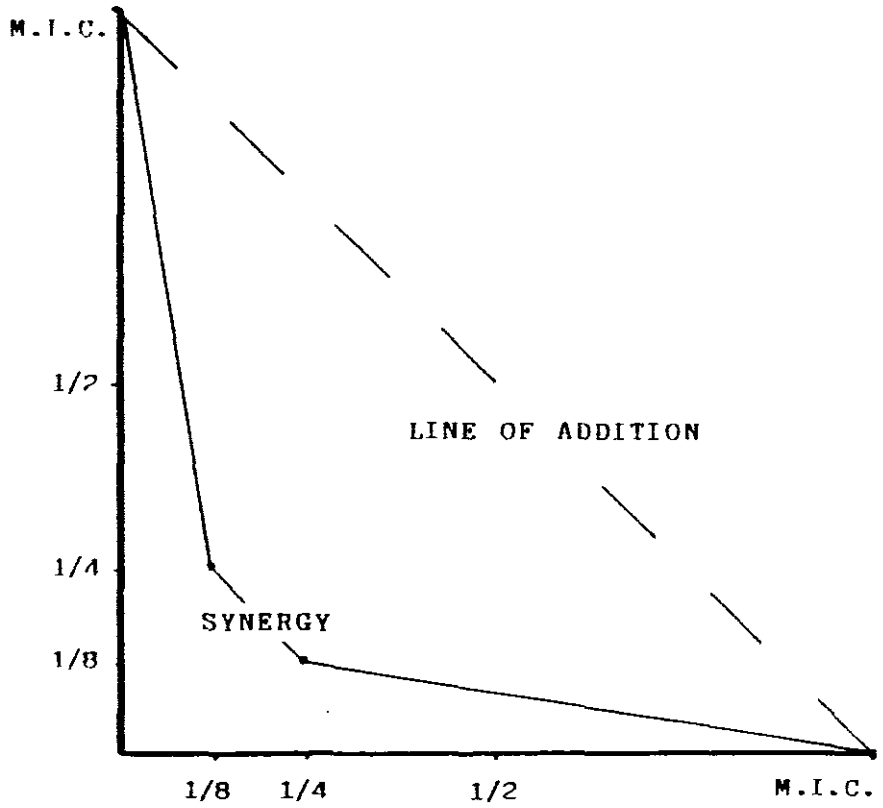
3.8 ISOBOLOGRAMS

By using the results obtained from the checkerboard titration, the interaction between the two antibiotics can be expressed in the form of a isobologram. Results were plotted on a arithmetric scale as described by the method of Loewe (1953) and reviewed by Lacey (1958). Increasing concentrations of amikacin and ceftazidime were plotted on the ordinate and abscissa respectively. Each point represented the lowest concentration of antibiotics required to inhibit growth. The line joining the points for each pair of antibiotics is termed an "isobol". Synergism was demonstrated if the line was concave (bowing towards the co-ordinates). An example of this principle is illustrated in figure 3.3.

Figure 3.2: Checkerboard titration worksheet

	AMIKACIN mg/L											
	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	0
128													
64													
32													
16													
8													
4													
2													
1													
0,5													
0,25													
0,125													
0,0625													
0													

Figure 3.3: Hypothetical isobologram



CHAPTER 4

RESULTS

4.1 DISC SUSCEPTIBILITY TESTS

A total of 128 G.N.B. were selected for disc susceptibility testing. The Kirby-Bauer method as described in sections 3.4.1 was used to determine the susceptibility of these organisms isolated from patients with nosocomial infections.

The zone size of each antibiotic tested was measured with a calipers to the nearest millimeter. Measurement of the zones included the entire diameter of the zone, including the disc. Table 3.2 contains the interpretive zone size information to determine susceptibility. The breakpoints were sensitive, intermediate and resistant.

It has been shown that an approximately linear relationship exist between log M.I.C. and zone size for organisms of comparable growth rate (Ericsson and Sherris 1971). Through this relationship the measurement of these zones can be used to predict *in vivo* response of the organism.

Regression lines expressing this relationship can be produced by performing minimum inhibition concentrations and disc susceptibility tests simultaneously on a large number of strains. Figure 4.1 illustrates such a regression analysis. The y- axis in the graph denotes the 2-fold M.I.C. susceptibility tests, whereas the x-axis is a non-log scale of the zone sizes. The scatter of values for most antimicrobials is linear and the formula of least squares will provide a mathematical computation of a regression line (Crosse et al. 1981). The zone size breakpoints relate to clinically achievable serum concentrations of antibiotics. Every antimicrobial has its own regression line and this information is contained in the Interpretive Standards Chart.

The results of the disc susceptibility tests of the G.N.B. chosen for this study are shown in tables 4.1 to 4.6.

4.2 MINIMUM INHIBITION CONCENTRATION

The test plates as inoculated in section 3.5.2 are read against a template to indicate the position of each strain tested. In reading end-points, a barely visible haze of growth or single colony was disregarded (Ericsson and Sherris 1971). Growth was recorded onto a worksheet. An example of such a recording of growth on a worksheet is shown in figure 4.2.

Figure 4.1: Regression line for ceftazidime

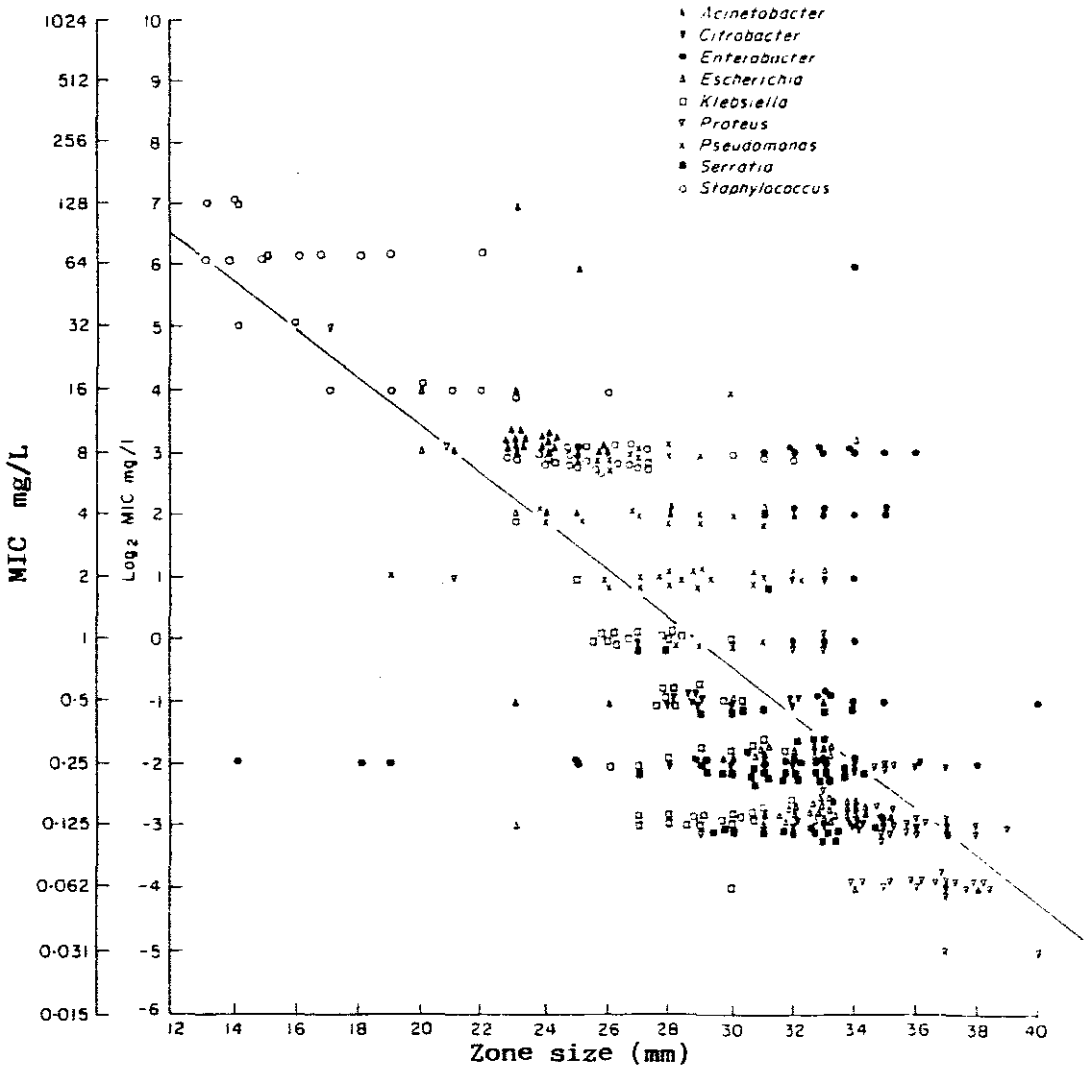


Figure 4.2: Worksheet for determining M.I.C. of amikacin against Escherichia coli

	Amikacin mg/L												
Strain	256	128	64	32	16	8	4	2	1	0,5	0.25	0.125	0.625
E. coli no. 6	-	-	-	-	-	-	-	+	+	+	+	+	+

\+ = indicates growth

\- = indicates no growth

M.I.C. = 4 mg/L

The M.I.C.'s of amikacin and ceftazidime against the test organisms are shown in table 4.7. to 4.12. In table 4.13 the inhibitory activities of amikacin and ceftazidime are shown.

4.3 COMBINED ANTIBACTERIAL ACTION

4.3.1 DIFFUSION METHOD

As mentioned in section 3.6.1 the diffusion method for testing synergy between two antimicrobials was not used. To illustrate this phenomenon, however, a selected strain of Pseudomonas aeriginosa was used and is shown in figure 4.3.

4.3.2 CHECKERBOARD TITRATION

Selected organisms with resistance to either amikacin or ceftazidime or both antibiotics were used for the checkerboard titration. The test plates were read against a template and plotted on a checkerboard chart as shown in figure 3.2. In figure 4.4 an example of a checkerboard titration is illustrated.

Synergy was reported when the concentration of each agent in combination was reduced to less than 25% of the amount required for each agent alone to inhibit growth (Norden et al. 1979). A less than four-fold reduction in the M.I.C. for both antibiotics was considered additive. Indifference was when neither drug exhibited a decrease in the M.I.C. Antagonism was when the concentration of each antibiotic increased when in combination to inhibit growth. Results of the checkerboard tirations on the test organisms are shown in table 4.14.

4.4 FRACTIONAL INHIBITORY CONCENTRATION INDEX

The results obtained from the checkerboard titration can be used algebraically to determine the interaction between the two antibiotics (Berenbaum 1978).

Figure 4.3: Illustration of synergism between amikacin and ceftazidime against Ps. aeruginosa by use of diffusion method.

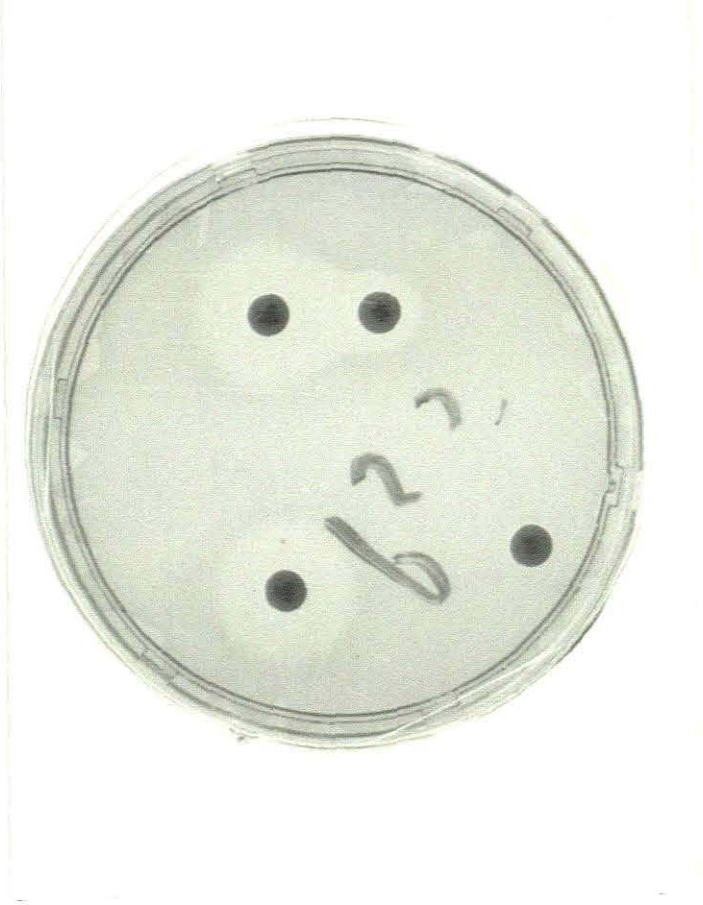


Figure 4.4: Illustration of a checkerboard titration of amikacin and ceftizidime against serratia marcescens

CEFTAZIDIME mg/L	AMIKACIN mg/L													
	128	64	32	16	8	4	2	1	0	0,5	0,25	0,125	0,0625	0
128														
64														
32									-	-	-	-	-	-
16							-	-	+	+	+	+	+	+
8					-	-	+	+	+	+	+	+	+	+
4				-	+	+	+	+	+	+	+	+	+	+
2				-	+	+	+	+	+	+	+	+	+	+
1				-	+	+	+	+	+	+	+	+	+	+
0,5				-	+	+	+	+	+	+	+	+	+	+
0,25				-	+	+	+	+	+	+	+	+	+	+
0,125				-	+	+	+	+	+	+	+	+	+	+
0,0625				-	+	+	+	+	+	+	+	+	+	+
0				-	+	+	+	+	+	+	+	+	+	+

M.I.C. of Amikacin : 16mg/L

M.I.C. of Ceftazidime : 32mg/L

\+ : Indicates growth

\- : No growth

Combined action : Amikacin and Ceftazidime

M.I.C. mg/L : 4 and 8

The equation as described in section 3.8 was used to determine synergy. Results are shown in table 4.15.

4.5 ISOBOLOGRAMS

The results obtained from the checkerboard titration can also be used to construct isobolograms. The M.I.C. results were plotted on a arithmetic scale according to the method described in section 3.7.

Results of these constructed isobolograms are shown in figure 4.5 to 4.27.

TABLE 4.1

ANTIBIOTIC SENSITIVITY PATTERNS OF *ESCHERICHIA COLI*

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
E. coli	1	R	R	S	R	R	S	S	S	S
	2	R	R	S	R	R	S	S	S	S
	3	R	R	S	R	R	S	S	S	S
	4	R	R	S	R	R	R	R	R	S
	5	R	R	S	R	R	R	R	R	S
	6	R	R	S	R	R	R	R	R	S
	7	R	R	S	R	R	R	R	R	S
	8	R	R	S	R	R	S	S	S	S
	9	R	R	S	R	R	S	S	S	S
	10	R	R	S	R	R	S	S	S	S
	11	R	S	S	S	S	S	S	S	S
	12	R	R	R	S	R	S	S	S	S

S - sensitive

R - resistant

I - intermediate

TABLE 4.2

ANTIBIOTIC SENSITIVITY PATTERNS OF
KLEBSIELLA PNEUMONIAE

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
Klebsiella	1	R	R	S	S	R	R	R	S	S
pneumoniae	2	R	R	S	S	R	S	S	S	S
	3	R	R	S	S	R	R	R	S	S
	4	R	R	S	S	R	R	R	S	S
	5	R	R	I	S	R	R	R	S	S
	6	R	R	S	S	R	R	R	S	S
	7	R	R	S	R	R	R	R	S	S
	8	R	R	S	S	R	R	R	S	S
	9	R	R	R	R	R	R	R	S	S
	10	R	R	S	R	R	S	S	S	S
	11	R	R	S	S	R	R	R	S	S
	12	R	R	S	S	R	R	R	S	S
	13	R	R	S	S	R	R	R	S	S
	14	R	R	S	R	R	S	S	S	S
	15	R	R	S	R	R	S	S	S	S
	16	R	R	R	R	R	S	S	S	S
	17	R	S	S	R	R	S	S	S	S

S - sensitive

R - resistant

I - intermediate

TABLE 4.3

ANTIBIOTIC SENSITIVITY PATTERNS OF *SERRATIA MARCESCENS*

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
Serratia	1	R	R	R	R	S	S	S	S	S
marcescens	2	R	R	I	R	S	S	S	S	S
	3	R	R	R	R	S	S	I	S	S
	4	R	R	I	R	S	S	S	S	S
	5	R	R	R	R	S	S	S	S	S
	6	R	R	I	R	S	S	S	S	S
	7	R	R	I	R	S	S	S	S	S
	8	R	R	R	R	S	S	S	S	S
	9	R	R	I	R	S	S	S	S	S
	10	R	R	I	R	S	S	S	S	S
	11	R	R	R	R	R	R	R	R	S
	12	R	R	R	R	R	R	R	R	S
	13	R	R	R	R	R	R	R	R	S
	14	R	R	R	R	S	S	I	R	S
	15	R	R	R	R	R	S	S	S	S
	16	R	R	R	R	R	S	S	S	S
	17	R	R	R	R	R	S	I	I	S
	18	R	R	R	R	R	R	R	R	S
	19	R	R	R	R	R	R	R	R	S
	20	R	R	R	R	R	R	R	R	S
	21	R	R	R	R	R	R	R	R	S
	22	R	R	R	R	R	S	S	S	S
	23	R	R	R	R	R	S	S	S	S
	24	R	R	R	R	R	S	I	R	S

S - sensitive

R - resistant

I - intermediate

TABLE 4.4

ANTIBIOTIC SENSITIVITY PATTERNS OF *ENTEROBACTER SPP.*

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
Enterobacter	1	R	R	R	S	S	S	S	S	R
spp.	2	R	R	R	S	S	S	S	S	R
	3	R	R	R	S	S	S	S	S	S
	4	R	S	R	R	R	S	S	S	S
	5	R	R	R	S	S	S	S	S	I
	6	R	R	R	S	S	S	S	S	R
	7	R	R	R	S	R	R	R	S	R
	8	R	S	R	R	R	S	S	S	S
	9	R	R	R	S	R	R	R	S	S
	10	R	R	R	R	R	R	R	S	S
	11	R	R	R	S	R	R	R	S	R
	12	R	R	R	R	R	R	R	S	S
	13	R	R	R	S	R	R	R	S	S
	14	R	S	R	R	R	S	S	S	S
	15	R	R	R	S	R	R	R	R	R
	16	R	R	R	S	S	S	S	S	R
	17	R	R	R	S	R	R	R	S	S
	18	R	S	R	R	R	S	S	S	S
	19	R	R	R	R	R	R	R	S	S
	20	R	R	R	R	R	R	R	S	S

S - sensitive

R - resistant

I - intermediate

TABLE 4.5

ANTIBIOTIC SENSITIVITY PATTERNS OF
PSEUDOMONAS AERIGINOSA

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
Pseudomonas	1	R	R	R	R	R	S	S	S	S
aeruginosa	2	R	R	R	S	R	S	S	S	S
	3	R	R	R	R	R	S	S	S	S
	4	R	R	R	R	R	S	S	S	S
	5	R	R	R	S	R	S	S	S	S
	6	R	R	R	R	R	S	S	S	S
	7	R	R	R	R	R	S	S	S	S
	8	R	R	R	R	R	S	S	S	S
	9	R	R	R	R	R	S	S	S	S
	10	R	R	R	R	R	S	S	S	S
	11	R	R	R	R	R	S	S	S	R
	12	R	R	R	R	R	R	R	R	S
	13	R	R	R	R	R	S	S	S	S
	14	R	R	R	R	R	R	R	R	S
	15	R	R	R	R	R	R	R	S	S
	16	R	R	R	R	R	R	R	I	S
	17	R	R	R	S	S	S	S	S	S
	18	R	R	R	R	R	R	R	S	S
	19	R	R	R	R	R	S	S	S	S
	20	R	R	R	R	R	S	S	S	S
	21	R	R	R	R	S	S	S	S	S
	22	R	R	R	R	R	R	R	R	S
	23	R	R	R	R	R	R	R	R	S
	24	R	R	R	R	R	R	R	R	R
	25	R	R	R	R	R	R	R	R	R

S - sensitive

R - resistant

I - intermediate

TABLE 4.6

ANTIBIOTIC SENSITIVITY PATTERNS OF
ACINETOBACTER ANITRATUS

ORGANISM		AMP	MA	FOX	TET	SXT	GNT	TOB	AM	CTZ
Acineto-	1	R	R	R	R	R	S	S	S	S
bacter ani-	2	R	R	I	R	S	S	S	S	S
tratus	3	R	R	R	R	R	R	I	R	I
	4	R	R	R	R	R	S	S	S	S
	5	R	R	R	R	R	S	S	S	S
	6	R	R	R	R	S	S	S	S	S
	7	R	R	R	R	R	S	S	S	S
	8	R	R	R	R	R	R	S	S	S
	9	R	R	R	R	R	S	S	S	I
	10	R	R	R	R	R	R	R	R	R
	11	R	R	R	R	R	R	R	S	R
	12	R	R	R	R	R	R	R	S	R
	13	R	R	R	R	R	R	R	S	S
	14	R	R	R	R	R	R	S	S	S
	15	R	R	R	R	R	S	S	S	S
	16	R	R	R	R	R	S	S	S	R
	17	R	R	R	R	R	I	I	S	S
	18	R	R	R	R	R	R	R	R	R
	19	R	R	R	R	S	S	S	S	S
	20	R	R	R	R	R	R	R	S	R
	21	R	R	R	R	R	R	R	S	R
	22	R	R	R	R	R	S	S	S	R
	23	R	R	R	R	R	R	R	R	R
	24	R	R	I	I	S	S	S	S	S
	25	R	R	R	R	R	R	R	R	R
	26	R	R	R	R	R	R	R	S	R
	27	R	R	R	R	R	S	S	S	R
	28	R	R	R	R	R	R	R	R	R
	29	R	R	R	R	R	S	S	S	R
	30	R	R	R	R	R	R	R	R	R

S - sensitive

R - resistant

I - intermediate

TABLE 4.7

M.I.C. mg/L. OF ESCHERICHIA COLI

ORGANISM		AMIKACIN	CEFTAZIDIME
E. coli	1	1	0,0625
	2	1	0,0625
	3	1	0,0625
	4	1	0,0625
	5	0,5	0,0625
	6	1	0,125
	7	2	0,125
	8	0,50	0,0625
	9	2	0,25
	10	1	0,0625
	11	1	0,0625
	12	2	0,125

TABLE 4.8

M.I.C. mg/L OF KLEBSIELLA PNEUMONIAE

ORGANISM		AMIKACIN	CEFTAZIDIME
Klebsiella	1	2	0,25
pneumoniae	2	1	0,5
	3	2	0,5
	4	1	0,25
	5	1	0,25
	6	2	0,5
	7	1	0,25
	8	2	0,5
	9	0,5	0,5
	10	0,5	0,25
	11	0,5	0,5
	12	2	2
	13	1	1
	14	0,5	0,5
	15	1	1
	16	0,5	0,5
	17	1	0,0625

TABLE 4.9

M.I.C. mg/L OF SERRATIA MARCESCENS

ORGANISM		AMIKACIN	CEFTAZIDIME
Serratia marcescens	1	1	0,125
	2	2	0,0625
	3	4	0,25
	4	1	0,0625
	5	1	0,0625
	6	2	0,0625
	7	2	0,0625
	8	2	0,25
	9	1	0,0625
	10	1	0,125
	11	64	4
	12	64	8
	13	128	8
	14	16	2
	15	4	0,25
	16	8	0,125
	17	16	2
	18	32	2
	19	128	8
	20	64	4
	21	64	4
	22	4	0,25
	23	2	0,25
	24	16	1,0

TABLE 4.10

M.I.C. mg/L OF ENTEROBACTER SP.

ORGANISM		AMIKACIN	CEFTAZIDIME
Enterobacter spp.	1	2	32
	2	2	64
	3	2	4
	4	4	8
	5	4	16
	6	2	32
	7	4	32
	8	2	4
	9	2	0,25
	10	2	32
	11	4	128
	12	4	4
	13	0,5	0,0625
	14	0,5	0,0625
	15	32	128
	16	4	1
	17	4	2
	18	2	4
	19	0,5	0,0625
	20	0,5	0,125

TABLE 4.11

M.I.C. mg/L OF PSEUDOMONAS AERUGINOSA

ORGANISM		AMIKACIN	CEFTAZIDIME
Pseudomonas	1	0,5	0,0625
aeruginosa	2	1	0,0625
	3	0,5	0,0625
	4	2	0,5
	5	1	0,125
	6	1	0,0625
	7	0,5	0,0625
	8	1	0,125
	9	2	0,25
	10	1	0,0625
	11	4	2
	12	16	2
	13	1	0,0625
	14	16	2
	15	4	4
	16	16	4
	17	4	2
	18	8	1
	19	2	0,25
	20	2	0,125
	21	4	2
	22	32	2
	23	32	2
	24	32	32
	25	32	32

TABLE 4.12

M.I.C. mg/L OF ACINETOBACTER ANITRATUS

ORGANISM		AMIKACIN	CEFTAZIDIME
Acinetobacter	1	2	4
anitratus	2	2	8
	3	32	16
	4	4	8
	5	2	4
	6	4	4
	7	2	4
	8	2	8
	9	8	16
	10	64	64
	11	16	32
	12	4	16
	13	4	8
	14	4	4
	15	2	4
	16	4	16
	17	0,5	8
	18	128	64
	19	2	8
	20	16	32
	21	8	32
	22	8	32
	23	256	64
	24	2	4
	25	64	32
	26	16	32
	27	16	32
	28	64	16
	29	16	32
	30	64	16

TABLE 4.13

COMPARITIVE ANTIBACTERIAL ACTIVITIES OF AMIKACIN AND CEFTAZIDIME

BACTERIAL SPECIES	NO. OF STRAINS TESTED	M.I.C (mg/L) AMIKACIN RANGE	AMIKACIN GEOMETRIC MEAN	CEFTAZIDIME RANGE	CEFTAZIDIME GEOMETRIC MEAN
Escherichia coli	12	0,5- 2	1,2	0,0625- 0,25	0,09
Klebsiella pneumoniae	17	0,5- 2	1,1	0,0625- 2	0,5
Serratia marcescens	24	1 -128	26,1	0,0625- 8	1,8
Enterobacter sp.	20	0,5- 4	3,9	0,0625-128	24,5
Pseudomonas aeruginosa	25	0,5- 32	8,6	0,0625- 32	3,5
Acinetobacter anitratus	30	2-256	27,2	4 - 64	20,6

TABLE 4.14

RESULTS OF CHECKERBOARD TITRATION

ORGANISM		AMIKACIN M.I.C.mg/L	CEFTAZIDIME M.I.C.mg/L	COMBINATION M.I.C. mg/L AMIKACIN & CEFTAZIDIME	CRITERION
Serratia marcescens	11	64	4	16	2
Serratia marcescens	12	64	8	8	4
Serratia marcescens	13	128	8	8	4
Serratia marcescens	14	16	2	2	0,5
Enterobacter sp.	2	2	64	0,5	32
Enterobacter sp.	10	2	32	0,25	4
Enterobacter sp.	11	4	128	1,0	16
Enterobacter sp.	15	32	128	8	8
Ps. aeruginosa	12	16	2	1	1
Ps. aeruginosa	23	32	2	16	0,5
Ps. aeruginosa	24	32	32	8	16
Ps. aeruginosa	25	32	32	8	16
Ac. anitratus	3	32	16	8	8
Ac. anitratus	10	64	64	8	32
Ac. anitratus	11	16	32	8	8
Ac. anitratus	18	128	64	32	32
Ac. anitratus	23	256	64	32	32
Ac. anitratus	25	32	16	8	8
Ac. anitratus	26	16	32	4	8
Ac. anitratus	27	8	32	4	8
Ac. anitratus	28	64	16	16	8
Ac. anitratus	29	16	32	4	8
Ac. anitratus	30	64	16	16	8

TABLE 4.15

RESULTS OF FRACTIONAL INHIBITION CONCENTRATION INDEXES

ORGANISM		SUM OF FRACTIONS
Serratia marcescens	11	0,75
Serratia marcescens	12	0,63
Serratia marcescens	13	0,56
Serratia marcescens	14	0,37
Enterobacter sp.	2	0,75
Enterobacter sp.	10	0,25
Enterobacter sp.	11	0,37
Enterobacter sp.	15	0,5
Ps. aeruginosa	12	0,56
Ps. aeruginosa	23	0,75
Ps. aeruginosa	24	0,75
Ps. aeruginosa	25	0,75
Ac. anitratus	3	0,75
Ac. anitratus	10	0,75
Ac. anitratus	11	0,75
Ac. anitratus	18	0,75
Ac. anitratus	23	0,6
Ac. anitratus	25	0,75
Ac. anitratus	26	0,52
Ac. anitratus	27	0,75
Ac. anitratus	28	0,75
Ac. anitratus	29	0,5
Ac. anitratus	30	0,75

Fig. 4.5 Isobologram

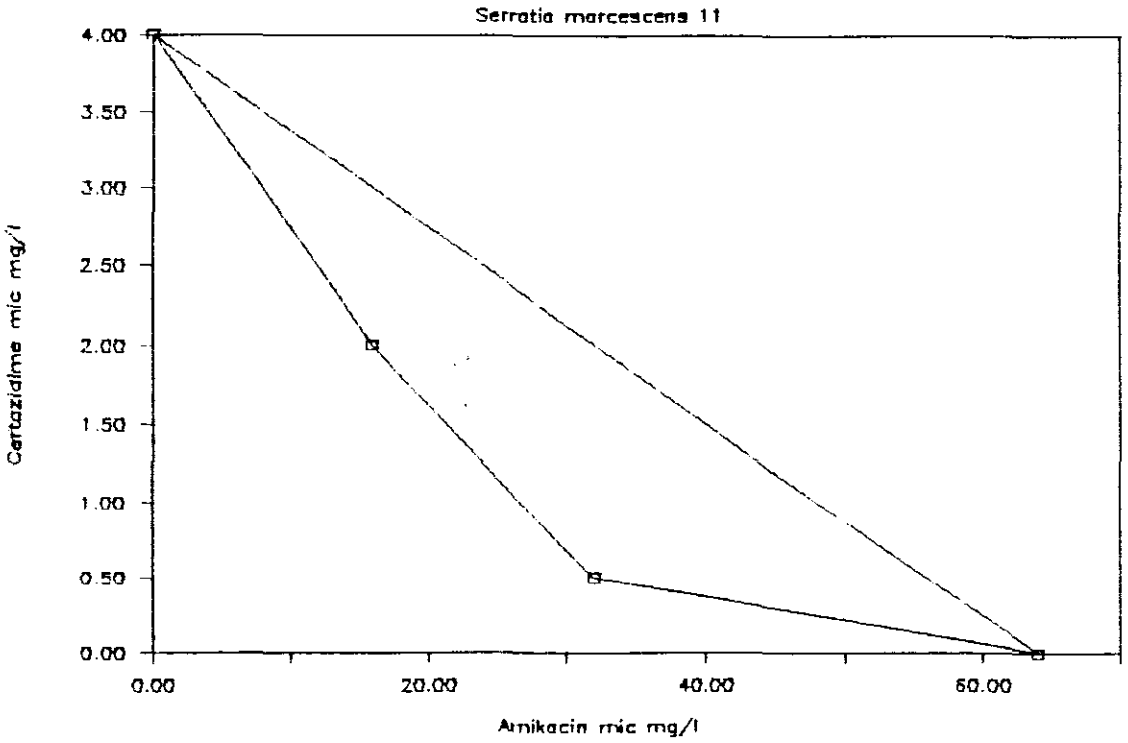
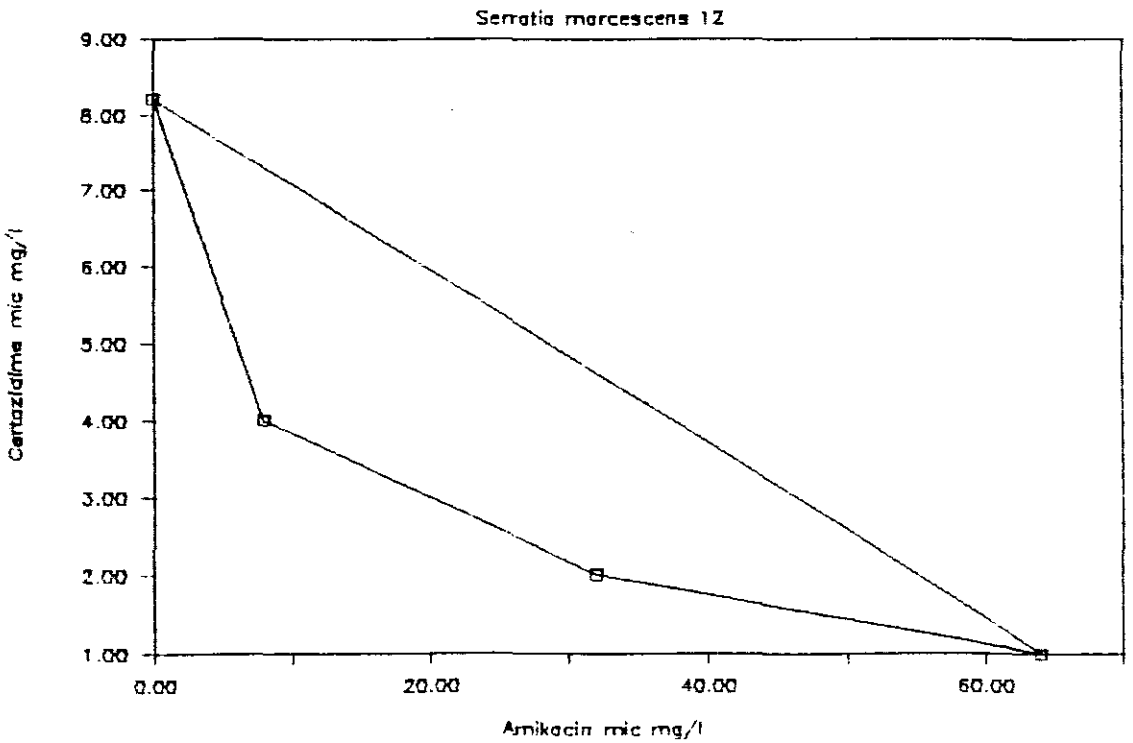


Fig. 4.6 Isobologram



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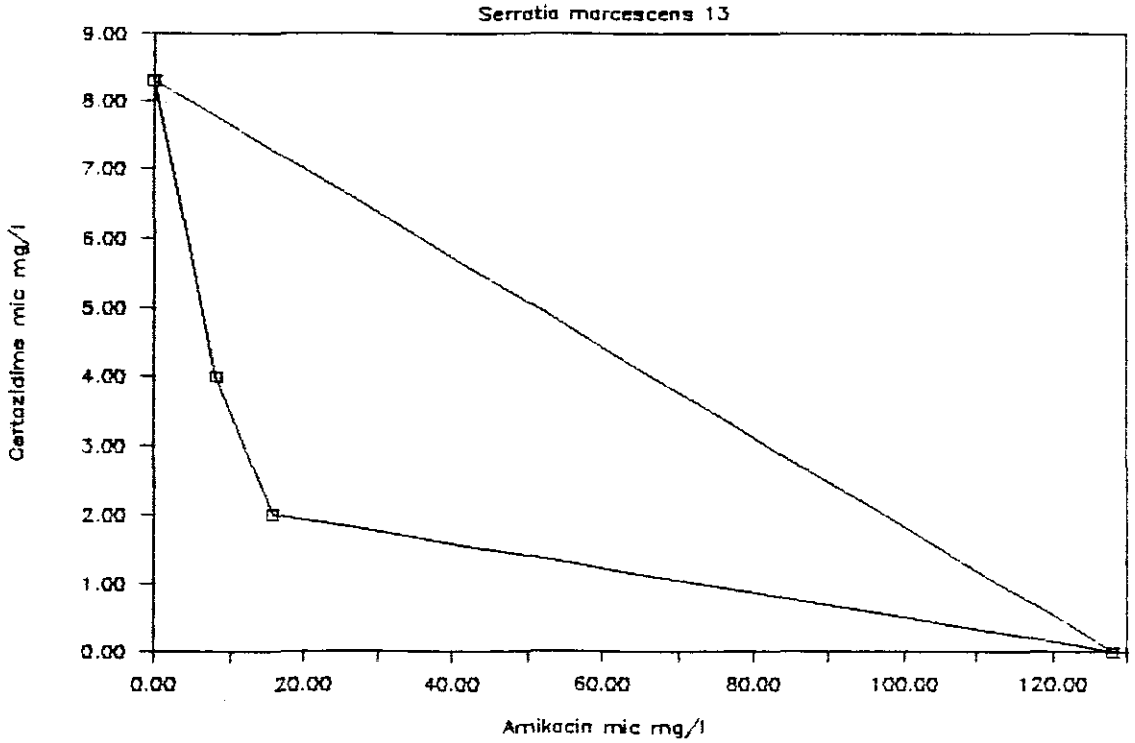

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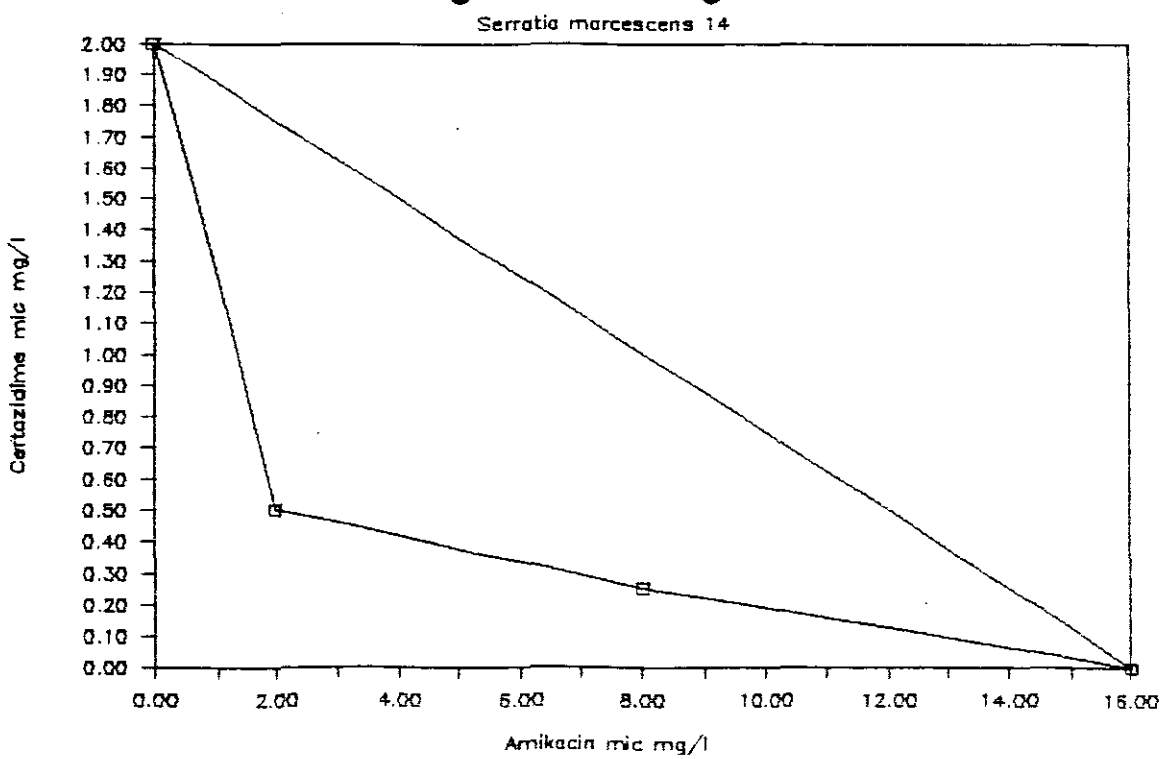

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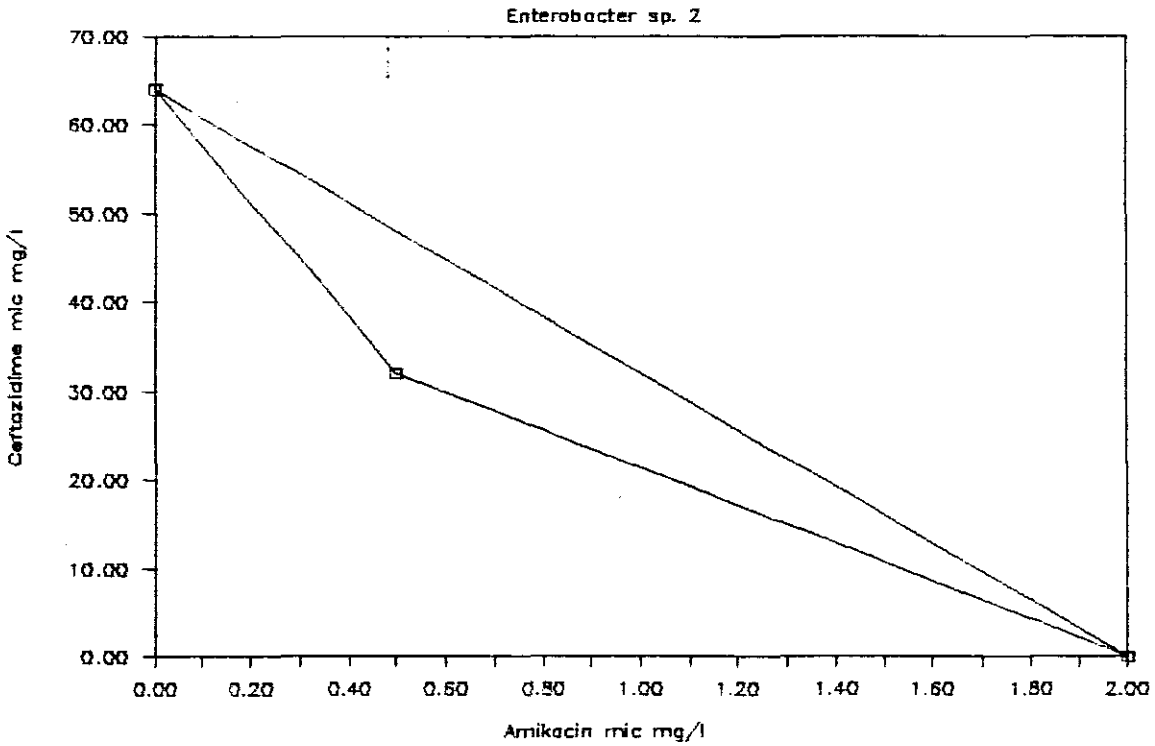

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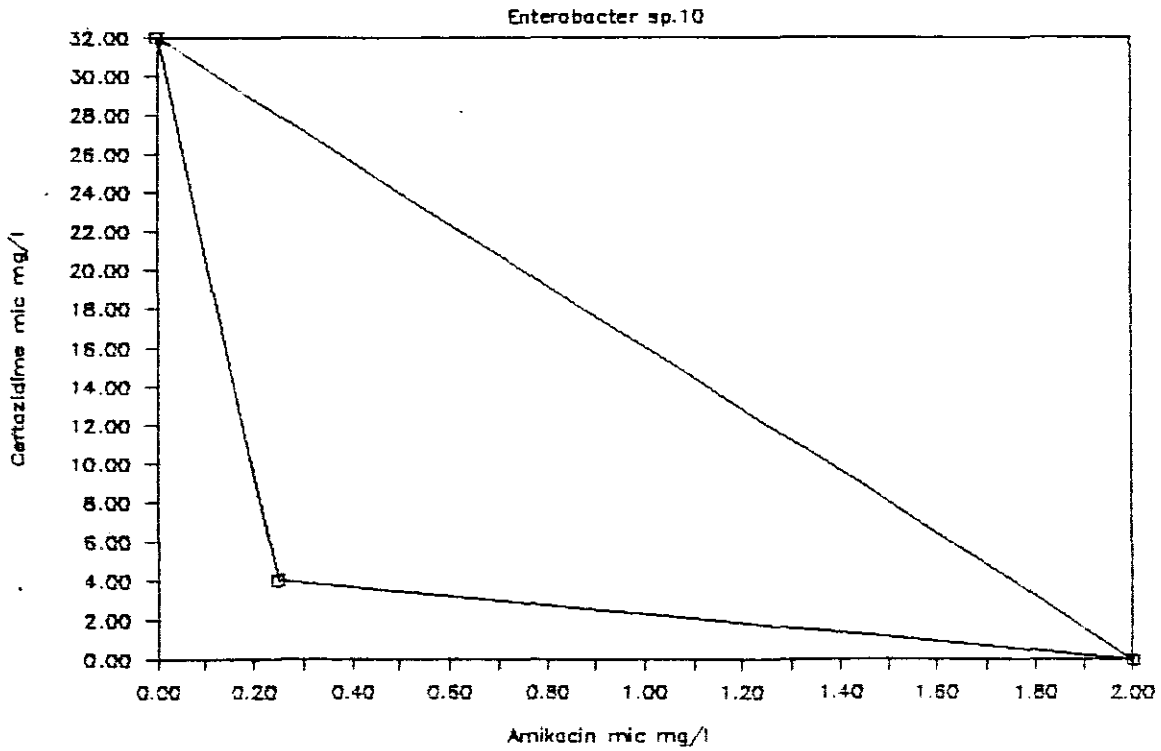

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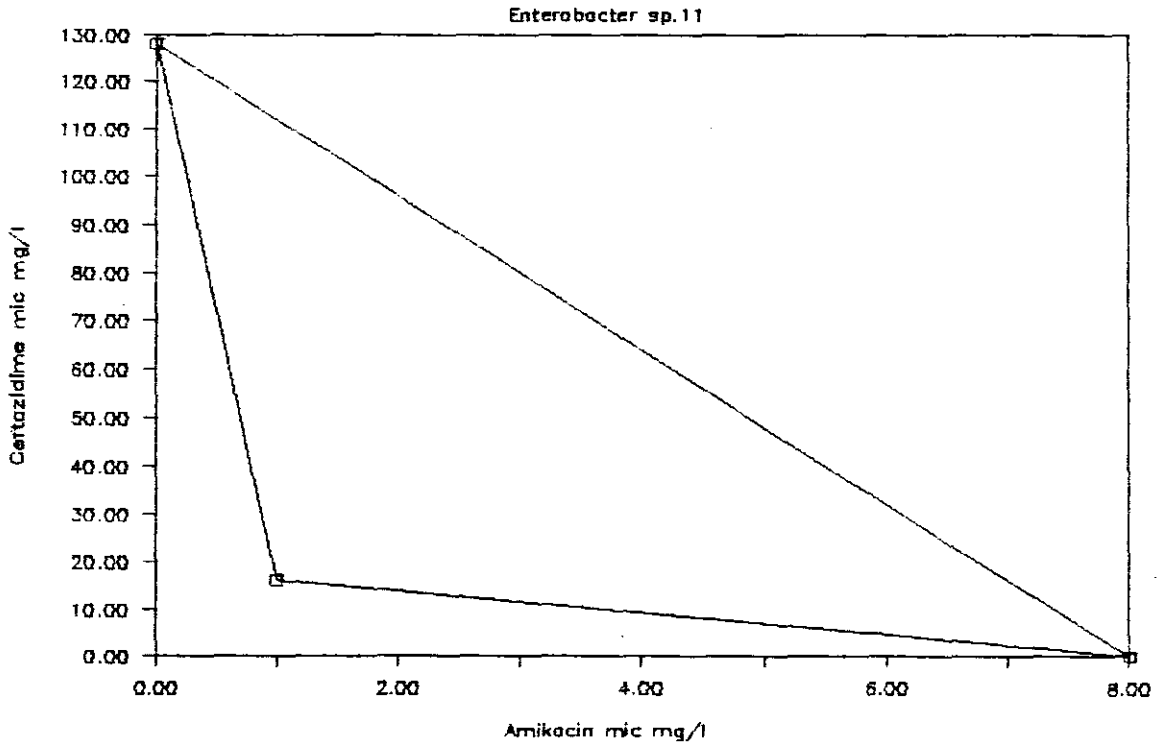
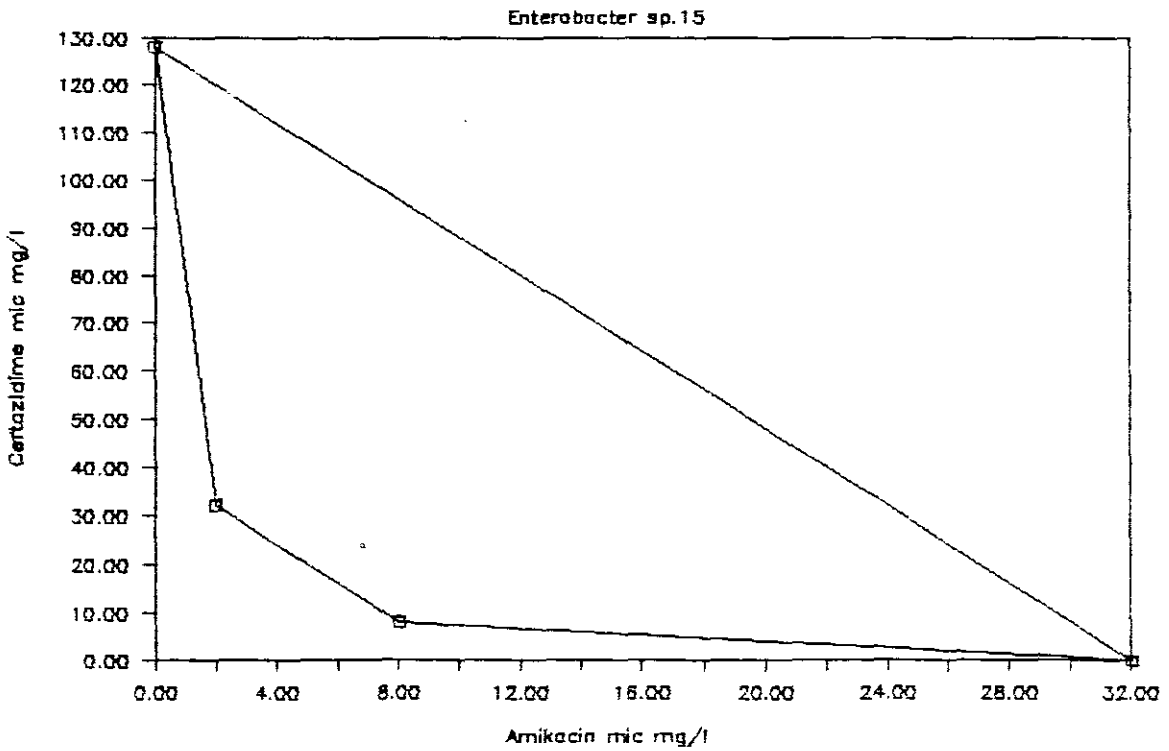


Fig. 4.12 Isobologram



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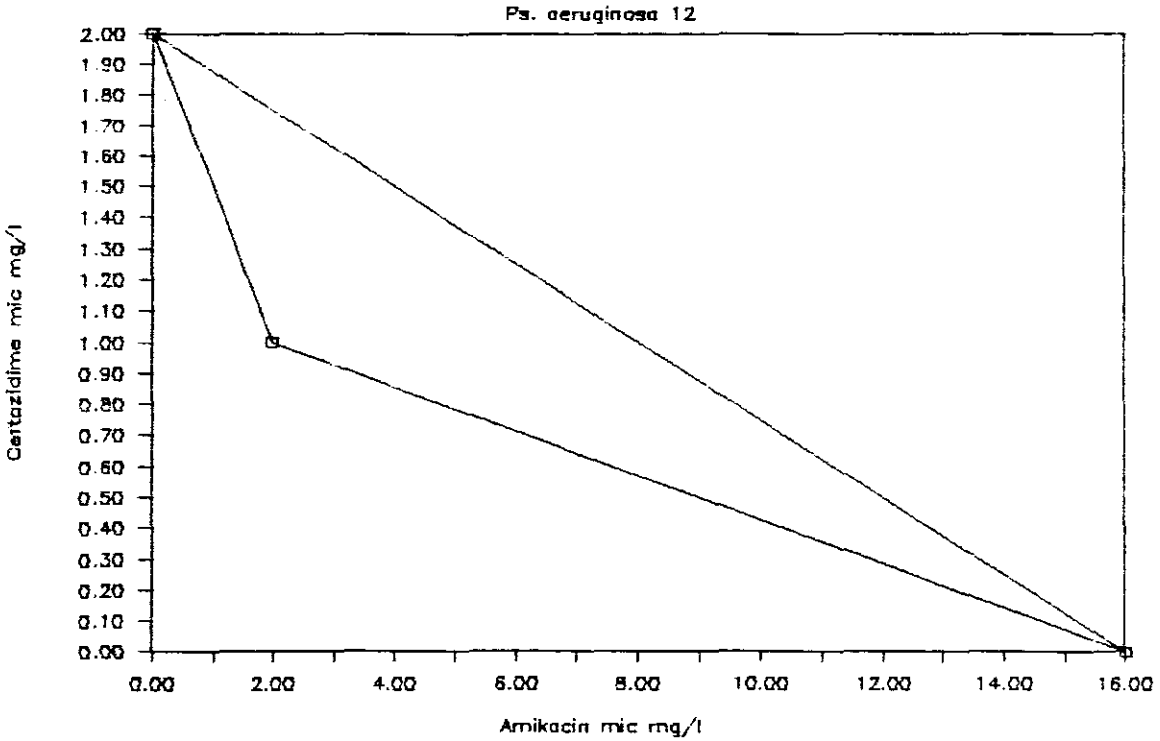
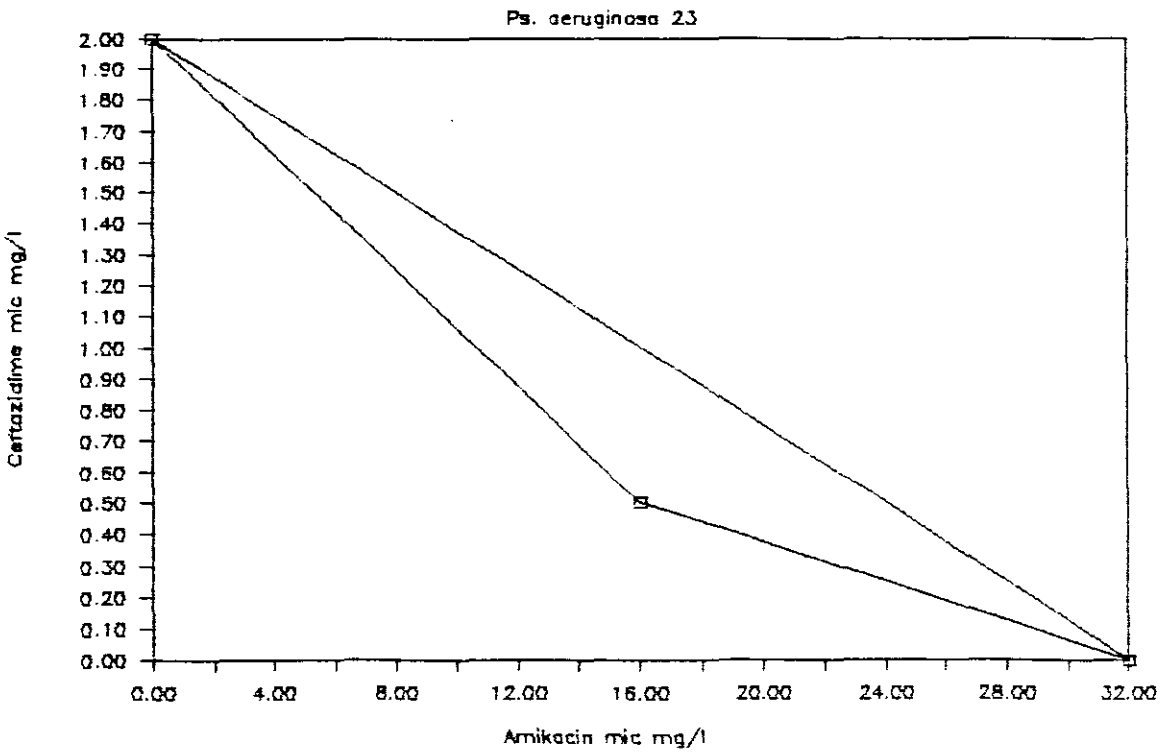


Fig. 4.14 Isobologram



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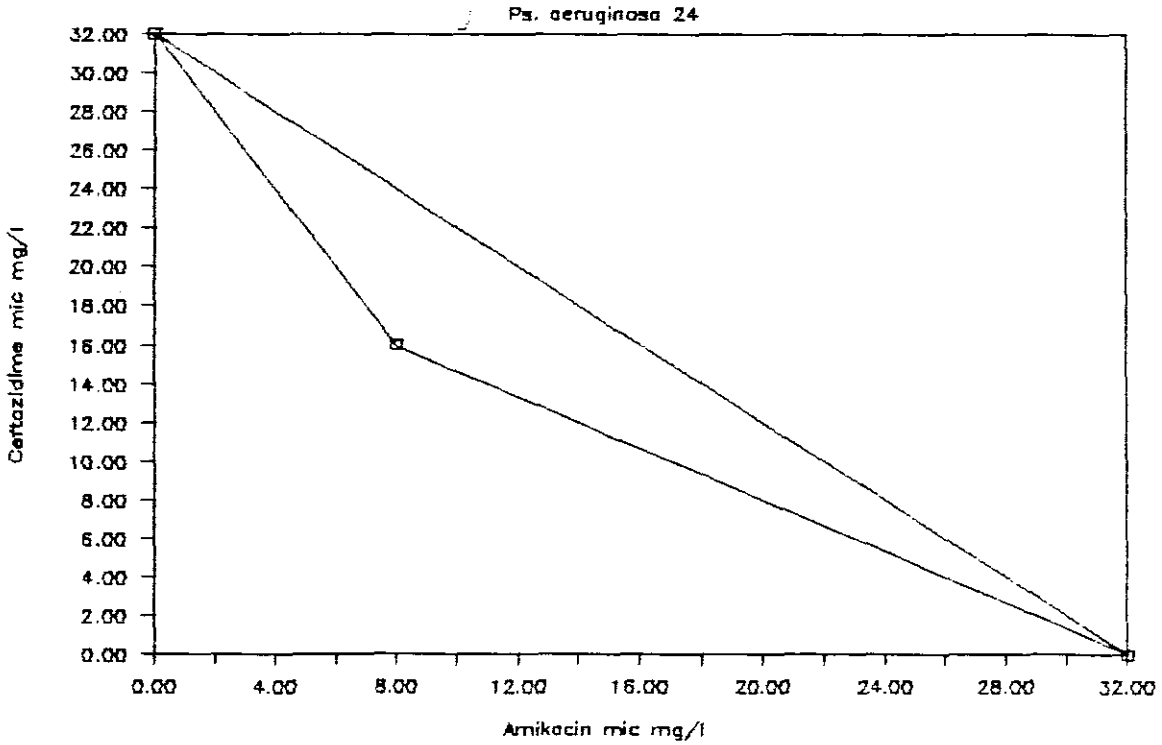


Fig. 4.16 Isobologram

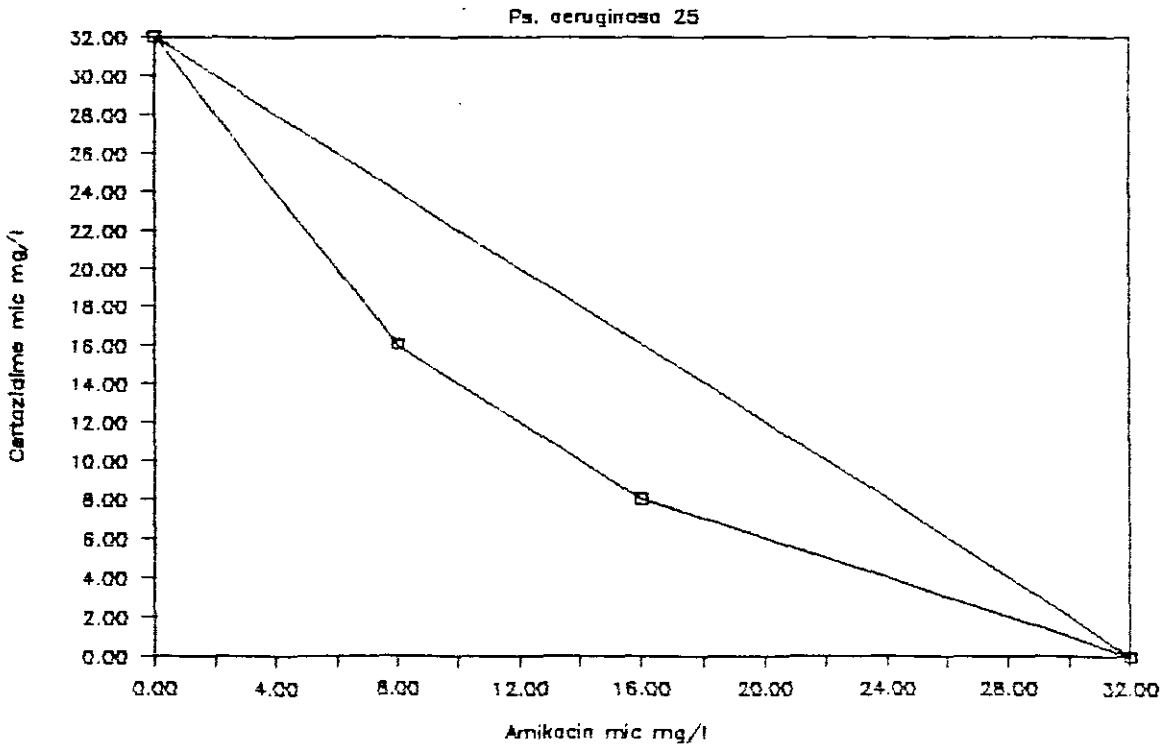


Fig. 4.17 Isobologram

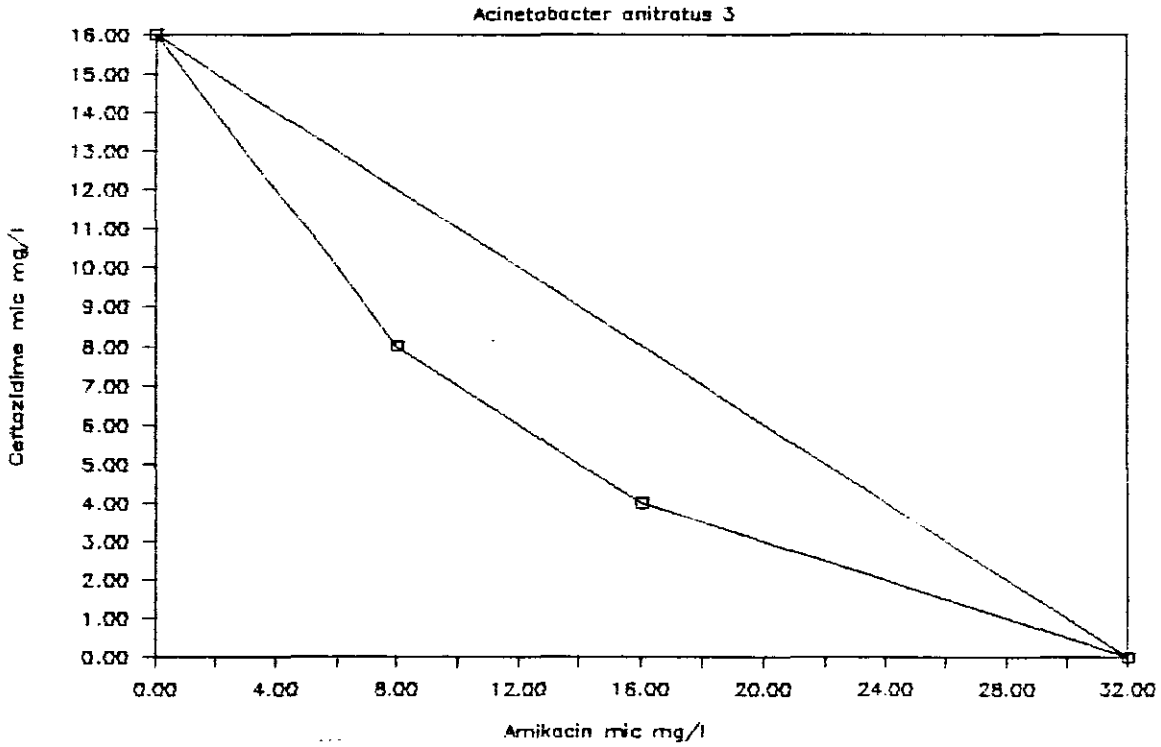


Fig. 4.18 Isobologram

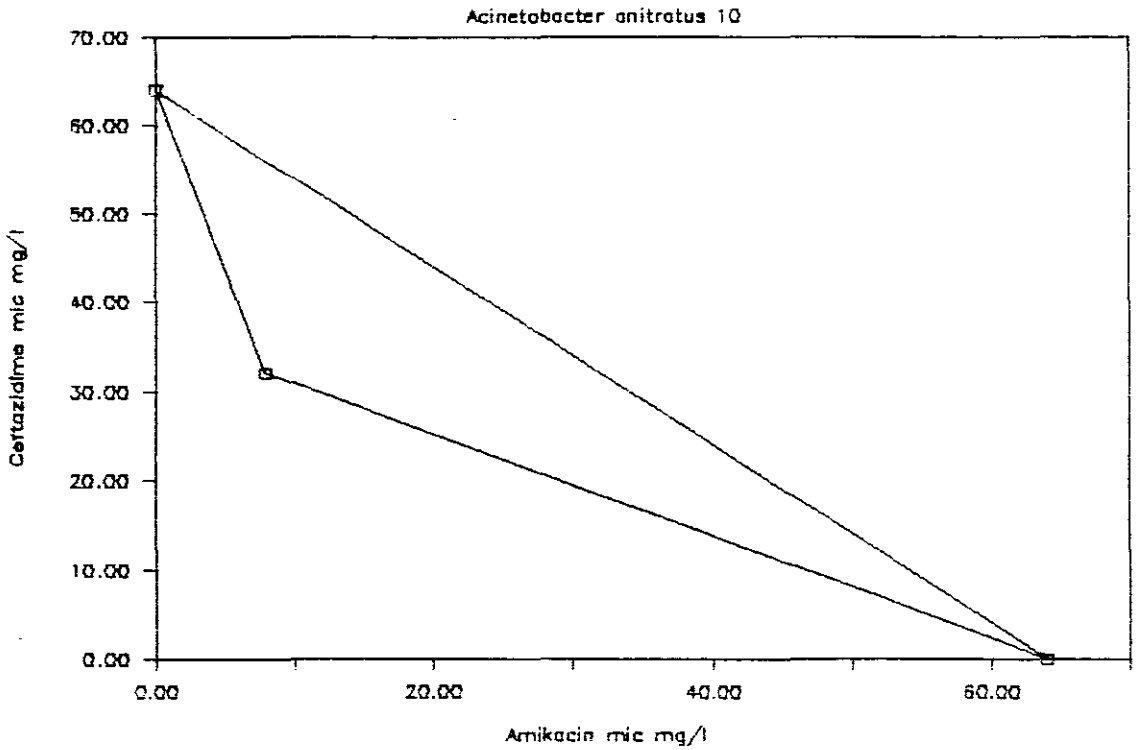


Fig. 4.19 Isobologram

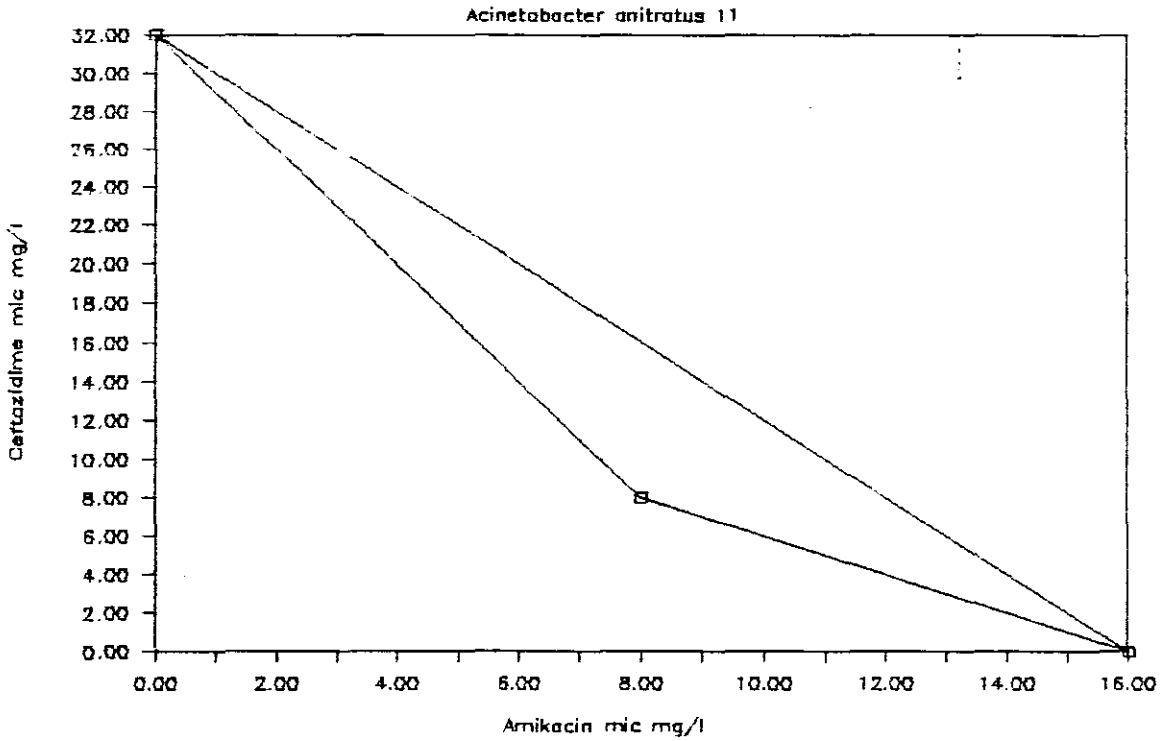
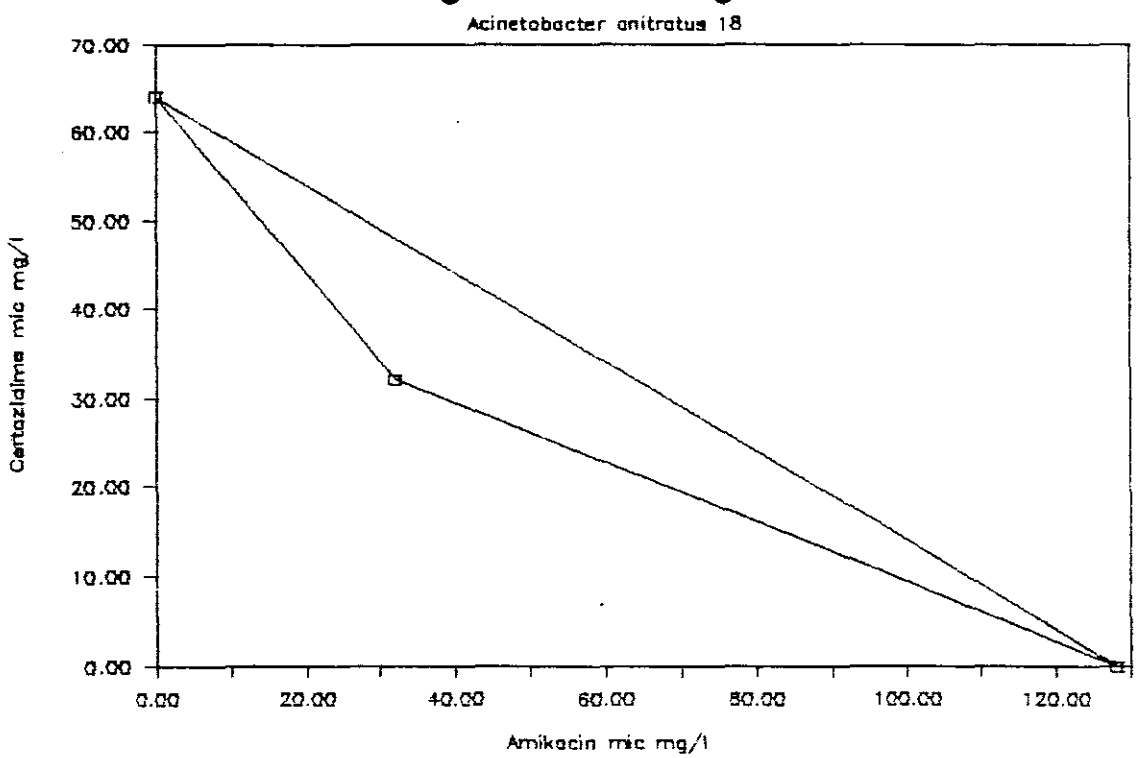


Fig. 4.20 Isobologram



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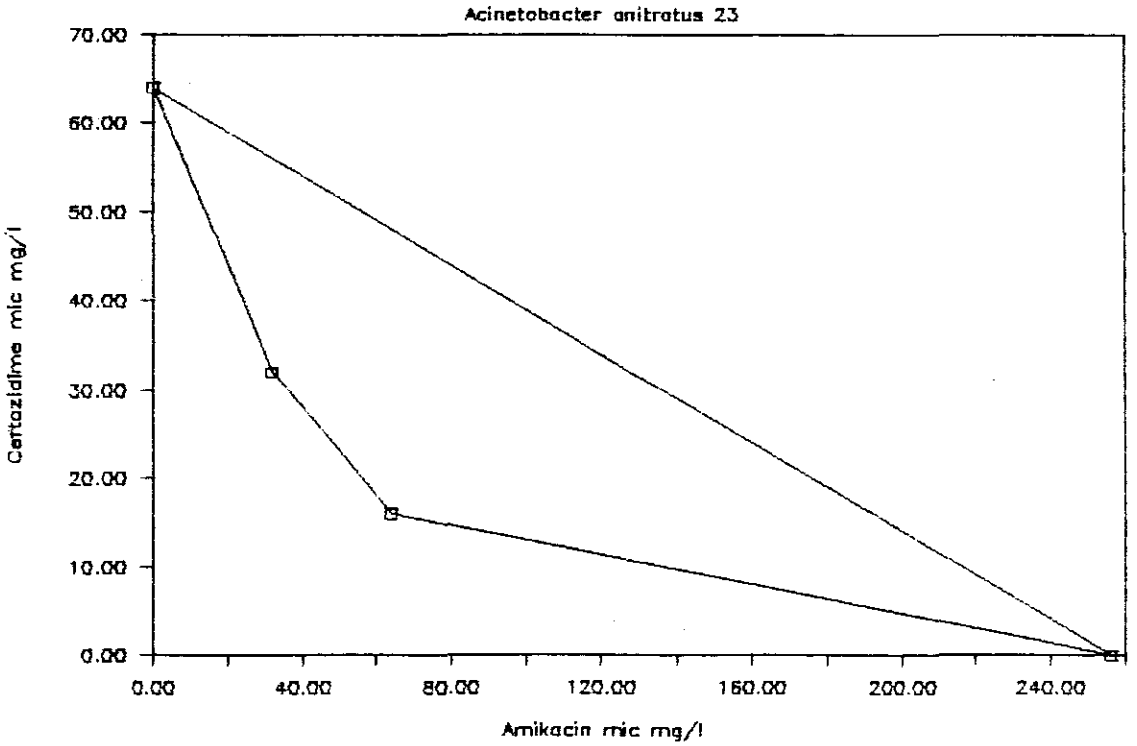

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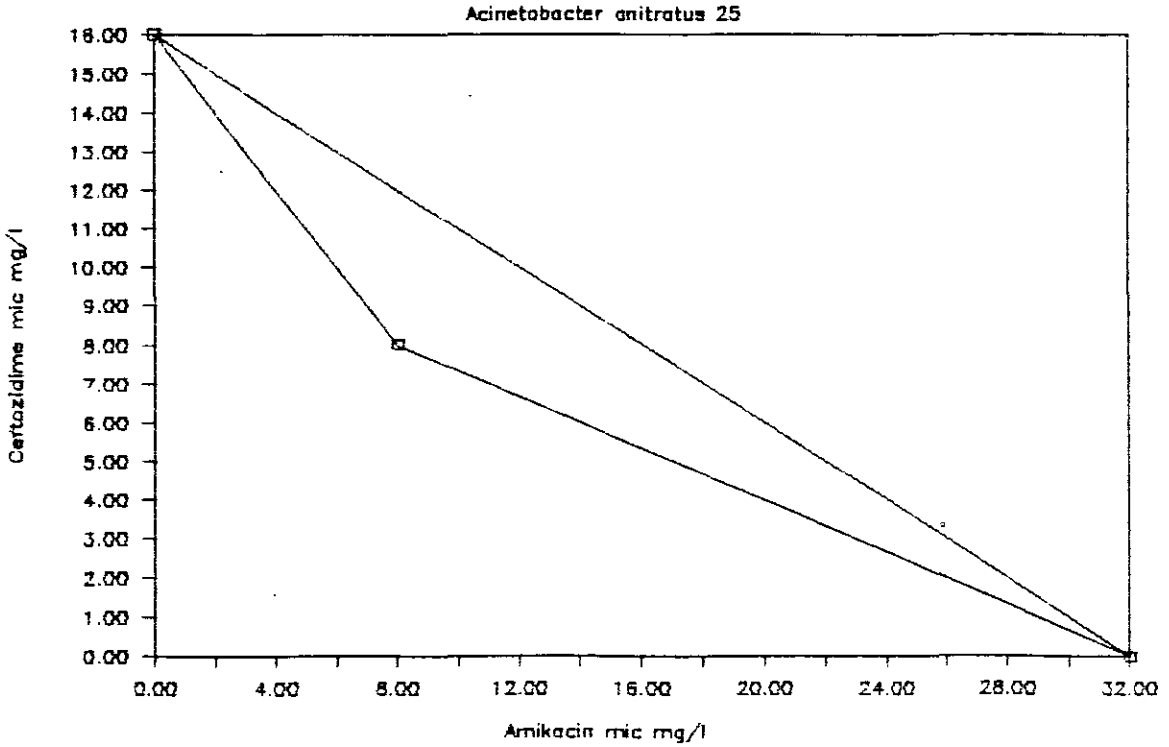


Fig. 4.23 Isobologram

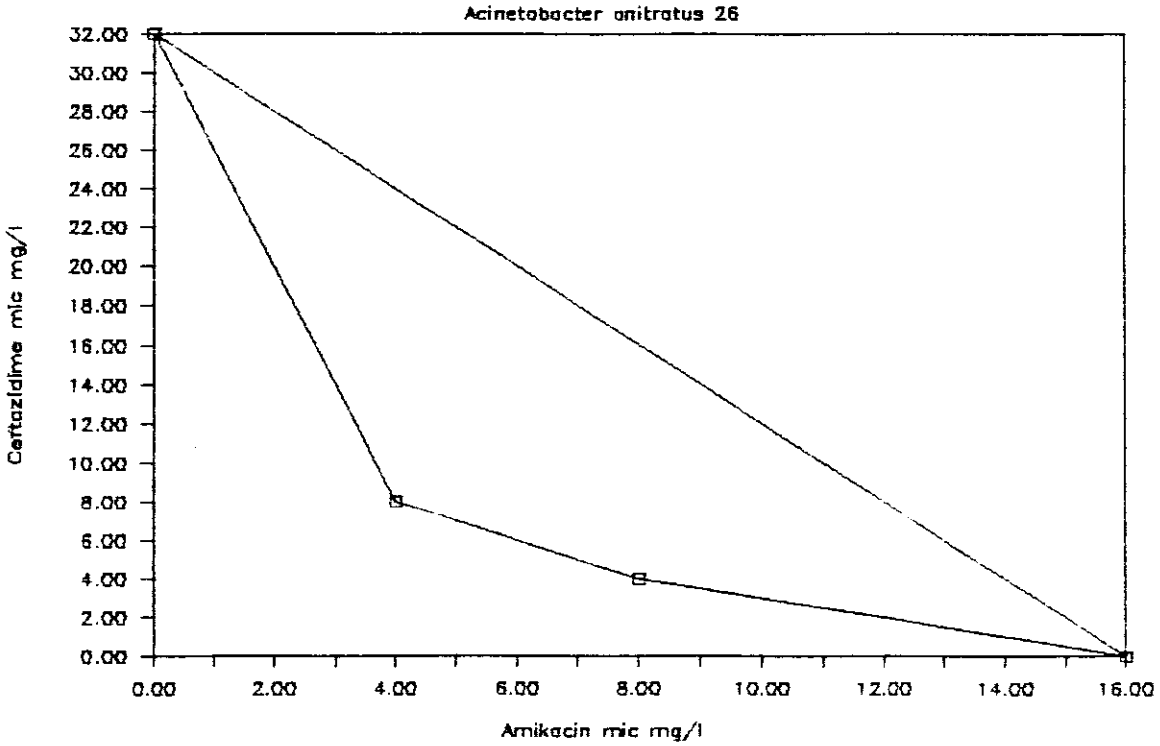


Fig. 4.24 Isobologram

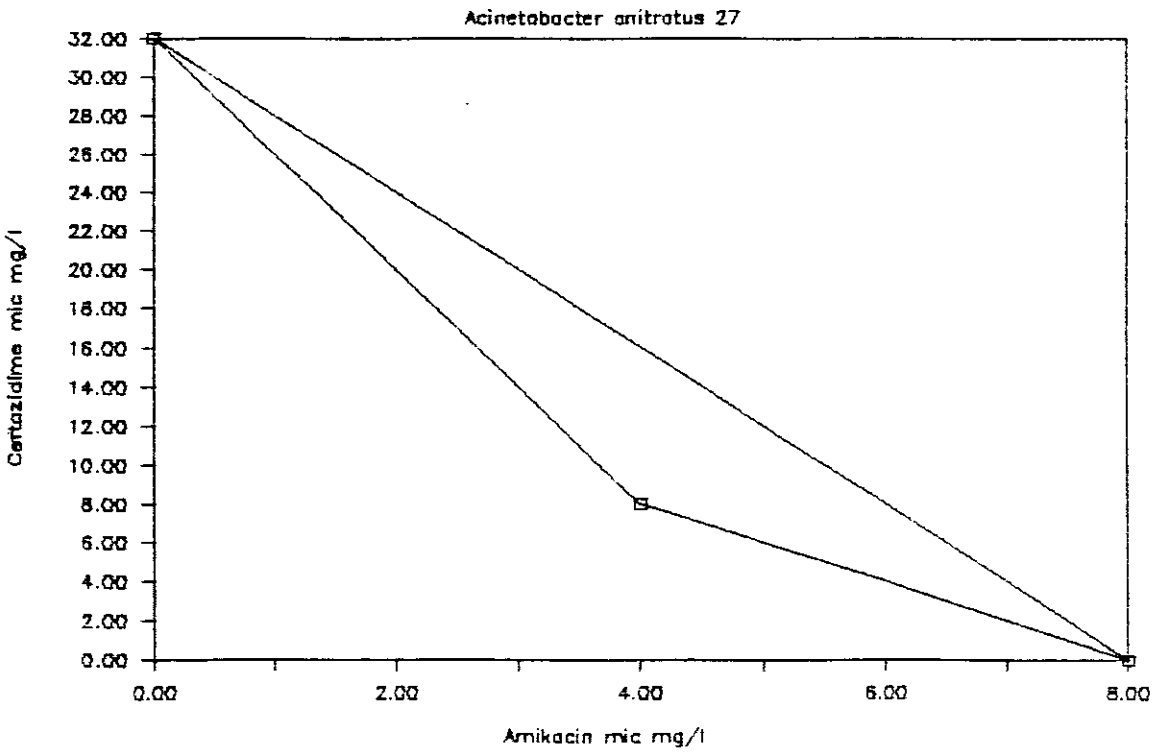


Fig. 4.25 Isobologram

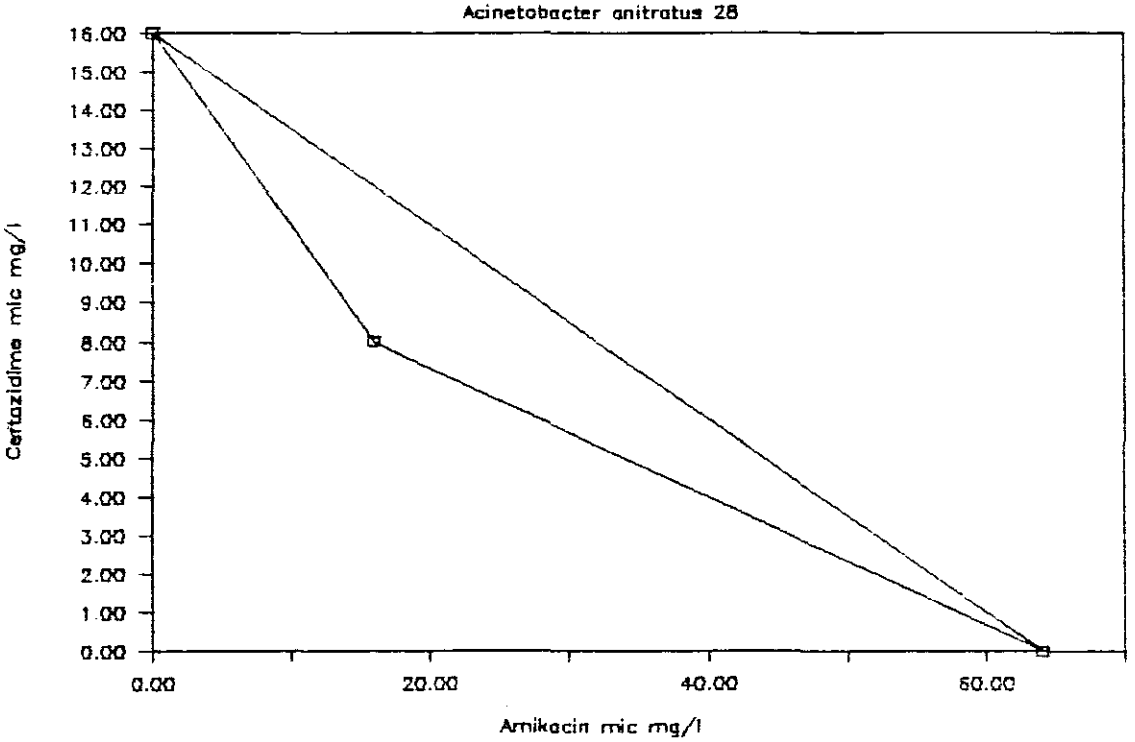


Fig. 4.26 Isobologram

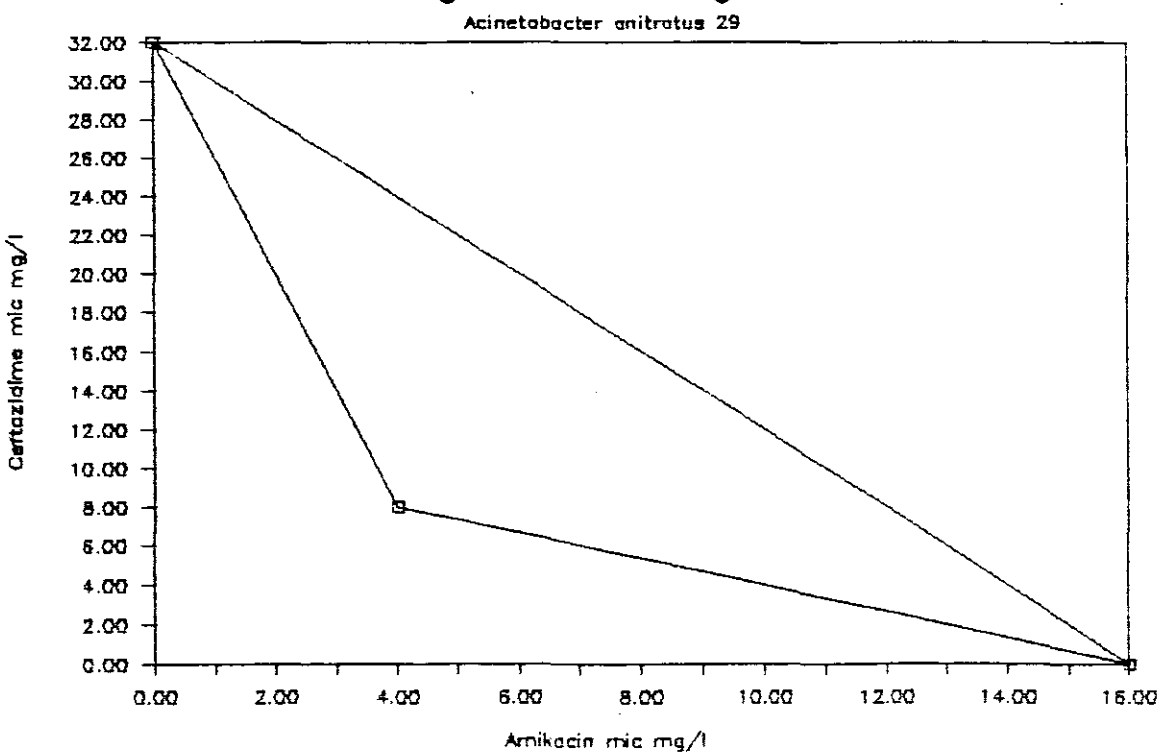
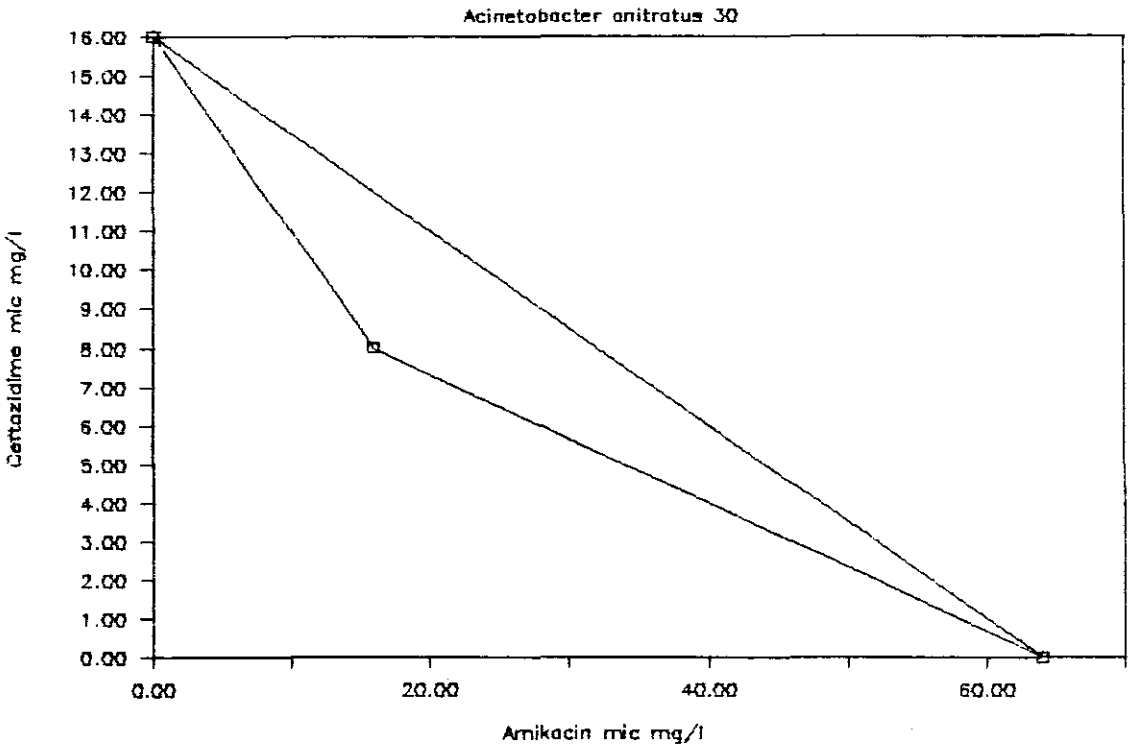


Fig. 4.27 Isobologram



CHAPTER 5

DISCUSSION

5.1 SUSCEPTIBILITY TESTS

It must be stressed that the organisms used in this trial were specially selected for their unusual resistance as well as their occurence in serious life-threatening infections.

The *in vitro* activity of amikacin and ceftazidime were very impressive against all strains of *Escherichia coli* and *Klebsiella pneumoniae*. Considering the multiple resistance of these organisms, the activity of these two antibiotics are even more remarkable. This is clearly indicated by the low geometric mean M.I.C.'s in table 4.13.

Against *Serratia marcescens* ceftazidime had excellent activity, (table 4.9) with no resistance encountered. Amikacin was however, less active and all the strains with gentamicin resistance were resistant to amikacin as well. Amikacin had excellent activity against the highly resistant strains of *Enterobacter* spp., (table 4.10). The geometric mean M.I.C of 3,9 mg/L is low

compared to the 24,5 mg/L of ceftazidime. Of the twenty strains tested 35% were resistant to ceftazidime with M.I.C.'s as high as 128 mg/L.

The antipseudomonal activity of ceftazidime was excellent against the multiple-resistant strains of Pseudomonas aeruginosa. Resistance to all the antibiotics used in this study was not uncommon (table 4.5). Of the 25 strains tested only two were resistant to ceftazidime with M.I.C.'s of 32 mg/L. The activity of amikacin was less impressive with 24 percent of the strains resistant to amikacin. The geometric mean M.I.C. of 8,6 mg/L was however, favourable (table 4.13). The geometric mean M.I.C. of 3,5 mg/L for ceftazidime clearly proves the superiority against Pseudomonas aeriginosa.

Of all the test organisms Acinetobacter anitratus had the highest incidence of resistance to both the antibiotics (table 4.6). These organism continually cause problems in intensive care units at Tygerberg Hospital and they are becoming highly resistant to most antimicrobials (table 4.6). Resistance to amikacin and ceftazidime were the highest compared to the other test organisms. The geometric mean M.I.C.'s of amikacin (27,2 mg/L) and ceftazidime (20,6 mg/L) are high and of great concern.

Studies by Brumfitt and Hamilton-Miller (1981) as well

as by Wilkinson and Gentry (1981) clearly prove ceftazidime to be very effective against hospital strains of gram-negative bacilli. Their results differ from this study as they did not have the same high level of resistance in Enterobacter spp and Acinetobacter anitratus strains.

The excellent activity of ceftazidime against Pseudomonas strains compare favourably with other workers (Scully and Neu 1984, Rusconi et al. 1984, Clumeck et al. 1983 and Gozzard et al. 1982). These authors also suggest ceftazidime as an alternative to aminoglycosides in the treatment of multiple-resistant Pseudomonas infections.

The activity of amikacin against the gram-negative bacilli were all favourable except against Serratia marcescens and Acinetobacter anitratus. Similar observations were made in studies by Wilkinson and Gentry (1981) who also encountered high resistance against amikacin by Serratia marcescens. According to an overview by Ristuccia and Cunha (1985) amikacin is effective against gentamicin-resistant isolates of Enterobacteriaceae and Pseudomonas spp.

Amikacin and ceftazidime had the same high level of activity against Excherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa with ceftazidime, the superior antibiotic against Pseudomonas strains.

Against Enterobacter spp. amikacin had far greater activity than ceftazidime, but the opposite was encountered against Serratia marcescens where ceftazidime was clearly far superior. Against Acinetobacter anitratus, however, both antibiotics met with resistance. Amikacin was slightly superior to ceftazidime with 23 percent resistance compared to the fifty percent of ceftazidime.

From these results it is clear that in serious life-threatening hospital infections, especially in the neutropenic patient, the highly resistant gram-negative bacilli have to be treated with broad spectrum antibiotics. When the infecting organisms are not known, it is therefor essential that combination therapy with the broadest anti-bacterial spectrum be used.

5.2 COMBINED ANTIBACTERIAL ACTIVITY

From the results obtained it is clear that monotherapy with ceftazidime and amikacin is not always possible and that combination therapy as an alternative should be used. As has been stated by Klastersky et al. (1980) that the use of an aminoglycoside plus a cephalosporim to treat serious systemic infections is common practice. Combination antimicrobial chemotherapy for a wide variety of infections has become routine at Tygerberg Hospital. Because of the increased bacte-

rial resistance to gentamicin and tobramycin, amikacin is commonly used as the aminoglycoside in serious nosocomial infections.

As stated before in section 2.6, antimicrobial combinations are mainly used to broaden the spectrum of antimicrobial activity, to obtain synergism, to prevent the emergence of resistance, and also to decrease the expected toxicity of each single drug.

The aim of this study was therefore to see how synergistic the combination of amikacin and ceftazidime were against the multiple-resistant gram-negative bacilli isolated from serious nosocomial infections at Tygerberg Hospital.

The criterion for synergy in the checkerboard titration is a four-fold reduction in the M.I.C. of each antibiotic used as described in section 4.3.2. According to these criteria the majority of the test organisms indicate an additive effect. However, according to the criteria for the F.I.C. indexes the interpretation differs, for example, in the case of Serratia marcescens no. 11 the M.I.C. reduction of amikacin was four-fold but the ceftazidime reduction was only two-fold (table 4.14). According to the criteria as described by Norden et al. (1979) this reaction is additive. In determining the F.I.C. index as described in section 3.8, this reaction is regarded as synergistic.

The results from the constructed isoboles as shown in figure 4.5 to 4.27, all indicate inward bowing and are regarded as synergistic. The results of the three methods of synergy testing are shown in table 4.16. These seems to be a wide variety of applied methods and interpretive criteria. It does seem that most laboratories interpret the checkerboard titration as a four-fold reduction of each antibiotic for synergy. According to the interpretations of Berenbaum (1978) a F.I.C. index of less than one is regarded as synergistic, however, Jones (1986) states that more than fourty percent of laboratories regard a F.I.C. index equal to or less than 0.5 as synergistic. These discrepancies make the interpretation of these results extremely difficult and depends on the methods employed.

To allow for these discrepancies it would be better to use the term favourable inhibitory effect to describe synergism or additive reactions. If one applies this term to the results obtained in this project, it would appear that the combined activity of amikacin and ceftazidime had favourable inhibitory effects. According to Jawitz (1967) there are no universally accepted definitions to separate additive from synergistic actions, and that additive actions are slightly smaller increases in activity than synergy.

This study was, however, not to evaluate the different methods for testing antimicrobial synergism. These

TABLE 4.16

RESULTS OF SYNERGY TESTS OF AMIKACIN AND CEFTAZIDIME AGAINST G.N.B.

ORGANISM		A	B	C
Serratia marcescens	11	A	S	S
Serratia marcescens	12	A	S	S
Serratia marcescens	13	A	S	S
Serratia marcescens	14	S	S	S
Enterobacter sp.	2	A	S	S
Enterobacter sp.	10	S	S	S
Enterobacter sp.	11	S	S	S
Enterobacter sp.	15	S	S	S
Ps. aeruginosa	12	A	S	S
Ps. aeruginosa	23	A	S	S
Ps. aeruginosa	24	A	S	S
Ps. aeruginosa	25	A	S	S
Ac. anitratus	3	A	S	S
Ac. anitratus	10	A	S	S
Ac. anitratus	11	A	S	S
Ac. anitratus	18	A	S	S
Ac. anitratus	23	A	S	S
Ac. anitratus	25	A	S	S
Ac. anitratus	26	S	S	S
Ac. anitratus	27	A	S	S
Ac. anitratus	28	A	S	S
Ac. anitratus	29	S	S	S
Ac. anitratus	30	A	S	S

A - Criterion as determined in checkerboard titration.

B - Criterion as determined with F.I.C. index.

C - Criterion as determined with isobolograms.

S - synergy

A - additive

O - antagonism or no synergy

discrepancies are mentioned as there have to date been no definate standardizations of methods for testing synergy. In this study the methods and interpretation as described will be used throughout.

One of the aims of combination therapy is the reduction of the dosage, especially the more toxic aminoglycosides. In this study, exactly that was achieved. Against Serratia marcescens the high M.I.C.'s of amikacin were reduced to achievable serum levels. Similar reductions were obtained against Pseudomonas aeruginosa and Acinetobacter anitratus.

Against Acinetobacter anitratus no. 18 and no. 23 additive levels could only be obtained in vitro. (table 4.14). There was marked synergy between amikacin and ceftazidime against Enterobacter spp. (table 4.14). Of interest was that these organisms were susceptible to amikacin and resistant to ceftazidime. In general all the selected pathogens were inhibited favourably, either synergistic or additive, by the combination of amikacin and ceftazidime. No antaginism or indifference was found against any of the strains.

These results are in accordance with those by Gombert and Aulicino (1986). The results of this study are also in accordance with those of Glew and Pavuke (1984) who found synergy between amikacin and six beta-lactam antibiotics against Enterobacteriaceae.

Marked synergy was also found when one or both antibiotics inhibited the bacterial strain.

In table 4.14 synergy or addition was possible in cases where there was resistance to both antibiotics. Similar observations were made by Neu and Fu (1978), who speculated that the organisms with resistance to aminoglycosides, attributed to a permeability barrier, were synergistically inhibited by the combination containing ureidopenicillins that disrupted the cell wall and facilitated the trasport of aminoglycosides to the ribosomes. Bryan and Kwan (1983) proposed a new mechanism of synergism. They demonstrated that the aminoglycosides also exert their bactericidal effect by disrupting the cytoplasmic membrane, acting in concert with the beta-lactams to lyse the cells. In another study Scudamore and Goldner (1982) concluded that synergy between beta-lactams and aminoglycosides are not mediated by the outer membrane.

Bayer et al. (1984) evaluated the synergistic potential of the third-generation cephalosporins with amikacin and gentamicin. They found enhanced bactericidal activity for amikacin and gentamicin combined with ceftriaxone, ceftrizoxime or ceftazidime. Synergy was found in eighty five to ninety percent of the cases. They concluded that the beta-lactam plus aminoglycoside combinations resulted in an enhanced frequency of killing and a rapid bactericidal interaction.

On the basis of this study it is evident that the combination of amikacin plus ceftazidime was inhibitory *in vitro* to the multiple-resistant organisms. Whether these *in vitro* synergistic combinations will prove superior to non-synergistic combinations in clinical practice is not clear. Anderson *et al.* (1978), Chadwick *et al.* (1986) have reported a good response when synergistic combinations were used especially in neutropenic patients.

As hospital flora become more and more resistant, the role of aminoglycosides such as amikacin and the third generation cephalosporins will become important.

In vitro synergy studies can be very helpful in defining the most effective antibiotic combinations, especially in the immuno-compromised host. Progress in newer and standardized methodologies should improve the correlation between *in vitro* and *in vivo* data.

This study has shown amikacin and ceftazidime to offer a broad antibacterial spectrum against highly resistant strains encountered at Tygerberg Hospital. Based on the information presented in this study, ceftazidime plus amikacin should be considered as initial single therapy for serious life-threatening infections.

THE IN VITRO ANTIMICROBIAL ACTIVITY OF AMIKACIN AND CEFTAZIDIME AGAINST MULTIPLE RESISTANT GRAM-NEGATIVE BACILLI IN NOSOCOMIAL INFECTIONS.

BY

MARIUS JOHANNES JOOSTE

INTERNAL EXAMINER : MR. A.W. VAN RIJSWIJK

EXTERNAL EXAMINER : DR. R. ROBSON

SUMMARY

The problem of hospital-acquired infection is an on-going one. Infections by gram-negative bacilli with increased resistance to most antibiotics available

today have become a major threat to hospitalized patients throughout the world. Patients often at risk are those in intensive care units and post-operative surgery wards. One of the major factors contributing to the development of bacterial resistance is the repeated exposure of bacteria to antimicrobials.

In this study these highly resistant gram-negative bacilli were isolated from patients with nosocomial infections and selected for further susceptibility studies. These organisms were tested against a variety of antibiotics including the two test drugs amikacin and a new third-generation cephalosporin, ceftazidime. The standardized disk diffusion method of Kirby-Bauer was used to determine the susceptibility of these organisms. The minimum inhibition concentrations (M.I.C.'s) of these selected organisms were tested against amikacin and ceftazidime using the agar dilution method.

The test organisms were all highly resistant to the various antibiotics, but favourable results were obtained against the two test drugs as the M.I.C.'s indicate. From these results organisms resistant to one or both of the two test drugs were selected for further study.

Combination therapy against life-threatening infections is a routine procedure in most hospitals. The

selected organisms were tested against the combinations of amikacin and ceftazidime to see if there were any synergistic reactions. The methods used to determine synergism was the checkerboard titration, Fractional Inhibition Concentration indexes and the construction of isoboles.

The combined activity of amikacin and ceftazidime against the selected organisms were either synergistic on additive. No antagonism or any indifference was found.

The results obtained from this study prove that amikacin and ceftazidime were effective against these highly resistant gram-negative bacilli. The in vitro combined activity of amikacin and ceftazidime was very satisfactory considering the resistance of the organisms.

The results and conclusions obtained from this study prove that amikacin and ceftazidime have superior in vitro broad-spectrum antibacterial activity against these highly resistant gram-negative bacilli often encountered in life-threatening infections.

DIE IN VITRO ANTIMIKROBIESE AKTIWITEIT VAN AMIKASIEN EN KEFTASIDIEM TEEN WEERSTANDIGE GRAM-NEGATIEWE BAKTERIEë IN NOSOKOMIALE INFEKSIES.

DEUR

MARIUS JOHANNES JOOSTE

INTERNE EKSAMINATOR : MNR. A.W. VAN RIJSWIJK

EKSTERNE EKSAMINATOR : DR. R. ROBSON

OPSOMMING

Nosokomiale infeksies is 'n ernstige probleem in hospitale en hierdie infeksies word veroorsaak deur gram-negatiewe bakterieë wat hoogs weerstandig teen die meeste antimikrobiese middels is. Pasiente in

intensiewe eenhede asook post-operatiewe chirurgiese sale is gewoonlik dié wat 'n hoë risiko loop om hierdie infeksies op te doen. Een van die faktore wat lei tot hierdie weerstandigheid by organismes is die aanhoudende blootstelling aan antimikrobiese middels.

Hierdie hoogs weerstandige gram-negatiewe bakterieë was geïsoller vanaf pasiënte met nosokomiale infeksies en gebruik vir verder gevoeligheidstoetse. Gevoeligheidstoetse teen verskeie antimikrobiese middels, insluitend amikasien en keftasidiem, was bepaal. Die gestandardiseerde metode van Kirby-Bauer was gebruik om hierdie gevoeligheid van die toetsorganismes te bepaal. Verder was die minimum inhibisie konsentrasies (M.I.K.) van amikasien en keftasidiem teen hierdie organismes bepaal deur middel van die agar verdunningsmetode.

Die toets organismes was almal hoogs weerstandig teen die meeste antimikrobiese middels, maar gunstige resultate was verkry met amikasien en keftasidiem. Organismes met weerstandigheid teen een of albei middels was geselekteer vir verdere toetse.

In die geval van lewensdreigende infeksies word daar gewoonlik kombinasieterapie toegepas in die vorm van 'n aminoglikosiede met beta-laktam antibiotikum. In hierdie geval was amikasien en keftasidien teen hierdie geselekteerde gram-negatiewe bakterie gebruik om

te sien of daar enige sinergistiese reaksies was. Die volgende metodes was gebruik om hierdie reaksies te bepaal, naamlik die "checkerboard" titrasie, die Fraksionele inhibisie konsentrasies en die konstruksie van isobole.

Die resultate verkry vanaf hierdie toetse het almal gedui op sinergisme of aanvullende aktiwiteit. Geen antagonisme of neutraliteit was waargeneem nie.

Die bevindinge van hierdie studie dui daarop dat amikasien en keftasidiem effektief was teen hierdie hoogs weerstandige gram-negatiewe bakterieë en dat die reaksies van die kombinasie van hierdie twee middels hoogs bevredigend was.

Die resultate en bevindige van hierdie studie dui daarop dat die breë-spektrum antibakteriële aktiwiteit van amikasien en keftasidiem <u>in vitro</u> hoogs effektief is in die behandeling van pasiente met lewensdreigende infeksies.

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INSTRUMENTATION

The following instruments were used.

Denley Multipoint Inoculator

Waterbath-Heidolph

Incubator-Gallenkamp

Steam Steriliser-Almor

Sartoruis Analytical balance

Calipers