THE IN VITRO ACTIVITY OF ANTIMICROBIAL AGENTS ALONE AND IN COMBINATION AGAINST CLINICAL ISOLATES OF GRAM-POSITIVE BACTERIA

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DECLARATION

This is to certify that this dissertation is my own, unaided work. It is submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg, and has not been submitted at any other university.

Alan van den Berg

October, 1993

ABSTRACT

Analysis of organisms involved in hospital infections has shown that Gram-positive bacteria have assumed an increasingly important role. Examples that have been recognised as important pathogens are staphylococci, enterococci, streptococci, *Corynebacterium jeikeium* and *Leuconostoc* species. Methicillin resistance in staphylococci has become a major problem in certain hospitals. Viridans streptococci continue to be the most frequent cause of native valve endocarditis. *Leuconostoc* species are being increasingly isolated from blood culture specimens. Strains of Gram-positive bacteria have become resistant to specific antibiotics; e.g. staphylococci to methicillin, enterococci to ampicillin, and viridans streptococci to penicillin. JK corynebacteria are sensitive only to vancomycin and resistant to other antimicrobials normally used for treating infection caused by Gram-positive bacteria.

In this study various combinations of antimicrobials against 35 clinical isolates of Gram-positive bacteria obtained from three hospitals in the Johannesburg area (Johannesburg, Hillbrow, and Baragwanath) from 1987-

1988 were investigated.

The MIC / MBC results conformed to others described in world-wide studies.

Results when different methodologies for determining synergy were used, varied. This emphasizes the need for standardization, especially with regard to the time-kill studies.

Most antimicrobial combinations tested against *Leuconostoc* species demonstrated synergy using the checkerboard method, but these results were not confirmed by time-kill procedures, which showed mainly indifference.

Synergy was also obtained when gentamicin plus ciprofloxacin was combined against isolates of *Corynebacterium jeikeium*.

Because of increasing resistance and the fact that Gram-positive bacteria cause serious infections, various and new combinations of antimicrobials need to be tested before treating these infections.

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For Francis, Janine and Nicole

with much love

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CHAPTER ONE

INTRODUCTION

Gram-positive bacteria, including strains resistant to the commonly used antimicrobial agents, have assumed an increasingly important place as causes of nosocomial infections (1). Many of these infections are serious, and require treatment with a synergistic combination of antimicrobial agents (2,3,4,5). Important Gram-positive bacteria include staphylococci, both *Staphylococcus aureus* and coagulase-negative staphylococci, enterococci, viridans streptococci, *Leuconostoc* species, and *Corynebacterium jeikeium* (6,7,8,9,10,11,12).
In this dissertation, Gram-positive bacteria that are commonly isolated in hospitalized patients and which present antimicrobial management problems have been chosen for study.

1.1 STAPHYLOCOCCI

Both *Staphylococcus aureus* and coagulase-negative staphylococci are important causes of serious infections which may require treatment with a combination of antimicrobial agents. Methicillin-resistant strains of *Staphylococcus aureus* were recognised as pathogens as early as 1962 (13), and the prevalence of infection

caused by methicillin-resistant strains is increasing. Hospital epidemiology studies have revealed that methicillin resistance in both *Staphylococcus aureus* and *Staphylococcus epidermidis* have become a major problem in certain hospitals (7,14,15,16,17).

1.1.1 *Staphylococcus aureus*

Staphylococcus aureus is a coagulase-producing, non-sporing, non-motile, facultative Gram-positive coccus ( ). It is an important causative agent of severe infections including infective endocarditis, septicaemia, osteomyelitis, puerperal sepsis, wound infections, meningitis, mediastinitis, and urinary and respiratory tract infections (19,20). *Staphylococcus aureus* infections are frequently associated with complications, such as metastatic abscesses, while less common events such as rapid cardiac valve destruction in cases of acute endocarditis can be devastating. (21,22).

1.1.2 Coagulase-negative staphylococci

Coagulase-negative staphylococci are normal inhabitants of the skin. They are however, being increasingly recognised as pathogens. They have been incriminated in causing sepsis in neonates and immuno-compromised

patients such as patients with granulocytopenia (23,24) and recipients of bone marrow transplants (25). In addition they cause infections related to prosthetic devices such as intravenous and intra-arterial catheters (26,27,28), prosthetic heart valves, vascular reconstructional grafts (29), CNS shunt materials (30), haemodialysis catheters (31), and orthopaedic prostheses. The pathogenesis of these infections has been related to slime production, a complex glycoconjugate identified as a mucoid exopolysaccharide which assists in these organisms adhering to the biopolymers (32). The slime also inhibits phagocytosis and protects the staphylococci from antibiotics (33). Infections caused by coagulase-negative staphylococci may only manifest months after insertion of prosthetic devices e.g. heart valves (34), or hip prostheses (35).

1.1.3 Antimicrobial agents used in the treatment of staphylococcal infections

Antimicrobial agents which have activity against staphylococci include some of the beta-lactams, such as cloxacillin and imipenem, aminoglycosides, macrolides, rifampicin, fusidic acid, quinolones and vancomycin. Some staphylococcal strains do however demonstrate resistance to the above-mentioned agents.

1.1.4 Staphylococcal antimicrobial resistance mechanisms

Mechanisms of resistance to staphylococci are similar to those encountered in other Gram-positive bacteria.

1.1.4.1 Resistance to beta-lactam antimicrobial agents

There are two main mechanisms by which staphylococci exhibit resistance to the beta-lactam antibiotics. Firstly, by beta-lactamase production, and secondly due to production of altered penicillin binding proteins.

1.1.4.2 Staphylococcal beta-lactamases

The introduction of benzylpenicillin (penicillin G) temporarily solved the problem of staphylococcal infections. However, the continued use of this agent resulted in the selection of resistant strains, which produced ß-lactamases. This production is mediated by chromosomal genes or by plasmids, which for staphylococci are transferred by transduction (36).
Here plasmid DNA is transferred by a bacteriophage from a penicillin-resistant to a penicillin-susceptible *Staphylococcus*.
The beta-lactamases are responsible for inactivating the penicillins by cleaving the amide bond in the ß-lactam

ring, thus altering penicillin to penicillinoic acid, which displays no antibactericidal properties. This is demonstrated in Figure 1.1.

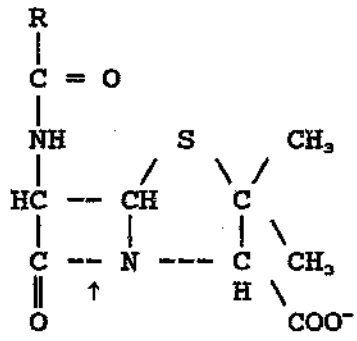

Figure 1.1 **Site of hydrolysis of penicillins by ß-lactamases**

According to Richmond, there are four immunologically distinct types of ß-lactamases produced by *Staphylococcus aureus* (37). Although their activity is similar, they are serologically divided into four types viz. A, B, C and D, all of which have different degrees of activity on penicillin (37,38,39). This division was confirmed kinetically and by isoelectric focusing by Zygmunt *et.al.* (40). Some strains of *Staphylococcus aureus* are hyperproducers of ß-lactamase. These strains demonstrate in vitro borderline resistance to the penicillinase-resistant semisynthetic penicillin methicillin, and the isoxazolyl penicillins oxacillin, cloxacillin, dicloxacillin flucloxacillin and nafcillin. In these cases ß-lactamase production is inducible

rather than constitutive, i.e. elevated ß-lactamase synthesis occurs in the presence of a ß-lactam drug, and enzyme synthesis returns to normal low levels when the drug is removed (41,42,43).

1.1.4.3 Staphylococcal penicillin-binding proteins

Penicillin-binding proteins (PBPs) are cytoplasmic membrane proteins which specifically bind penicillin and other ß-lactam antibiotics. They act as enzymes in transpeptidation reactions in peptidoglycan synthesis. Inhibition of transpeptidation results from co-valent binding of ß-lactams to these penicillin-binding proteins. Changes in the affinity of PBPs for ß-lactam antibiotics form the basis of development of resistance to many of these antimicrobial agents, notably methicillin and the other isoxazolyl antibiotics.

In *Staphylococcus aureus*, four penicillin-binding proteins are present, viz. 1,2,3,4. Studies indicate that killing occurs with inhibition of penicillin-binding protein 2 and penicillin-binding protein 3 (44). Resistance to ß-lactam antibiotics results when these penicillin-binding proteins are altered, resulting in decreased affinity for the antibiotic with retention of their enzymatic activity. Studies have shown strains of *Staphylococcus aureus* in which penicillin-binding protein 3 was missing or modified, have a lower affinity

for ß-lactam antibiotics than strains containing the regular penicillin-binding protein 3 (45,46,47). Methicillin resistance has also been related to the acquisition of a new penicillin-binding protein, penicillin-binding protein 2a, which too displays a low affinity for ß-lactams (47,48). This protein, penicillin-binding protein 2a, has also been detected in a methicillin-resistant strain of coagulase-negative staphylococcus (49).

1.1.5 Resistance to vancomycin

This antibiotic also exhibits bactericidal effects against staphylococci by inhibiting peptidoglycan synthesis (50,51). Vancomycin binds the terminal D-alanyl-D-alanine of the peptidoglycan during cell wall synthesis of Gram-positive organisms. By forming a hydrogen bond with the acyl-D-alanyl-D-alanine terminus it inhibits the transglycosylation step by which glycan units are polymerised within the peptidoglycan (52).
In the past few years there have been occasional reports of development of resistance to vancomycin by strains of coagulase-negative staphylococci producing MICs ranging from 2 to >16µg/ml (53). The resistance mechanism is not yet fully understood, and alteration of the target site is thought to be the cause (54). Although vancomycin resistant strains of *Staphylococcus aureus* have not been

reported, increasing MICs ranging from 2μg/ml to 8μg/ml have been observed (55,56). Tolerance (MBC/MIC ≥32 to vancomycin may occur with strains of *Staphylococcus aureus*.

Enterococci may also demonstrate high-level resistance to vancomycin (MIC 64- >2000μg/ml). This transferable vancomycin resistance is plasmid-mediated. (57). Although the effectiveness of vancomycin alone for therapy of enterococcal endocarditis is questionable, the combination of vancomycin and gentamicin consistently showed enhanced bacterial killing (2).

1.1.6 Resistance to other antimicrobial agents

1.1.6.1 Aminoglycosides

Resistance to aminoglycosides occurs in three ways in many bacteria including staphylococci (58):

(a) The aminoglycosides are modified at various sites by three classes of aminoglycoside-modifying enzymes, viz. O-phosphotransferases, N-acetyltransferases and adenylyltransferases. These enzymes are plasmid-mediated.

(b) Ribosomal resistance whereby mutations result in antimicrobial agents being unable to attach to the ribosomes found in genes coding for ribosomal proteins.

(c) Diminished uptake of the aminoglycoside. (Uptake of

aminoglycosides can be studied by incubation of the organism in a growth medium containing radioactive aminoglycoside. Samples are filtered through membrane filters which retain the bacteria and the radioactivity on the filters is measured).

1.1.6.2 Quinolones

Most staphylococci, including strains of *Staphylococcus aureus* and coagulase-negative staphylococci are susceptible to ciprofloxacin being inhibited by concentrations of ≤1μg/ml (59). Ciprofloxacin resistance, when it occurs, is probably due to an altered DNA gyrase subunit A (60). This occurs when one of the strands in the double helix has a phosphodiester bond broken (61). This form is mutational and not plasmid mediated (62).

1.1.6.3 Fusidic Acid

Resistance to fusidic acid is due to chromosomal mutation or to the presence of plasmids. Chromosomal mutants invariably have a modified G factor (i.e. a translocation factor protein) that has a reduced affinity for fusidic acid, (54,62), while in *Staphylococcus aureus*, plasmid-mediated resistance sometimes causes reduced uptake of the antibiotic across

the cytoplasmic membrane (63).

1.1.6.4 Rifampicin

Resistance to rifampicin is on the basis of an altered DNA-directed RNA polymerase (54,62). RNA polymerase contains four polypeptide chains and resistance is associated with changes in one of them, the ß-subunit (64).

1.1.7 Multiple resistance

Although disk tests often indicate that cephalosporins are active against methicillin-resistant *Staphylococcus aureus* strains, clinical results have been unsatisfactory (65,66).

The incidence of methicillin-resistant coagulase-negative staphylococci is high (16). Of 500 isolates looked at by Richardson and Marples over a three year period, 38% were resistant to methicillin and 30% were resistant to gentamicin (67).

At the time methicillin-resistant *Staphylococcus aureus* strains were first isolated, methicillin resistance was linked to at least four resistance determinants. The most frequent were ß-lactamase production, tetracycline resistance, streptomycin resistance and inducible erythromycin resistance. Approximately 90% of

methicillin-resistant *Staphylococcus aureus* strains are resistant to erythromycin (68). Tetracyclines and chloramphenicol were also proving to be ineffective. In 1975 emergence and spread of strains resistant to all aminoglycosides, including gentamicin, netilmicin and amikacin were observed in a large number of hospitals (69). Since then, methicillin-resistant *Staphylococcus aureus* strains resistant to aminoglycosides have become endemic in many hospitals. In vitro studies of time killing curves have revealed that the effect of vancomycin was slow, limited and not related to the concentration of the drug (51). Alternatives to vancomycin show varying results.

1.1.8 Tolerance

Tolerance occurs when the minimum bactericidal concentration (MBC) exceeds the MIC by five or more 2-fold dilutions, i.e. MBC/MIC ratios of ≥ 32 (after 24-48 hour incubation) (70,71). In other words the organisms are inhibited but not killed by a particular antibiotic (72). This tolerance progressively declines (over a period of a year) if isolates are refrigerated at 4°C or frozen at -70°C (70). Clinical isolates of ß-lactam-tolerant *Staphylococcus aureus* appear to be deficient in autolytic activity due to the presence of an excess of unidentified autolysin inhibitor according to Sabath

et.al. (73). The actual clinical relevance of tolerance is yet to be established (74). Several factors can influence the degree of tolerance in *Staphylococcus aureus*, leading to inconsistent results. These include the methods and media used when performing broth dilution studies and time-kill curves (75,76).

1.1.9 Treatment

Current antimicrobial therapy for patients with serious infections caused by methicillin-susceptible *Staphylococcus aureus* consists of administration of a beta-lactamase-stable beta-lactam with or without an aminoglycoside. Vancomycin can be used as alternative therapy for penicillin-allergic patients (77,78). The recommended therapy for staphylococcal endocarditis is 4 to 6 weeks of a semi-synthetic penicillin, a cephalosporin, or vancomycin (79,80,81). Combinations of these drugs with an aminoglycoside were more rapidly bactericidal in vitro and in animal models of endocarditis than when used alone (82,83,84). However, such combinations given in a 4 to 6 week regimen have not proved superior to single-drug regimens for the treatment of *Staphylococcus aureus* endocarditis in humans (77,78,85). Serious infections caused by methicillin-resistant *Staphylococcus aureus* are usually treated with vancomycin either alone or in combination

with rifampicin or an aminoglycoside or both. Alternative regimens are required for the following reasons:

(a) High concentrations of aminoglycoside in combination with vancomycin are nephrotoxic.

(b) These antibiotics are also costly (17,86,87,88,89).

(c) In certain areas of the United States of America, among intra-venous drug abusers, endocarditis is most commonly caused by methicillin-resistant *Staphylococcus aureus* (90). These patients display disruptive behaviour, causing problems when applying extended intravenous treatment regimens.

(d) As stated before, tolerance to vancomycin can develop with strains of *Staphylococcus aureus* (91).

Rifampicin has excellent in vitro activity against methicillin-resistant *Staphylococcus aureus* with MIC_{90} (i.e. 90% of the strains are inhibited at the indicated concentration) of around 0,03µg/ml (92,93,94). However, when used alone, there is rapid development of resistance due to mutations (95). Other antistaphylococcal antibiotics which have been investigated as alternatives include rifampicin, fusidic acid and the quinolones. In vitro studies have shown that the interaction of rifampicin and fusidic acid is either synergistic or indifferent, as determined by checkerboard and/or time kill curves (96). Note that

hepatotoxicity can be increased by combining these two drugs. The treatment of deep seated prosthetic infections due to coagulase-negative staphylococci may usually be guided by results of sensitivity testing, but other infections (such as peritonitis in patients undergoing continuous ambulatory peritoneal dialysis) may require urgent empiric treatment. Vancomycin has been recommended for these circumstances and is effective (34,97). The choice of antibiotics should be influenced by the site of infection to ensure adequate penetration: neonatal infections due to coagulase-negative staphylococci have responded to netilmicin (which was more active than gentamicin) (98), and for other infections antibiotics such as rifampicin, chloramphenicol and fluoroquinolones (e.g. ciprofloxacin) also show good activity against coagulase-negative staphylococci in the laboratory and may have a place in treatment (99).

1.2 STREPTOCOCCI

Streptococci are Gram-positive coccal-shaped bacteria that usually appear in chains of various lengths. They are non-motile, nonsporing and may be encapsulated. The majority are facultative anaerobes, but there are species that are anaerobic or microaerophilic.

The Lancefield system of classification is based on the

carbohydrate antigens located in the cell wall of the streptococci, and are designated A through V.

Group A is then further divided by specific agglutinating sera into Griffith types according to their surface protein antigens - M, T and R.

Facultative anaerobic streptococci that do not produce soluble haemolysin may be divided into two broad categories according to their appearance when grown on blood agar. Those that cause a greenish pigmentation with a narrow zone of partial haemolysis are called α-haemolytic streptococci. Both the viridans streptococcus group and pneumococci are α-haemolytic. Those without effect on the blood-containing medium are called non-haemolytic, or gamma-haemolytic streptococci and include the enterococci. Whilst most of the Lancefield group A (*Streptococcus pyogenes*) produce ß-haemolysis (a clear zone of haemolysis on fresh blood agar), some variants are non-haemolytic. Conversely, a variant of *Enterococcus faecalis* (Lancefield group D) may be actively haemolytic on blood agar, although it does not produce a soluble haemolysin (100,101).

1.2.1 Enterococci

Enterococci have long been known to have the potential for causing serious disease. However, these organisms have often been considered relatively non-virulent, and

their pathogenicity disputed (102,103). Studies have shown a significant mortality rate for patients with enterococcal bacteraemia and infective endocarditis. There has been a growing use of broad-spectrum antibiotic agents in the treatment of serious infections, especially cephalosporins and aminoglycosides to which enterococci are resistant (104,105,106). Although increased cephalosporin use has been blamed for the apparent increase in the rate of enterococcal infections, it is also possible that the increase may be due in part to factors other than exposure to antibiotic agents, such as greater reliance on indwelling urinary catheters and intravascular devices in the case of the hospitalised patients. The most recent Nosocomial Infection Surveillance Survey (107), has indicated that the enterococcus is the third commonest cause of nosocomial infections reported to the Centres for Disease Control.

Infections of soft tissue (abdomen, pelvis, biliary and urinary tracts), associated with enterococcal bacteraemia, as well as endocarditis, are now common. Enterococci are usually susceptible to some penicillins, e.g. ampicillin, while being resistant to cephalosporins (108). Resistance of *Enterococcus faecalis* to a large number of antibiotics has complicated the antibiotic treatment and management of these infections.

Only a few strains of *Enterococcus faecalis* produce β-

lactamases (109). ß-Lactam resistance is usually mediated by altered penicillin-binding proteins, of which five are detected in *Enterococcus faecalis*. Penicillin-binding protein 1 and penicillin-binding protein 3 are proposed as target proteins as they bind ß-lactams at concentrations comparable to minimum inhibitory concentrations (110,111), while penicillin-binding protein 5 appears to be least sensitive to ß-lactams. There is variable resistance to gentamicin. So-called high-level resistance occurs when the MIC ≥2000µg/ml (112).

Resistance to aminoglycosides is plasmid-mediated, the plasmids coding for production of aminoglycoside-modifying enzymes. As described for staphylococci, the aminoglycosides are inactivated by three classes of aminoglycoside-modifying enzymes, viz. O-phosphotransferases, N-acetyltransferases and adenylyltransferases. However only the first two have been detected in strains of *Enterococcus faecalis* (113). Other mechanisms of resistance are firstly diminished uptake of the aminoglycoside in energy-dependent phase I (58), and secondly ribosomal, whereby mutations result in antimicrobial agents being unable to attach to the ribosomes.

The development of resistance to all aminoglycosides in some strains of *Enterococcus faecalis* has rendered the treatment of patients with endocarditis extremely

difficult (114). It has been established that high-level resistance of streptococci and enterococci to aminoglycosides blocks the synergistic effect that normally occurs when aminoglycosides are combined with penicillin or vancomycin (115). *Enterococcus faecalis* is known to harbour a large number of plasmids encoding resistance to tetracyclines, macrolides, chloramphenicol and aminoglycosides. These plasmids appeared to be responsible for the dissemination of resistance genes encoding for a variety of aminoglycoside-modifying enzymes (116), and accounted for the dramatic increase both of the number of resistant strains and the number of resistant patterns in enterococci (114,116). Despite the question of the pathogenicity of the enterococcus, the basis for the antimicrobial treatment of enterococcal infection is well established. Studies have consistently shown that enterococci are more resistant to antibiotics than most other streptococci (117). The relatively high minimal inhibitory concentrations of some ß-lactams have been related to the decreased affinity of certain enterococcal penicillin-binding proteins for these agents (118,119). However, combinations of some of these agents with aminoglycosides do achieve bactericidal synergy (120,121). Although enterococci have become progressively more resistant to such antimicrobial agents such as erythromycin and the tetracyclines, it is

the evolution of aminoglycoside resistance that has been of clinical significance. High-level resistance to gentamicin has shown to be endemic to many regions of the United States. Studies have established that organisms with high-level resistance to gentamicin are generally resistant to all other aminoglycosides. Invariably, high-level resistance to gentamicin also entails the loss of bactericidal synergy with combinations of gentamicin and either penicillin or vancomycin (112,122,123). In addition to high-level resistance to aminoglycosides, in itself a cause for concern, beta-lactamase production by isolates of *Enterococcus faecalis* resistant to gentamicin has recently been described (124,125). Clinical isolates of enterococci with either high-level resistance to aminoglycoside agents (126,127,128) or vancomycin (129,130,131), as well as isolates producing ß-lactamase (132,133), have been recognised. As a result, treating patients with enterococcal infections has become difficult.

1.2.2 Viridans streptococci

The viridans streptococci are found as commensals in the oral cavity. They cause a transient bacteraemia following dental procedures and continue to be the most frequent cause of native valve endocarditis. In addition

they sometimes cause prosthetic valve endocarditis (134,135).

Much difficulty has been encountered in attempting to classify viridans streptococci. Species cannot be differentiated by serological tests (136), and discrepancies between investigators exists because each only uses specific physiological (biochemical) tests. Also certain of these biochemical tests are not sufficiently optimized for these bacteria (137).

This study included strains of *Streptococcus mitis* and *Streptococcus sanguis* II taken from the South African Institute for Medical Research which uses a combination of two classifications. Firstly the classification by French *et.al*. This system incorporates the API-20STREP system, which is a commercial test kit for the identification of streptococci, along with a series of additional biochemical reactions (138). Secondly a taxonomy used by Kilian *et.al*., which includes a series of biochemical tests and serological examinations (137).

These organisms had long been considered universally susceptible to penicillin. However, in the past few years there has been an emergence of penicillin-resistant viridan streptococci, with MICs $\geq 2\mu g/ml$ (intermediately resistant).

Farber *et al* (10), working on South African strains of viridans streptococci found the strains of *Streptococcus mitis* and *Streptococcus sanguis* to be resistant to

penicillin (MICs 2-16μg/ml) and streptomycin (MICs >2000 μg/ml).

Of the five penicillin-binding proteins detected in streptococci, the majority are essential and are targets for ß-lactam antibiotics. However, the organisms can survive relatively high concentrations of penicillin due to modifications of these penicillin-binding proteins. Farber *et.al.* (139) observed that there were major changes in penicillin-binding proteins of penicillin-resistant viridans streptococci compared with those of penicillin-susceptible strains.

When aminoglycoside-modifying enzyme activity was studied, their observations showed that no adenylating, phosphorylating or acetylating enzymes were produced by these bacteria. Since aminoglycoside resistance occurred without detectable plasmid transfer, it was suggested that resistance was chromosomally mediated (10).

Recently, high-level streptomycin resistance among viridans streptococci has been reported (140).

Resistance to vancomycin among Gram-positive cocci has been exceedingly rare (141,142,143,144), but there has been a recent observation of a serious infection caused by vancomycin-resistant *Streptococcus sanguis II*. Shlaes *et.al.* (145), reported a MIC and MBC >128 μg/ml. It is possible that this strain was wrongly identified and could have been a *Leuconostoc* species.

1.3 *LEUCONOSTOC* SPECIES

Leuconostoc species are Gram-positive, catalase-negative, non-motile, nonsporeforming facultative anaerobes found in plants and dairy products (146). The key reactions that differentiate *Leuconostoc* species from streptococci are gas production during glucose fermentation and the failure of *Leuconostoc* to hydrolyse arginine with the production of ammonia. Until recently, *Leuconostoc* species, which have been used in the dairy, wine and pickling industries, were not thought to be pathogenic for humans or animals (146,147). However, these bacteria have recently been recognised as causing serious infections. Among the reported diseases caused by *Leuconostoc* species are meningitis (148,149), bacteraemia and septicaemia (150,151,152,153,154,155). Reviews of all the latest literature concerning *Leuconostoc* species indicate that there is still much confusion and misidentification of this organism. (The isolate of Shlaes *et.al.* (145), may actually be a *Leuconostoc* species according to doubts of the existence of vancomycin resistant streptococci expressed by Thornsberry and Facklam (156)).

It is very likely that difficulty in distinguishing *Leuconostoc* species from streptococci has resulted in the underreporting of *Leuconostoc* infection. As *Leuconostoc* species are usually resistant to vancomycin,

the isolation of vancomycin-resistant streptococci should encourage further laboratory investigations to determine the identity of the isolates which may be *Leuconostoc* species or other nonstreptococcal bacteria. Isenberg *et.al.* isolated vancomycin-resistant Gram-positive cocci, which were later identified as *Leuconostoc* species, following the guidelines of Garvie (146,147). The main indicator would seem to be the fact that *Leuconostoc* species show a constant pattern of varied resistance to vancomycin. Resistance varies with MICs of 4μg/ml (150), to 1024μg/ml (157). Although vancomycin-resistance would seem to be the norm, most isolates are susceptible to penicillin and penicillin therapy combined with an aminoglycoside (tobramycin) has been successful (158).

1.4 CORYNEBACTERIA

Corynebacteria are Gram-positive rods of varying lengths, often containing volutin (polyphosphate) granules. They are non-motile, non-capsulate and non-acid-fast, grow best aerobically, though are facultatively anaerobic. They are catalase-positive, oxidase-negative and produce acid but not gas, both fermentatively and oxidatively from carbohydrates (159).

1.4.1 *Corynebacterium jeikeium*

Corynebacterium jeikeium is the name recently given to a group of coryneform rods, formerly known as the JK group. These rods grow on sheep blood agar to form very small, smooth white colonies.

Diphtheroids other than *Corynebacterium diphtheriae*, are increasingly being acknowledged as causing human infection (160,161). Most of these infections are acquired in hospitals and take place in immuno-compromised patients (e.g. patients with granulo-cytopenia associated with leukaemia or patients who have undergone cardiac surgery.

Corynebacterium jeikeium has been associated with bacteraemia, septicaemia and endocarditis, including prosthetic valve endocarditis (162,163,164).

Susceptibility patterns vary considerably (161).

Spitzer *et.al.* (165), found certain strains to be aminoglycoside (gentamicin and tobramycin) -resistant (MIC >1024µg/ml), and other strains to be aminoglycoside susceptible (MIC 0,125µg/ml). They also showed the glycopeptides (vancomycin and teicoplanin) to inhibit 90% of the strains they tested (MIC_{90} 1,0µg/ml). The latter results were confirmed by De Briel *et.al.* (163), who also found strains to be susceptible to pristinamycin, novobiocin and fusidic acid. Other antibiotics (ampicillin, cephalothin, gentamicin,

amikacin, nitrofurantoin, norfloxacin, ciprofloxacin, minocycline and rifampicin) demonstrated less consistent activity. Other in vitro studies by Philippon and Bimet (166) showed strains sensitive to fusidic acid (MIC 0,234µg/ml), pristinamycin (MIC 0,235µg/ml) and teicoplanin (MIC 0,557µg/ml). Combinations of glycopeptide and aminoglycoside antibiotics failed to show consistent synergistic killing of either aminoglycoside-susceptible or -resistant strains. However, glycopeptide antibiotics were more effective against aminoglycoside susceptible strains than against aminoglycoside-resistant strains (165). Some strains were resistant to most antibiotics except vancomycin (167,168).

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CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains and identification

The bacterial strains use were clinical isolates obtained from blood cultures from patients attending the Johannesburg, Hillbrow and Baragwanath Hospitals. The isolates were stored in liquid nitrogen until required.

2.1.2 Antimicrobial agents

Standard antimicrobial reference powders and their sources were as follows:

Ampicillin :Beecham Laboratories
Ceftriaxone :Hoffman-LaRoche
Chloramphenicol:Parke Davis & Company
Ciprofloxacin :Bayer-Miles Pty Ltd
Cotrimoxazole :Hoffman-LaRoche
Fusidic acid :Leo Laboratories
Gentamicin :Schering Corporation
Methicillin :Beecham Laboratories
Oxacillin :Bristol Laboratories

Penicillin :Glaxo Laboratories Ltd

Rifampicin :Gruppo Lepitit

Vancomycin :Eli Lilly and Company

Stock solutions were prepared according to NCCLS recommendations (1).

2.2 SUSCEPTIBILITY TESTING

2.2.1 Minimum Inhibitory Concentrations

Minimum inhibitory concentrations were determined by a microtiter broth dilution technique, using sterile, U-bottom, 200µl plastic microdilution trays (Dynatech).

Isolates were inoculated into cation-supplemented Mueller-Hinton broth (BBL Microbiology Systems). The cation supplement was prepared as defined by NCCLS (2), to give a final concentration of Ca^{2+} of 20mg/l and Mg^{2+} of 10mg/l.

Cultures were incubated for approximately 4h at 37°C. The suspensions were then adjusted to contain approximately $1x10^8$ CFU/ml by comparing culture turbidity with a 0,5 MacFarland (barium sulphate) standard.

Each antibiotic stock solution was diluted to a concentration of 128µg/ml. Final concentrations were derived from serial two-fold dilutions of the starting concentration of 128µg/ml, to give antibiotic

concentration ranges of 0,06 - 128µg/ml.

The wells were inoculated with the suspensions of bacteria to give a final inoculum of approximately $1x10^6$ CFU/ml. A positive control well which did not contain the antimicrobial agent was used to assess:

(a) Viability of the test organism,

(b) Culture turbidity for reading endpoints.

One well contained medium only to act as a negative / sterility control.

A 10µl sample of each inoculum and from the positive-control well was also streaked onto a blood agar plate to perform a colony count and to act as a culture purity control.

The trays were placed in plastic bags to prevent evaporation and incubated at 37°C for 18h.

Following incubation the trays were placed on a Micro-Shaker (Dynatech) for one minute to resuspend the mixture.

Using an illuminated plate reader MIC 2000 (Dynatech Laboratories Inc.), visible growth or no growth was recorded. The minimum inhibitory concentration was reported as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism. The growth and purity controls were also checked for viability and mixed growths respectively.

In all the tests, quality control was monitored using the reference strain *Staphylococcus aureus* ATCC® 29213

2.2.2 Minimum Bactericidal Concentrations

Blood agar plates were inoculated with 10μl from all the wells up to and including the first well in which growth was evident, and were incubated at 37°C for 18h.
The minimum bactericidal concentrations were reported as the lowest concentration which inhibited 90% growth of the organisms.

2.2.3 Checkerboard Synergy Studies

Synergy studies were performed by a microtiter checkerboard technique, using cation supplemented Mueller-Hinton broth. The concentration of each antimicrobial agent tested ranged from three dilution factors below the minimum inhibitory concentration to three dilution factors above the minimum inhibitory concentration (i.e. 8 times lower and 8 times higher than the minimum inhibitory concentration).

The checkerboard was comprised of different antibiotic combinations constructed as follows:
Antibiotic A is diluted along the horizontal rows (range one eighth to 8 times MIC), and vertically each well receives the same concentration of antibiotic B (range one eighth to 8 times MIC).

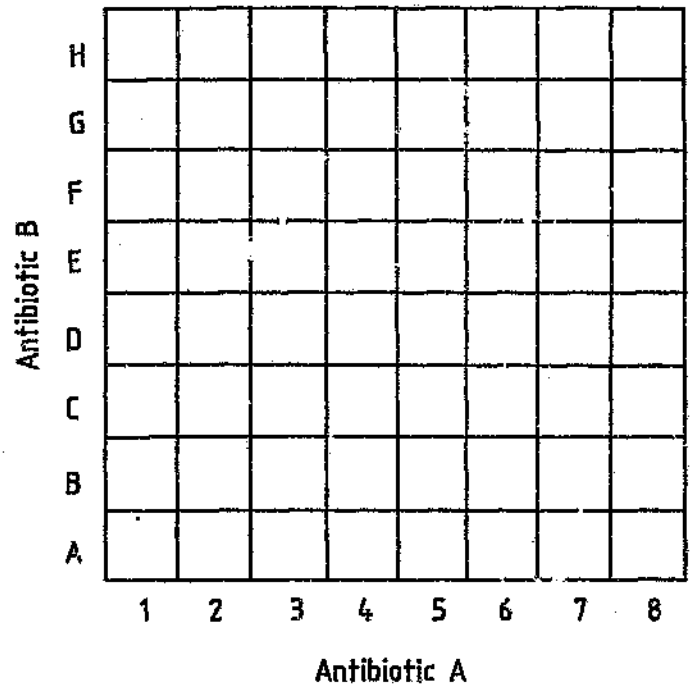

Figure 2.1 **Checkerboard with serial dilutions of two antibiotics whose concentrations are proportional to their MICs**

Checkerboard method example:

MIC A 8,0µg/ml; MIC B 8,0µg/ml

Concentrations of antibiotic A 1-64µg/ml in combination with antibiotic B 1-64µg/ml.

As the antimicrobial agent will be diluted four-fold on completion, the initial solutions are at 4 times the final concentrations when placed in the wells.

25µl of each antibiotic was placed in wells as follows:
Antibiotic A : wells 1-7: concentrations 1-64µg/ml respectively
Antibiotic B : wells A-G: concentrations 1-64µg/ml respectively
An extra 25µl of Mueller-Hinton broth was placed in rows 8 and H in order to maintain a constant volume of 50µl in the wells containing single antibiotic concentrations. As can be seen in Figure 2.1 well 8H contains only Mueller-Hinton broth and on addition of the inoculum will serve as the growth control.
Isolates were inoculated into cation supplemented Mueller-Hinton broth (previously described in 2.2.1), and placed in a waterbath at 37°C for approximately 4h. The cultures were then diluted to approximately $1x10^8$ CFU/ml (previously described in 2.2.1). A further 1:10 dilution was then made and 50µl of this culture suspension was placed in each well giving a final inoculum of $c.5x10^6$ CFU/ml. The trays were then placed in plastic bags to prevent evaporation and incubated at 37°C for 18h. Before reading, the trays were shaken and examined for growth (2.2.1). The results were plotted as isobolograms.

The results of checkerboard synergy tests were interpreted by examining the patterns of the isobolograms.

Synergy is defined as having a positive interaction: The combined effect of the drugs being examined is significantly greater than the expected result - based on their independent effects when they are used separately.

Antagonism is defined as having a negative interaction: The combined effect of the drugs being examined is significantly less than their independent effects when they are tested separately.

When the two drugs have a combined effect equal to the sum of their separate effects, they are regarded as additive.

Other interactions are considered indifferent.

These combinations are demonstrated in Figure 2.2 (3).

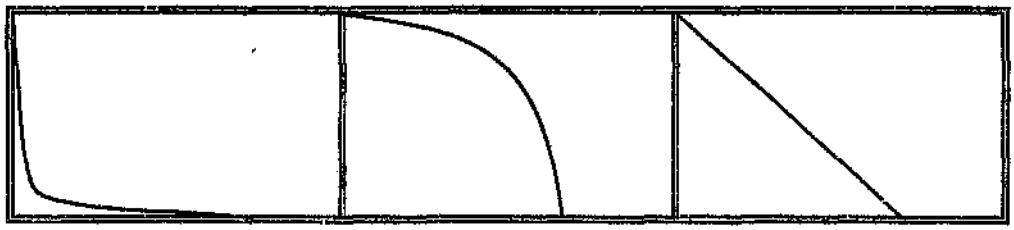

Figure 2.2 **Isobolograms demonstrating synergism, antagonism and indifference respectively**

2.2.4 Fractional Inhibitory Concentration Index

The fractional inhibitory concentration index (ΣFIC) is a mathematical restatement of the isobologram (4). The FIC of each antibiotic is derived by dividing the concentration of that antibiotic necessary to inhibit growth in a given row or column by the MIC of the test organism to that antibiotic alone. The ΣFIC is then calculated by adding the separate FICs for each of the antibiotics present in that well, and is calculated from the following formula:

$$\Sigma FIC = \frac{\text{MIC of a in the comb.}}{\text{MIC of a alone}} + \frac{\text{MIC of b in the comb.}}{\text{MIC of b alone}}$$

where a and b are two different antimicrobials. A combination of antimicrobials is synergistic when the ΣFIC is ≤0,5, and antagonistic when the ΣFIC is ≥2,0. In between is regarded as indifferent.

2.2.5 Killing Curve Synergy Studies

Time-kill studies were performed as set out by Krogstad and Moellering (5).

2.2.5.1 Inoculum

Inocula were prepared by one of two methods depending on which growth phase was to be challenged by the antibiotics tested.

(a) Stationary-phase cells:

From a blood agar plate 5 colonies were inoculated into 10cm^3 of Mueller-Hinton broth. The broth was incubated overnight at 37°C. The optical density of the culture was measured at 620nm using a spectrophotometer (LKB, Sweden) and viable cell counts were performed to standardise cell numbers for the time-kill experiments. For each experiment a fresh overnight culture was prepared to ensure only a short lag-phase following the addition of antibiotics.

(b) Early exponential-phase cells:

To an overnight culture grown in Mueller-Hinton broth, glycerol was added to give a final concentration of 10% (v/v). Aliquots of the culture suspension were stored at -70°C. One aliquot was retained and a viable cell count performed to estimate cell numbers. Cultures held at -70°C were diluted on the day of the experiment grown to early

exponential-phase (1,5h), before addition of the antibiotics.

4.2.5.2 Viable counts

Culture viability was estimated by the method of Miles and Misra (6). Ten-fold dilutions of cultures were made using Mueller-Hinton broth as diluent. 20µl samples from appropriate dilutions were spotted onto the surface of air dried blood agar plates. The plates were incubated overnight at 37°C. Between 20-200 colonies per spot were counted using a colony counter (Darkfield Quebec). CFU/ml were calculated as follows:

e.g. colonies per spot: 100, 105, 94, 102 & 99 at a dilution of 10^{-2}.

Average colony count x Dilution factor x sample 20µl conversion to ml

$$100 \times 10^{2} \times 50$$

$$= 5 \times 10^{5} \text{ CFU/ml}$$

If the culture was turbid samples were taken from 10^{-4} to 10^{-7} dilutions. If there was no visible growth samples were taken from the test flask (neat) and dilutions 10^{-1} to 10^{-4}. On sampling and performing each dilution series

20µl aliquots were delivered onto the agar plate immediately so as to obviate continuation of growth.

2.2.5.3 Time-kill method

The killing rates of bacteria using single antimicrobial agents and antibiotic combinations were determined according to previously described methods (7).

The medium was cation-supplemented Mueller-Hinton broth (2.2.1). Tests were carried out in 25ml flasks containing a final volume of 10ml. The culture inoculum prepared as described above (2.2.5.1) was diluted to give an initial viable count of about $5x10^5$ CFU/ml. Antibiotics were added immediately to stationary-phase cells or after a growth period of about 1½h for experiments on early exponential-phase cells. All experiments included antibiotic-free culture controls. The cultures were incubated at 37°C and 150 r.p.m. on an orbital environmental shaker (New Brunswick Scientific, N,J., U.S.A.). Viable counts (2.2.5.2) were performed on addition of the antibiotics and thereafter at 2h, 4h, 6h and 24h. Results were plotted on semilog paper and antibiotic interactions determined according to Lorian Figure 2.3 (5).

The kill curves were done in duplicate, each antibiotic in turn used at a concentration which just held the organisms growth.

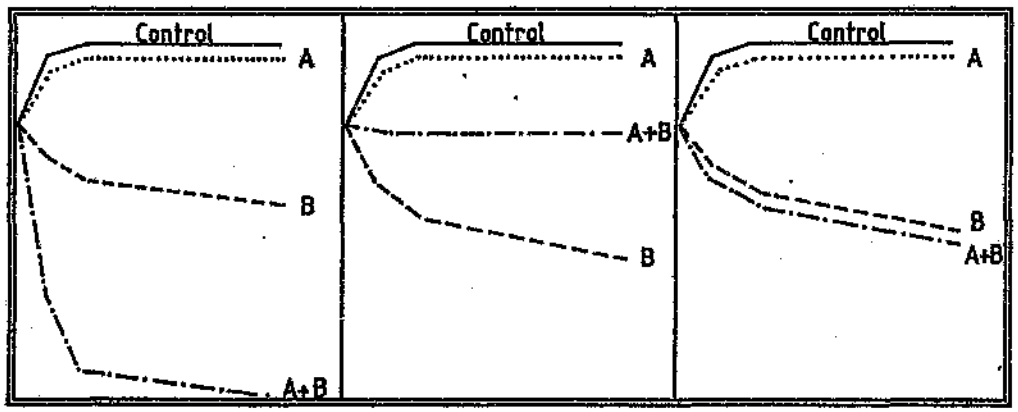

Figure 2.3 **Effects of antimicrobial combinations as measured by kill-curve method**

2.3 DISCUSSION

In contrast to the checkerboard method which typically provides only inhibitory data following overnight incubation, the killing curve technique measures the bactericidal activity of the antibiotic combination being tested at short intervals during a 24h period.
A major advantage of killing curves over checkerboard methodology is therefore that they provide a dynamic picture of antimicrobial action (9) and interaction over

time, as opposed to checkerboard which is usually examined after 16-12h of incubation.

As for selection for resistant strains, the methods chosen were standard methods used in most routine laboratories.

The only problem experienced was the lysing of streptococcal cells when performing killing curves so that no 24h readings could be obtained.

NOTE: All experiments were done in duplicate to validate results.

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CHAPTER THREE

RESULTS: STAPHYLOCOCCI

3.1 MINIMUM INHIBITORY CONCENTRATIONS / MINIMUM BACTERICIDAL CONCENTRATIONS

The MICs and MBCs of methicillin, ciprofloxacin, fusidic acid, rifampicin, vancomycin, and gentamicin were determined against five methicillin-sensitive and five methicillin-resistant strains of *Staphylococcus aureus* as described in Chapter two.

The results are given in Table 3.1.

The methicillin-sensitive strains were susceptible to methicillin (MICs 2μg/ml, MBCs 4μg/ml), ciprofloxacin (MICs 0,5-1μg/ml, MBCs 0,5-1μg/ml), fusidic acid (MICs 0,03-0,12μg/ml, MBCs 0,06-0,12μg/ml), rifampicin (MICs 0,0015-0,003μg/ml, MBCs 0,003μg/ml), and vancomycin (MICs 2μg/ml, MBCs 2-4μg/ml). Of the strains, 4 of the 5 were sensitive to gentamicin (MICs ≤4μg/ml, MBCs 2-8μg/ml), with gentamicin having an MIC of 8μg/ml and a MBC of 8μg/ml against the resistant strain.

Rifampicin and fusidic acid were the most active agents against methicillin-sensitive strains of *Staphylococcus aureus*, with both ciprofloxacin and vancomycin demonstrating good activity. Least active was

gentamicin.

The methicillin-resistant strains were resistant to gentamicin (MICs 128-256µg/ml, MBCs 128-256µg/ml), while being sensitive to ciprofloxacin (MICs 0,25-0,5µg/ml, MBCs 0,5µg/ml), rifampicin (MICs 0,0015-0,003µg/ml, MBCs 0,003-0,006µg/ml) and vancomycin (MICs 1-2µg/ml, MBCs 1-2µg/ml).

As with the methicillin-sensitive strains, rifampicin was the most active agent against the methicillin-resistant strains with vancomycin, fusidic acid and ciprofloxacin also demonstrating good activity. Again gentamicin was least active.

The greatest difference between the MICs and MBCs for all the strains tested was a one-fold dilution.

Table 3.1 **Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations**

Organism	:*Staphylococcus aureus* (Methicillin-sensitive) (n=5)
Antibiotic	:Methicillin (Breakpoint 8µg/ml)
MICs	:2 (5)
MBCs	:4 (5)
Antibiotic	:Ciprofloxacin (Breakpoint 1µg/ml)
MICs	:0,5 (3), 1 (2)
MBCs	:0,5 (2), 1 (3)
Antibiotic	:Fusidic acid
MICs	:0,03 (1), 0,06 (2), 0,12 (2)
MBCs	:0,06 (1), 0,12 (4)
Antibiotic	:Rifampicin (Breakpoint 1µg/ml)
MICs	:0,0015 (3), 0,003 (2)
MBCs	:0,003 (5)
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:2 (5)
MBCs	:2 (3), 4 (2)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:1 (1), 2 (2), 4 (1), 8 (1)
MBCs	:2 (1), 4 (2), 8 (2)

Table 3.1 (Continued)

Organism	:*Staphylococcus aureus* (Methicillin-resistant) (n=5)
Antibiotic	:Methicillin (Breakpoint 8µg/ml)
MICs	:16 (1), 32 (3), 64 (1)
MBCs	:32 (1), 64 (2), 128 (2)
Antibiotic	:Ciprofloxacin (Breakpoint 1µg/ml)
MICs	:0,25 (2), 0,5 (3)
MBCs	:0,5 (5)
Antibiotic	:Fusidic acid
MICs	:0,03 (3), 0,06 (2)
MBCs	:0,06 (5)
Antibiotic	:Rifampicin (Breakpoint 1µg/ml)
MICs	:0,0015 (3), 0,003 (2)
MBCs	:0,003 (3), 0,006 (2)
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:1 (3), 2 (2)
MBCs	:1 (2), 2 (3)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:128 (4), 256 (1)
MBCs	:128 (2), 256 (3)

3.2 CHECKERBOARD SYNERGY STUDIES

Checkerboard studies were performed on selected methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains using various combinations of antimicrobial agents. The results depicted in the form of isobolograms are shown in Figures 3.1.1 - 3.5.4 (Appendix Figures A.1 - A.8), and ΣFICs given in Table 3.2.

The combinations used were as follows:

Vancomycin combined with fusidic acid in Figures 3.1.1 and 3.1.2 (methicillin-sensitive *Staphylococcus* aureus) and 3.1.3 (methicillin-resistant *Staphylococcus aureus*), vancomycin combined with ciprofloxacin in Figures 3.2.1 and 3.2.2 (methicillin-sensitive *Staphylococcus aureus*) and 3.2.3 (methicillin-resistant *Staphylococcus aureus*), vancomycin combined with rifampicin in Figures 3.3.1 and 3.3.2 (methicillin-sensitive *Staphylococcus aureus*) and 3.3.3 (methicillin-resistant *Staphylococcus aureus*), and ciprofloxacin combined with rifampicin in Figures 3.4.1 to 3.4.3 (methicillin-sensitive *Staphylococcus aureus*) and 3.4.4 (methicillin-resistant *Staphylococcus aureus*).

All combinations demonstrated either indifference or weak antagonism as defined in Chapter two. Methicillin plus gentamicin demonstrated antagonism (Figure 3.5.1)

(ΣFIC 2). Figure 3.5.2 (methicillin-sensitive *Staphylococcus aureus*) demonstrated indifference (ΣFIC 1) while Figures 3.5.3 (methicillin-sensitive *Staphylococcus aureus*) and 3.5.4 (methicillin-resistant *Staphylococcus aureus*) demonstrated synergy (ΣFICs 0,2 and 0,1 respectively).

Table 3.2 Fractional Inhibitory Concentration Indices

Organism *Staphylococcus aureus* (Methicillin-sensitive)

Antimicrobial Combination			ΣFICs		
Methicillin	+	Gentamicin	2,0	1,0	0,2
Fusidic Acid	+	Vancomycin	1,0	1,0	2,0
Ciprofloxacin	+	Vancomycin	2,0	1,0	2,0
Rifampicin	+	Vancomycin	1,0	2,0	2,0
Rifampicin	+	Ciprofloxacin	1,0	2,0	2,0

Organism *Staphylococcus aureus* (Methicillin-resistant)

Antimicrobial Combination			ΣFICs	
Methicillin	+	Gentamicin	0,1	0,2
Fusidic Acid	+	Vancomycin	2,0	2,0
Ciprofloxacin	+	Vancomycin	2,0	2,0
Rifampicin	+	Vancomycin	2,0	2,0
Rifampicin	+	Ciprofloxacin	2,0	2,0

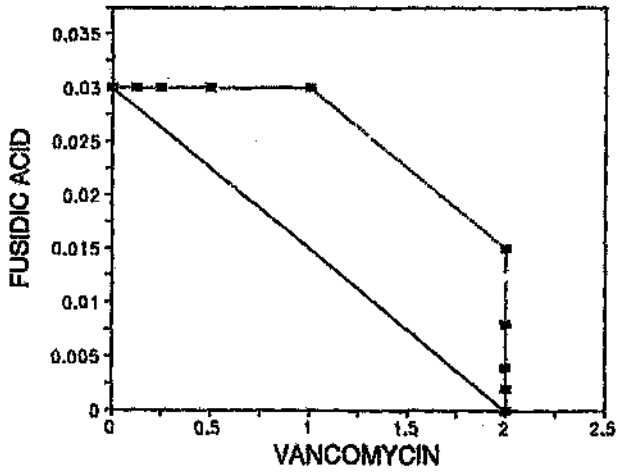

Figure 3.1.1 Activity of Fusidic acid (MIC 0,03µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

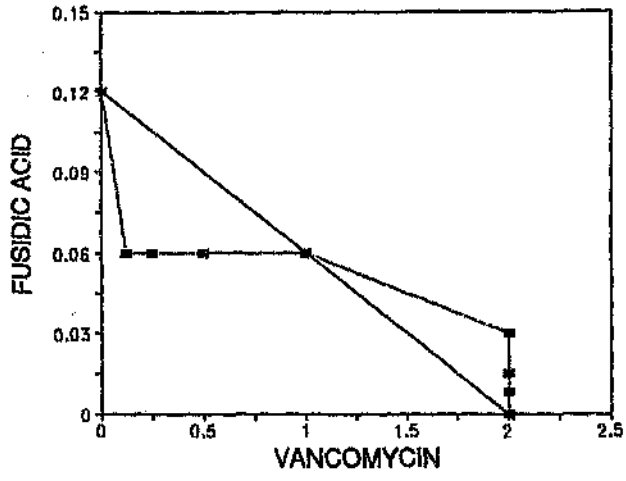

Figure 3.1.2 Activity of Fusidic acid (MIC 0,12µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

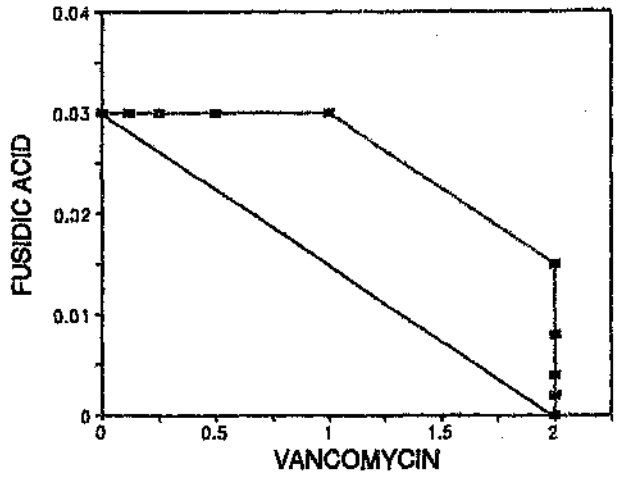

Figure 3.1.3 Activity of Fusidic acid (MIC 0,03µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

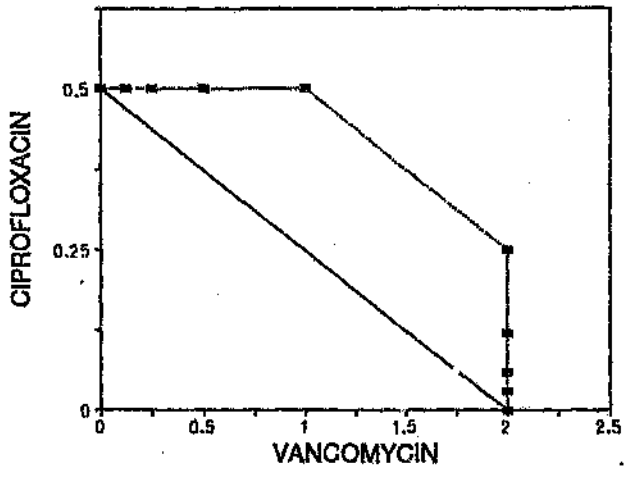

Figure 3.2.1 Activity of Ciprofloxacin (MIC 0,5µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

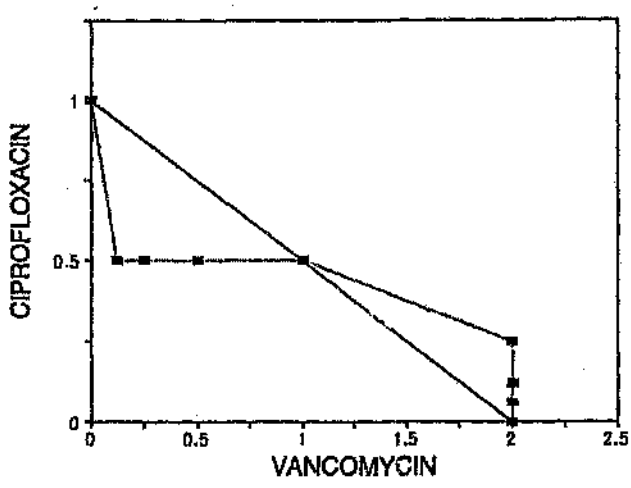

Figure 3.2.2 Activity of Ciprofloxacin (MIC 1,0µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

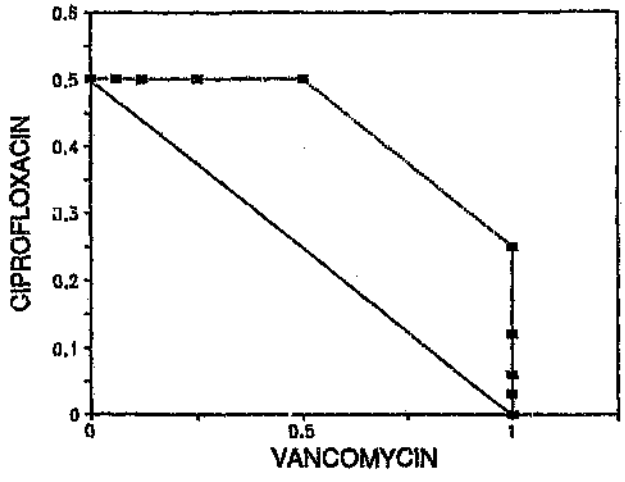

Figure 3.2.3 Activity of Ciprofloxacin (MIC 0,5µg/ml) plus Vancomycin (MIC 1µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

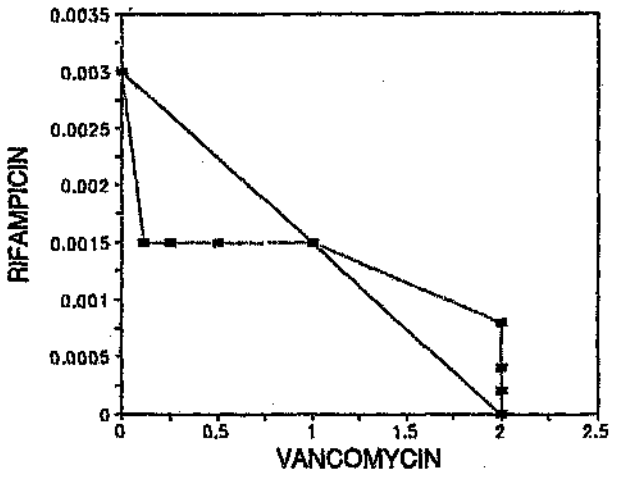

Figure 3.3.1 Activity of Rifampicin (MIC 0,003µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

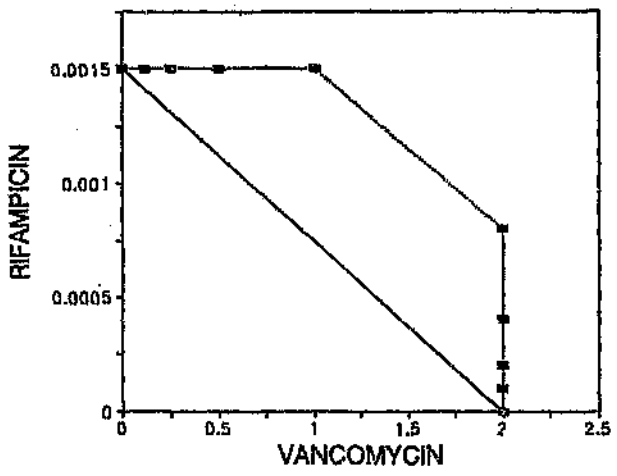

Figure 3.3.2 Activity of Rifampicin (MIC 0,0015µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

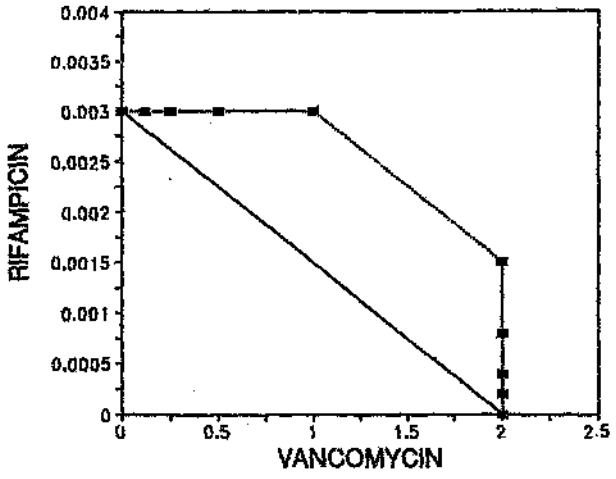

Figure 3.3.3 Activity of Rifampicin (MIC 0,003µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

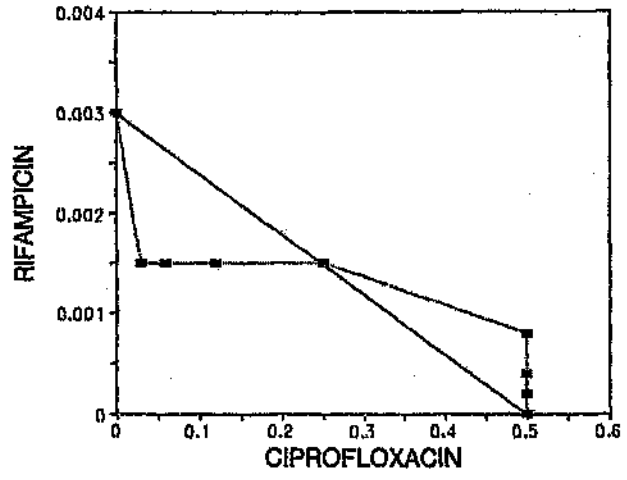

Figure 3.4.1 Activity of Rifampicin (MIC 0,003µg/ml) plus Ciprofloxacin (MIC 0,5µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

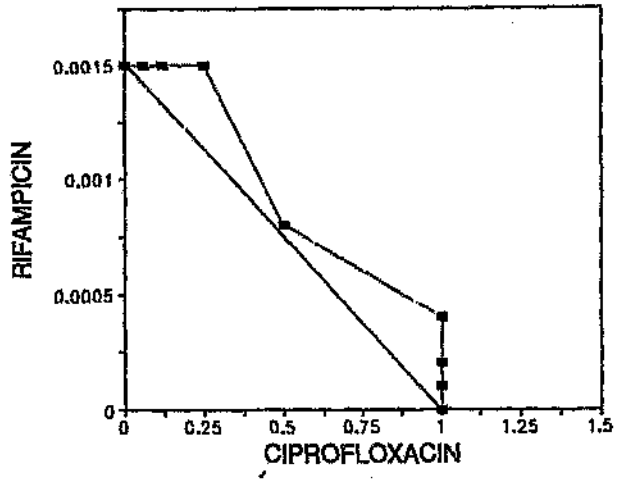

Figure 3.4.2 Activity of Rifampicin (MIC 0,0015µg/ml) plus Ciprofloxacin (MIC 1µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

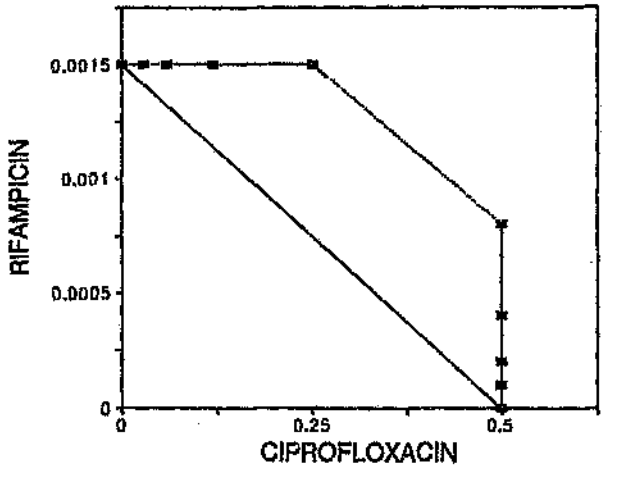

Figure 3.4.3 Activity of Rifampicin (MIC 0,0015µg/ml) plus Ciprofloxacin (MIC 0,5µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

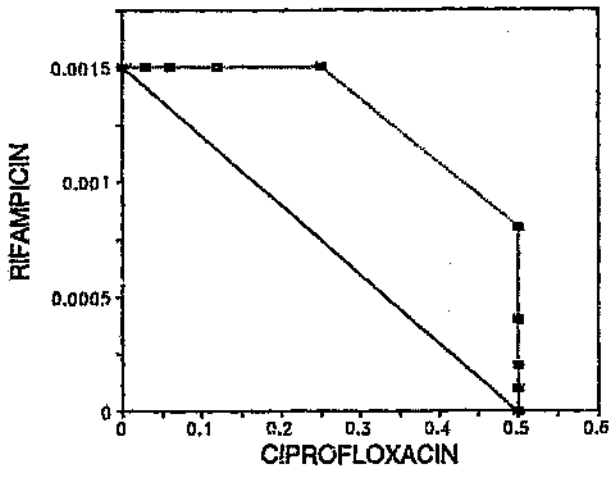

Figure 3.4.4 Activity of Rifampicin (MIC 0,0015µg/ml) plus Ciprofloxacin (MIC 0,5µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

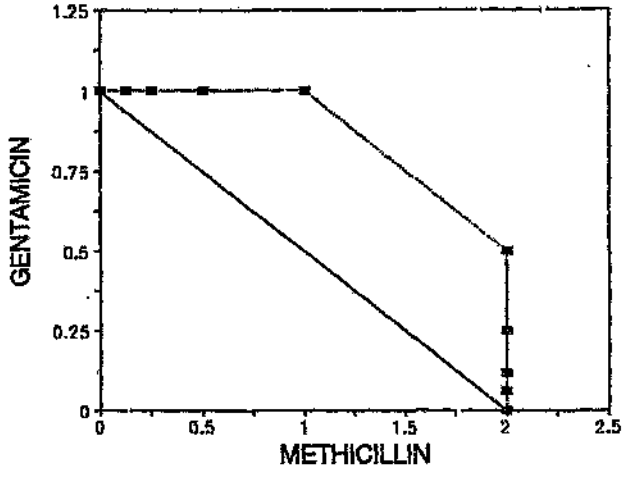

Figure 3.5.1 Activity of Gentamicin (MIC 1µg/ml) plus Methicillin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

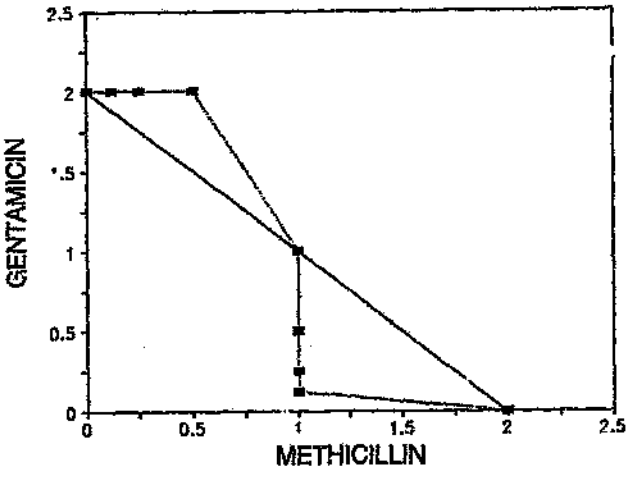

Figure 3.5.2 Activity of Gentamicin (MIC 2µg/ml) plus Methicillin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

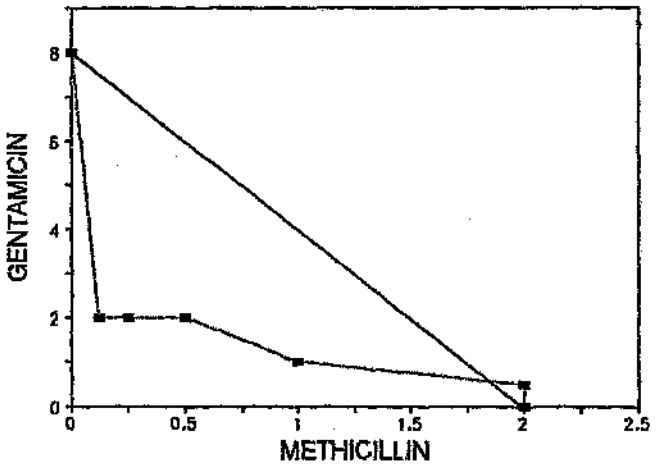

Figure 3.5.3 Activity of Gentamicin (MIC 8µg/ml) plus Methicillin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

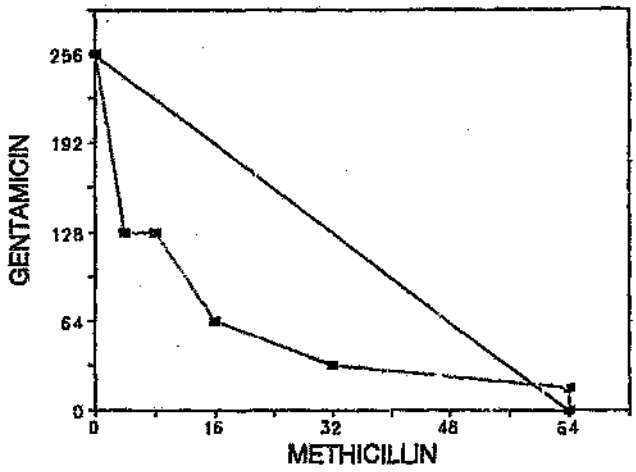

Figure 3.5.4 Activity of Gentamicin (MIC 256µg/ml) plus Methicillin (MIC 64µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

3.3 DISCUSSION

Of the 5 methicillin-sensitive and 5 methicillin-resistant isolates of *Staphylococcus aureus* included in this study, the MICs of methicillin of the sensitive and resistant strains were comparable with those reported in larger studies. Studies by Knox, 1960 (1) in England, John and McNeill in America, 1980 (2), and Benson *et al*, 1987 (3) reported MICs of between 2-4µg/ml for methicillin-sensitive *Staphylococcus aureus* and MICs of 4-128µg/ml for methicillin-resistant *Staphylococcus aureus* isolates. These studies show that over a period of 30 years there is still a group of isolates that remain sensitive to isoxazolyl penicillins.

All the *Staphylococcus aureus* strains investigated in this study were susceptible to ciprofloxacin with MICs ranging from 0,5-1µg/ml against methicillin-sensitive isolates and 0,25-0,5µg/ml for the methicillin-resistant isolates. Thus the MICs for ciprofloxacin against both groups of staphylococci were comparable. However, increasing resistance to ciprofloxacin due to the use of this agent, has been reported. A study in Michigan reported ciprofloxacin resistance in methicillin-resistant staphylococci (MIC values 6,25-50µg/ml) (4). Although resistance to ciprofloxacin was not reported in

two other studies performed in the United States of America (5,6), Schaefler (7), demonstrated high-level ciprofloxacin resistance against *Staphylococcus aureus* (MICs 12,5-100µg/ml). Similarly, Forstall *et.al.* (8), reported MICs of 8 to >128µg/ml for ciprofloxacin in methicillin-resistant *Staphylococcus aureus*.

Furthermore, a methicillin-sensitive *Staphylococcus aureus* was isolated from a lung abscess from a patient in Dublin. The patient was treated intravenously with ciprofloxacin and after seven days treatment the MIC for ciprofloxacin was 4µg/ml (9).

It is therefore important to monitor susceptibility to ciprofloxacin in South Africa.

Infections, especially osteomyelitis, caused by *Staphylococcus aureus*, including methicillin-resistant strains respond well treatment with fusidic acid. Only fusidic acid-sensitive strains were included in this study (0,03-0,12µg/ml).

However resistant strains have been reported. A study performed in Belgium by Van der Auwera *et.al.* (10), demonstrated resistance to fusidic acid with MICs ranging from 0,025-3,2µg/ml.

All the staphylococci selected for this study were susceptible to rifampicin, having MICs well below the breakpoint of 1µg/ml. These results were similar to

studies by Sabath *et.al.* (11), and Bartoloni *et.al.* (12), who reported MICs of 0,0003-0,005µg/ml. However, rifampicin-resistant isolates occur world wide. When rifampicin is used alone, resistance develops rapidly, but resistant mutants occur less frequently when rifampicin is used together with another antimicrobial agent (13).

Recently a HIV-positive intravenous drug abuser with right-sided endocarditis caused by methicillin-sensitive, ciprofloxacin- and rifampicin-sensitive *Staphylococcus aureus* was treated with intravenous cloxacillin. The patient was discharged on oral ciprofloxacin and rifampicin. He/she relapsed and after readmission, the *Staphylococcus aureus* isolated from blood cultures was now rifampicin- and ciprofloxacin resistant (MICs >128µg/ml and >8µg/ml respectively), while still being sensitive to cloxacillin (MIC ≤0,25µg/ml). The latter was administered intravenously and the patient was discharged with no symptoms (14).

All the *Staphylococcus aureus* isolates in this study were susceptible to vancomycin, this being consistent with other studies (12,15). To date there have been no reports of vancomycin-resistant *Staphylococcus aureus* isolates. Many studies attest to this clinical efficacy of vancomycin in serious *Staphylococcus aureus* infections (16,17,18,19).

While four of the five strains of methicillin-sensitive *Staphylococcus aureus* were susceptible to gentamicin (MICs 1-4μg/ml), all the methicillin-resistant *Staphylococcus aureus* strains were resistant (MICs 128-256μg/ml).

Strains of *Staphylococcus aureus* investigated by Norden and Keleti (20) were found to be vancomycin tolerant, however no strains of *Staphylococcus aureus* in this study demonstrated tolerance.

Serious infections caused by *Staphylococcus aureus* often require combination therapy (21).

Although the only combination which was found to be synergistic against isolates of *Staphylococcus aureus* used in this study was gentamicin and methicillin, many other studies performed on different isolates, from different countries and using different techniques have demonstrated synergy with other drug combinations. It must be noted however that different methodologies for determining synergy e.g. time-kill and checkerboard methods are not fully comparable (22).

In this study none of the ten isolates tested against rifampicin combined with vancomycin in checkerboard studies demonstrated synergy. Faville *et.al.* (23), reported two clinical cases where the combination of rifampicin plus vancomycin demonstrated synergy. In the

first case a methicillin-resistant *Staphylococcus aureus* was isolated from a patient with endocarditis and septicaemia. In the second case a methicillin-sensitive *Staphylococcus aureus* was isolated from a patient with endocarditis and bacteraemia. Both patients recovered fully after treatment. However in another study by Van der Auwera *et.al.* (24), only 61% of the 33 patients treated with the combination of rifampicin plus vancomycin were clinically cured.

In this study when rifampicin was combined with ciprofloxacin, indifferent results were obtained. Hackbarth *et.al.* (25) using the combination of ciprofloxacin and rifampicin against methicillin-sensitive *Staphylococcus aureus* found the effect of this combination to be additive. While synergy was demonstrated with a ciprofloxacin / rifampicin combination in a rabbit model used by Kaatz and co-workers (26), a study in Denmark using time-kill procedures found antagonism between these two agents (27).

In a study performed by O'Grady and Greenwood (28), fusidic acid used in combination with methicillin against methicillin-sensitive *Staphylococcus aureus* demonstrated antagonism. In the same study fusidic acid was combined with vancomycin which demonstrated either

indifference or antagonism. Although antagonism might not occur with all strains, the combination should not be used empirically, and only used after synergy studies on individual isolates have been performed. It is thus suggested that alternative combinations be sought.

Although it is suggested that vancomycin be combined with rifampicin for treating vancomycin-tolerant strains of *Staphylococcus aureus* (12), the combinations of vancomycin plus rifampicin in this study as well as fusidic acid and ciprofloxacin did not demonstrate synergy.

Many studies have demonstrated synergy with a combination of an aminoglycoside and a beta-lactam against many organisms e.g. enterococci and streptococci (29,30,31,32,33,34,35,36).

A penicillinase-resistant penicillin combined with an aminoglycoside usually exhibits in vitro synergy against methicillin-sensitive staphylococci (37). In this study synergy was demonstrated when gentamicin was combined with methicillin. One methicillin-sensitive *Staphylococcus aureus* and two methicillin-resistant *Staphylococcus aureus* isolates showed synergy when methicillin was combined with gentamicin. Against the remaining strains, which included methicillin-sensitive and methicillin-resistant isolates the combination

demonstrated indifference or antagonism.

In order to demonstrate synergy between gentamicin and methicillin a dose of 8μg/ml of gentamicin was required.

From these results, such a combination appears to be the most appropriate treatment. However, for treating penicillin-allergic patients, or patients with infections caused by methicillin-resistant strains, alternative combinations must be considered. As none of the other combinations tested against the isolates in this study resulted in synergy, it is recommended that each strain be investigated individually, and the patient treated according to the in vitro results.

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CHAPTER FOUR

RESULTS: STREPTOCOCCI

4.1 MINIMUM INHIBITORY CONCENTRATIONS / MINIMUM BACTERICIDAL CONCENTRATIONS

The MICs and MBCs of penicillin, gentamicin and vancomycin were determined against five strains of *Streptococcus sanguis* and six strains of *Streptococcus mitis* according to methods described in Chapter two. The results are given in Table 4.1.

All *Streptococcus sanguis* isolates were resistant to gentamicin (MICs 8-32µg/ml, MBCs 8-64µg/ml), while being susceptible to vancomycin (MICs 1-2µg/ml, MBCs 1-2µg/ml). Only one of the isolates tested was sensitive to penicillin (MIC 0,12µg/ml, MBC 0,12µg/ml) the rest were intermediately resistant (MICs 0,25-4µg/ml, MBCs 0,5-8µg/ml).

All six *Streptococcus mitis* isolates were resistant to gentamicin five showed low-level gentamicin resistance (MICs 8-32µg/ml, MBCs 16-64µg/ml), while one had high-level gentamicin resistance (MIC 1024µg/ml, MBC 1024µg/ml). All six *Streptococcus mitis* isolates were susceptible to vancomycin (MICs 1-2µg/ml, MBCs 1-2µg/ml). Only one strain was sensitive to penicillin

(MIC 0,12μg/ml, MBC 0,12μg/ml), the rest showed intermediate resistance with MICs of 0,5-4μg/ml and MBCs of 0,5-4μg/ml. The strain demonstrating high-level gentamicin resistance was also fully resistant to penicillin having a MIC of 32μg/ml and a MBC of 32μg/ml.

Table 4.1 **Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations**

Organism	:*Streptococcus sanguis* (n=5)
Antibiotic	:Penicillin (Breakpoint 0,12µg/ml)
MICs	:0,12 (1), 0,25 (1), 1 (1), 2 (1), 4 (1)
MBCs	:0,12 (1), 0,5 (1), 2 (1), 4 (1), 8 (1)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:8 (1), 16 (2), 32 (2)
MBCs	:8 (1), 32 (2), 64 (2)
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:1 (3), 2 (2)
MBCs	:1 (3), 2 (2)
Organism	:*Streptococcus mitis* (n=5)
Antibiotic	:Penicillin (Breakpoint 0,12µg/ml)
MICs	:0,12 (1), 0,5 (1), 1 (1), 2 (1), 4 (1)
MBCs	:0,12 (1), 0,5 (1), 2 (1), 4 (2)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:8 (2), 16 (1), 32 (2)
MBCs	:16 (2), 32 (2), 64 (1)
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:1 (3), 2 (2)
MBCs	:1 (3), 2 (2)
Organism	:*Streptococcus mitis* (Gentamicin-resistant) (n=1)
Antibiotic	:Penicillin (Breakpoint 0,12µg/ml)
MICs	:32
MBCs	:32
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:1024
MBCs	:1024
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:1
MBCs	:1

4.2 CHECKERBOARD SYNERGY STUDIES

Checkerboard studies were performed on selected *Streptococcus sanguis* and *Streptococcus mitis* isolates using various combinations of antimicrobial agents. The results depicted in the form of isobolograms are shown in Figures 4.1.1 to 4.2.3. (Appendix Figures B.1 - B.5), and ΣFICs given in Table 4.2.

The combinations tested were as follows:

Gentamicin plus penicillin.

Streptococcus sanguis demonstrated indifference in Figure 4.1.1 (ΣFIC 1,0) and synergy in Figures 4.1.2 and 4.1.3 (ΣFICs 0,1).

Streptococcus mitis demonstrated antagonism in Figure 4.1.4 (ΣFIC 2.0), indifference in Figure 4.1.5 (ΣFIC 1,0) and synergy in Figure 4.1.6 (ΣFIC 0,2).

Gentamicin plus vancomycin.

Streptococcus sanguis demonstrated antagonism in Figure 4.2.1 (ΣFIC 1,0), indifference in Figure 4.2.2 (ΣFIC 1,0) while *Streptococcus mitis* demonstrated synergy in Figure 4.2.3 (ΣFIC 0,5).

Table 4.2 Fractional Inhibitory Concentration Indices

Organism	*Streptococcus sanguis*		
Antimicrobial Combination	ΣFICs		
Penicillin + Gentamicin	0,1	1,0	0,1
Vancomycin + Gentamicin	2,0	1,0	2,0

Organism	*Streptococcus mitis*		
Antimicrobial Combination	ΣFICs		
Penicillin + Gentamicin	2,0	1,0	0,2
Vancomycin + Gentamicin	2,0	2,0	0,5

Organism	*Streptococcus mitis* (Gentamicin-resistant)
Antimicrobial Combination	ΣFICs
Penicillin + Gentamicin	2,0
Vancomycin + Gentamicin	2,0

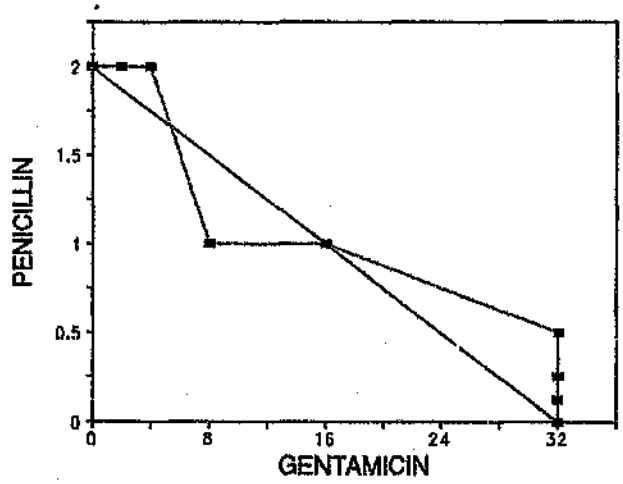

Figure 4.1.1 Activity of Penicillin (MIC 2µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus sanguis*

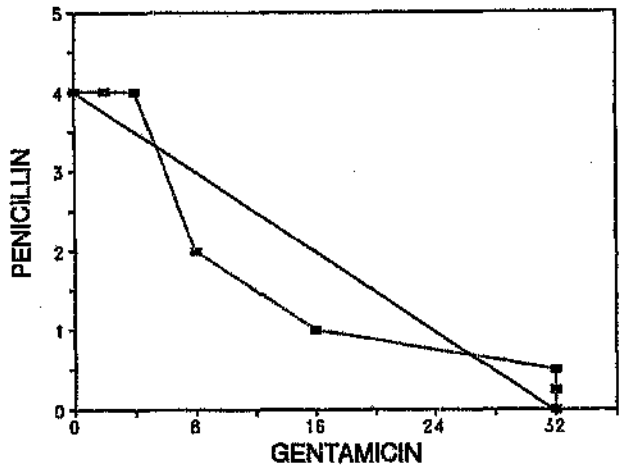

Figure 4.1.2 Activity of Penicillin (MIC 4µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus sanguis*

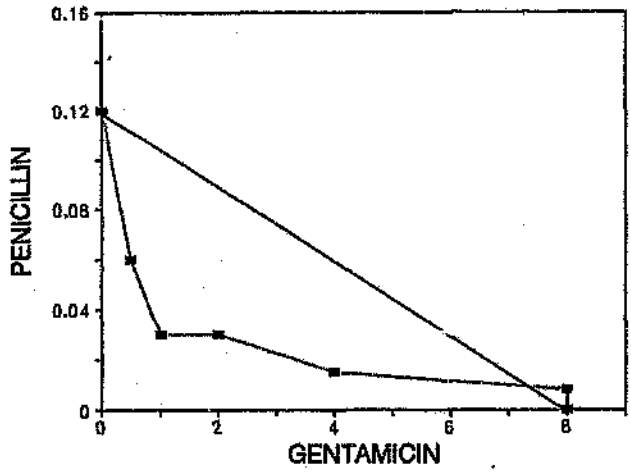

Figure 4.1.3 Activity of Penicillin (MIC 0,12µg/ml) plus Gentamicin (MIC 8µg/ml) in combination against *Streptococcus sanguis*

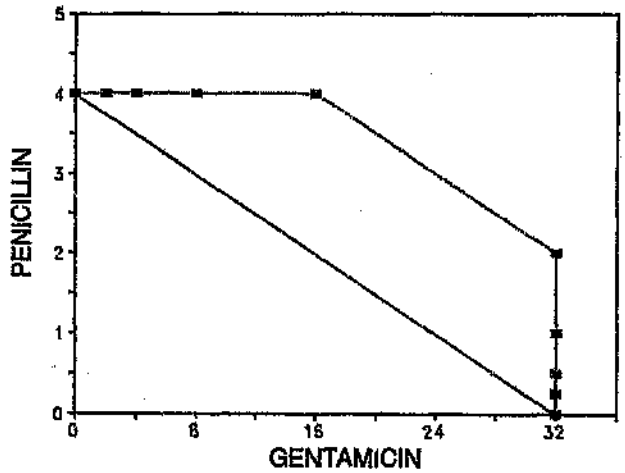

Figure 4.1.4 Activity of Penicillin (MIC 4µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus mitis*

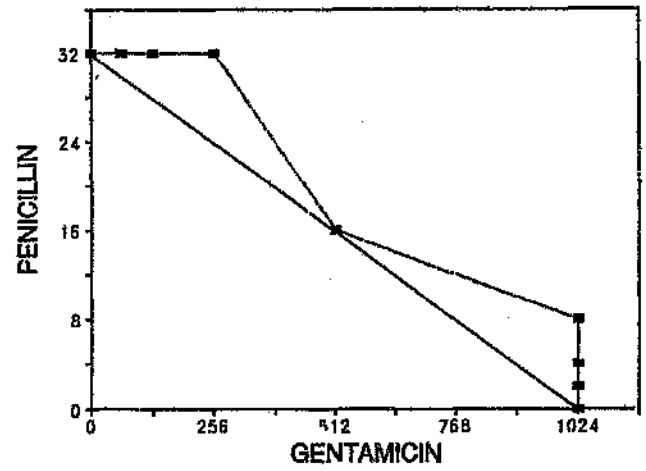

Figure 4.1.5 Activity of Penicillin (MIC 32µg/ml) plus Gentamicin (MIC 1024µg/ml) in combination against *Streptococcus mitis*

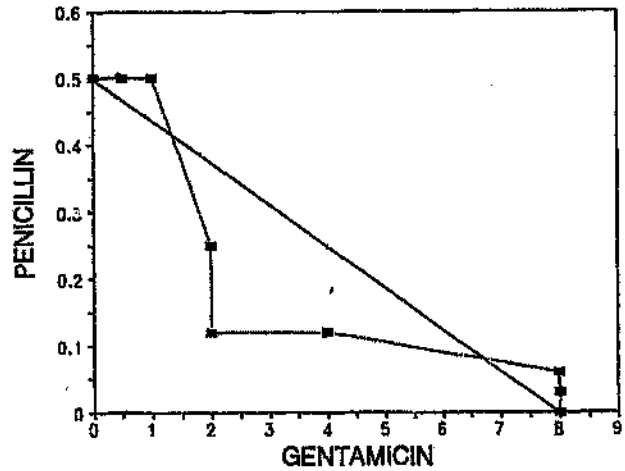

Figure 4.1.6 Activity of Penicillin (MIC 0,5µg/ml) plus Gentamicin (MIC 8µg/ml) in combination against *Streptococcus mitis*

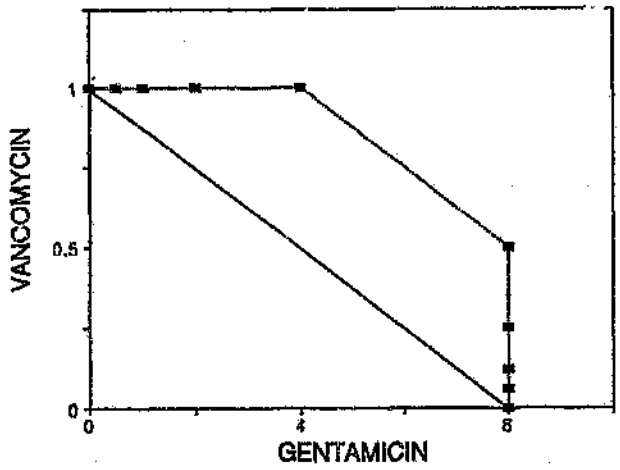

Figure 4.2.1 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 8µg/ml) in combination against *Streptococcus sanguis*

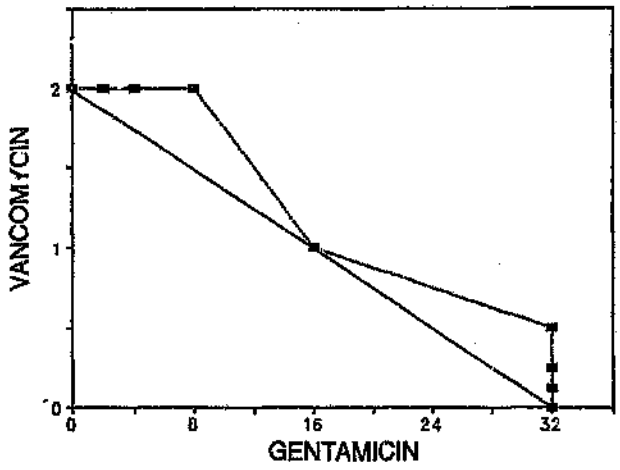

Figure 4.2.2 Activity of Vancomycin (MIC 2µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus sanguis*

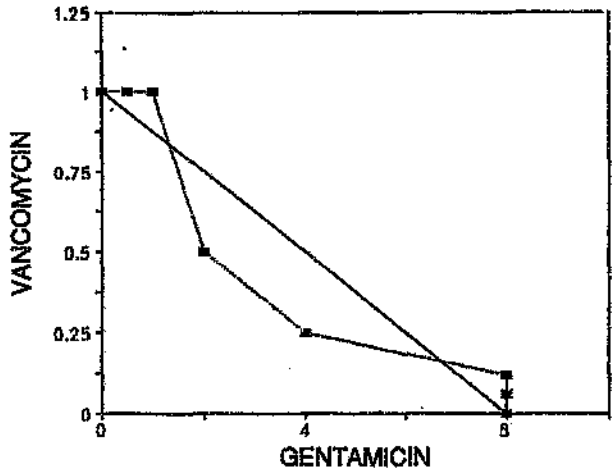

Figure 4.2.3 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 8µg/ml) in combination against *Streptococcus mitis*

4.3 TIME KILL STUDIES

Time kill techniques as described in Chapter two were performed with the combination of penicillin plus gentamicin against two strains of penicillin-resistant *Streptococcus mitis*. This combination proved to be indifferent against both strains (Figures 4.3, 4.4, 4.5 and 4.6).

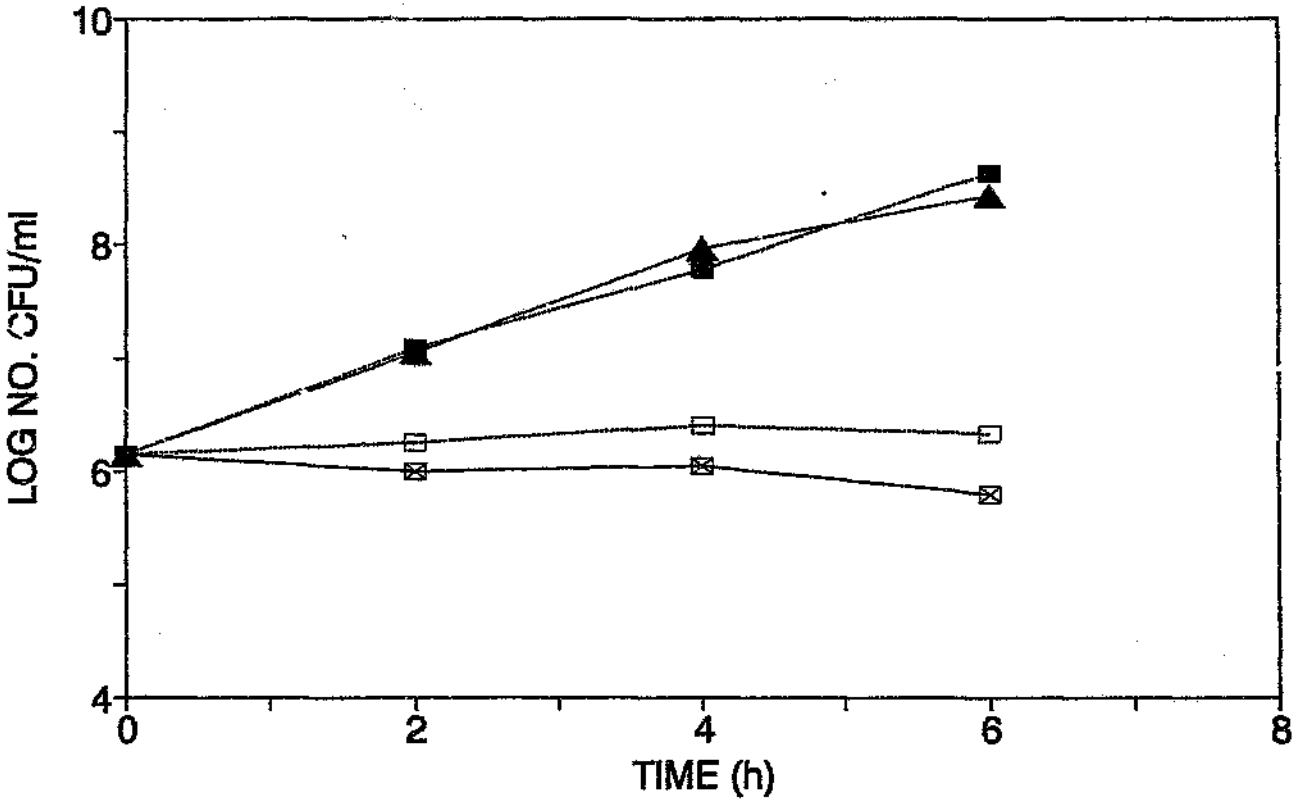

Figure 4.3 Activities of penicillin and gentamicin alone and in combination against *Streptococcus mitis* strain A. Symbols: □, penicillin 2,0µg/ml; ■, gentamicin 1,0µg/ml; ⊠, penicillin 2,0µg/ml + gentamicin 1,0µg/ml; ▲, control.

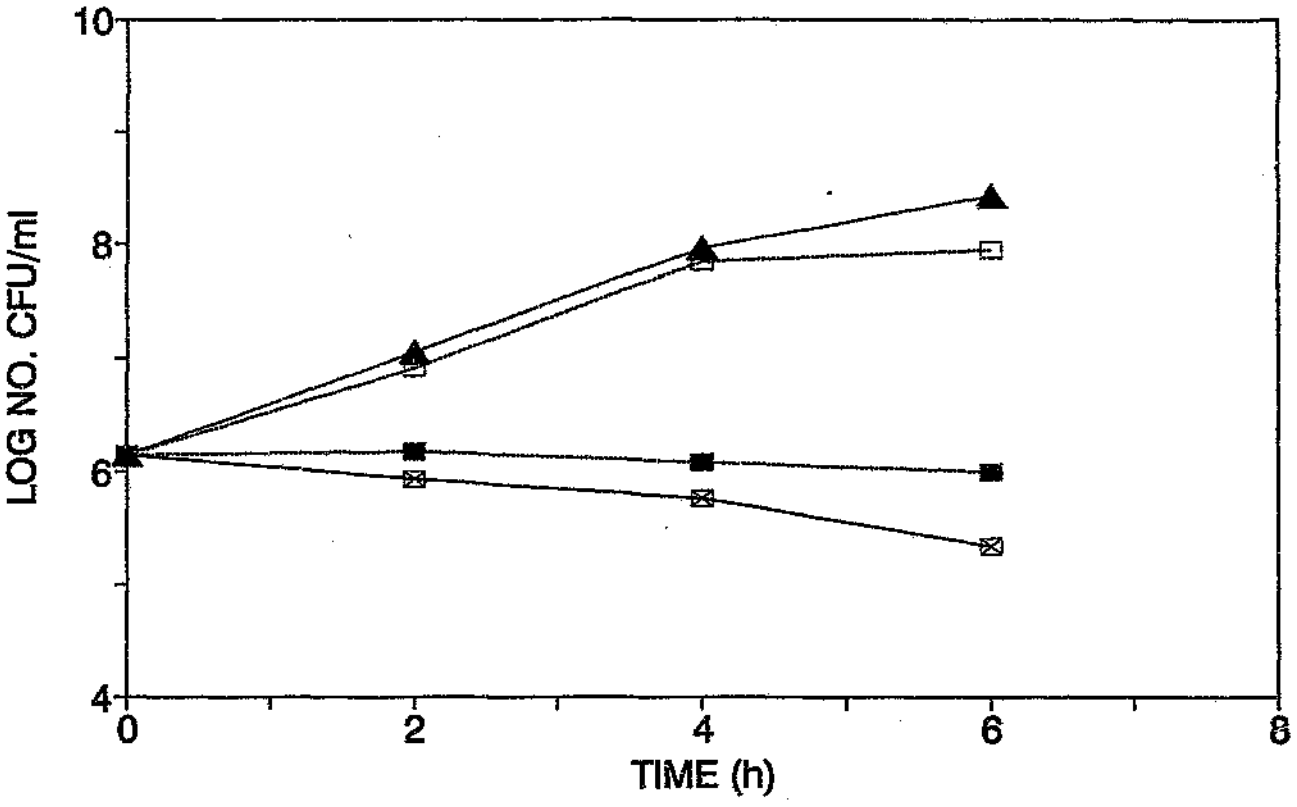

Figure 4.4 Activities of penicillin and gentamicin alone and in combination against *Streptococcus mitis* strain A. Symbols: □, penicillin 0,25µg/ml; ■, gentamicin 8,0µg/ml; ⊠, penicillin 0,25µg/ml + gentamicin 8,0µg/ml; ▲, control.

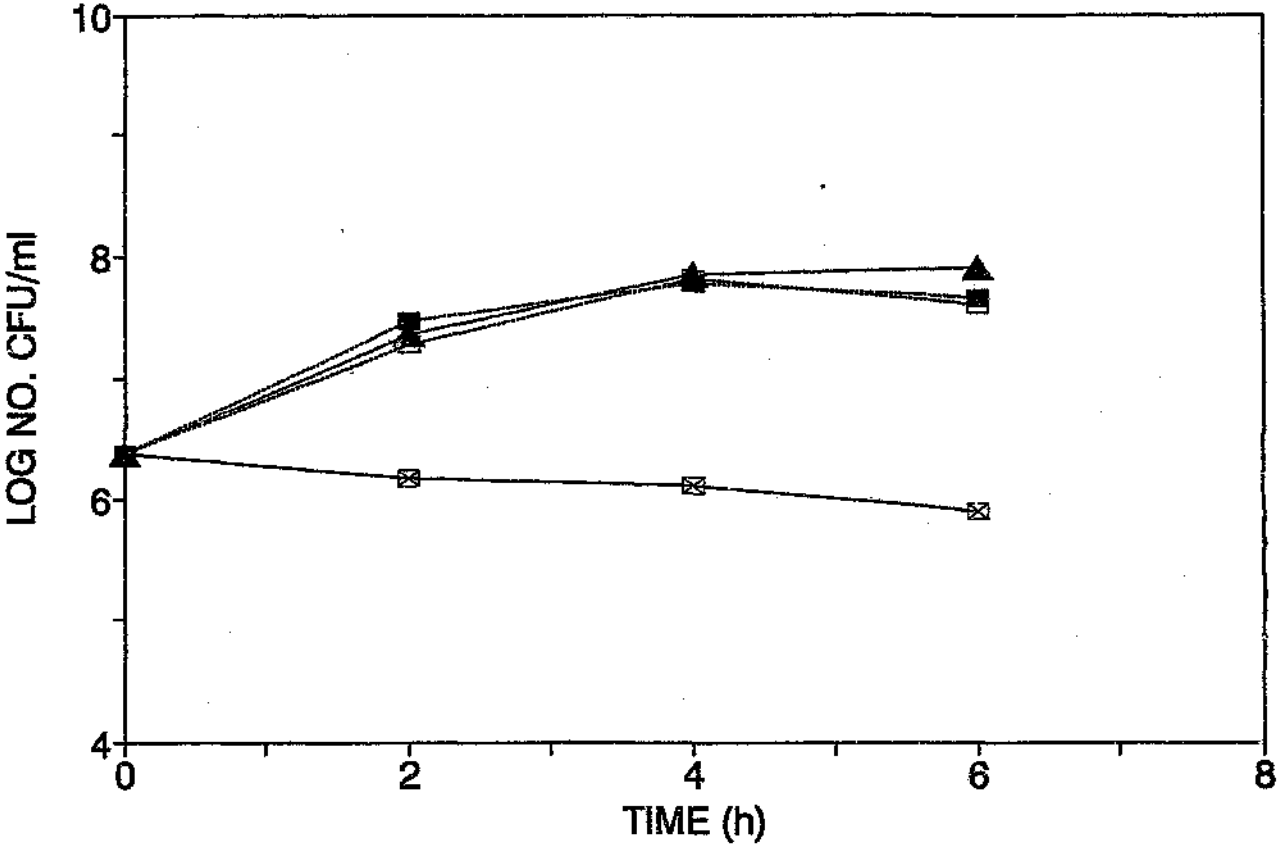

Figure 4.5 Activities of penicillin and gentamicin alone and in combination against *Streptococcus mitis* strain C. Symbols: □, penicillin 1,0µg/ml; ■, gentamicin 1,0µg/ml; ⊠, penicillin 1,0µg/ml + gentamicin 1,0µg/ml; ▲, control.

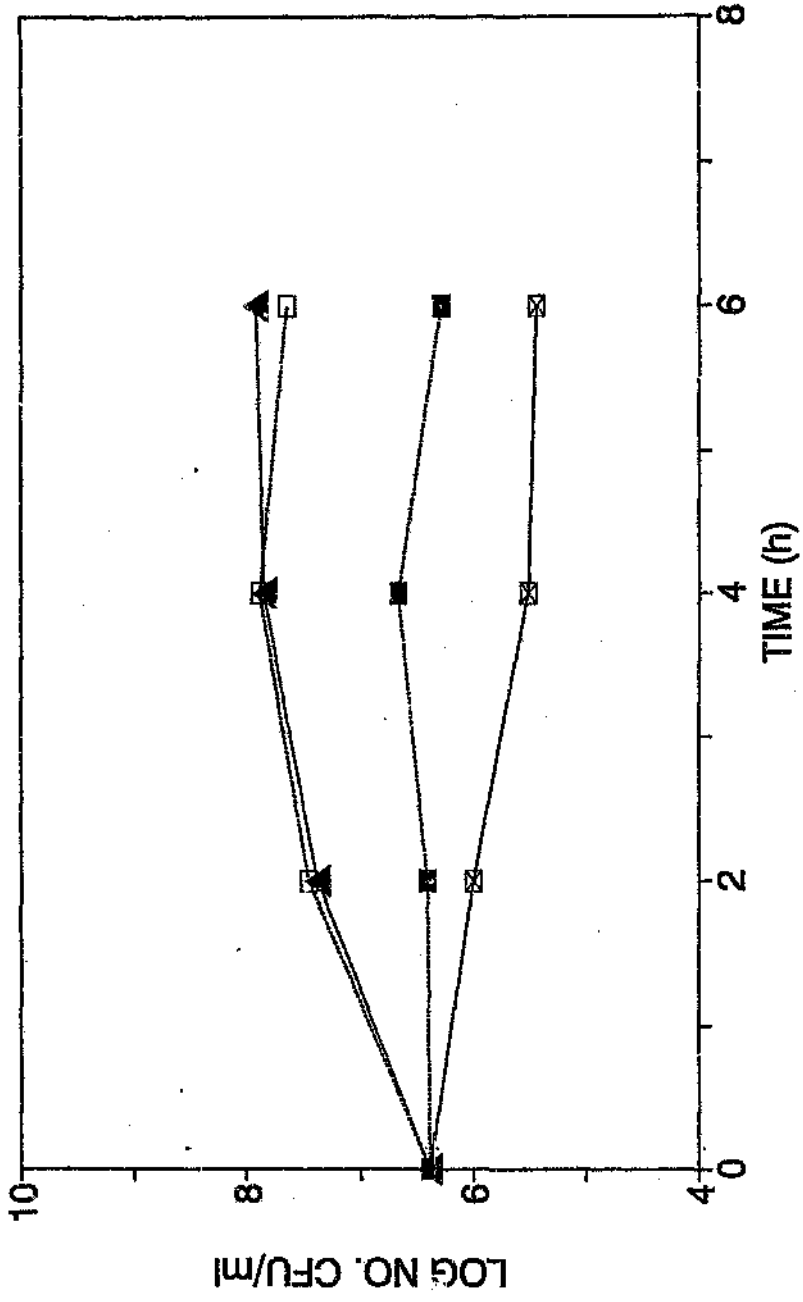

Figure 4.6 Activities of penicillin and gentamicin alone and in combination against *Streptococcus mitis* strain C. Symbols: □, penicillin 0,03µg/ml; ■, gentamicin 6,0µg/ml; ⊠, penicillin 0,03µg/ml + gentamicin 6,0µg/ml; ▲, control.

4.4 DISCUSSION

The MICs and MBCs of penicillin, vancomycin and gentamicin were determined against five *Streptococcus sanguis* and six *Streptococcus mitis* isolates and were comparable with those reported in larger studies around the world. All the viridans streptococci (including the high-level gentamicin-resistant *Streptococcus mitis* strain), were susceptible to vancomycin with MICs ranging from 1-2µg/ml. The findings on vancomycin susceptibility were similar to those in studies in America and Europe (1,2,3). However, resistance to vancomycin has been reported (4,5). In the United States of America in 1984, Shlaes *et.al.* (6), reported a serious infection caused by vancomycin-resistant *Streptococcus sanguis* II, with MIC values >128µg/ml. The identity of their organism has however been questioned by authoritative taxonomists from the Centres for Disease Control, Atlanta and may very well be a *Leuconostoc* sp. (7).

One penicillin-sensitive isolate of *Streptococcus sanguis* and one penicillin-sensitive isolate of *Streptococcus mitis* were used in this study (MICs 0,12µg/ml), the rest of the strains were resistant to penicillin with MICs ranging from 0,25-32µg/ml.

All the strains were resistant to gentamicin, with the

low-level gentamicin resistant strains having MICs 8-32μg/ml, and a high-level gentamicin-resistant strain having a MIC 1024μg/ml. These strains were selected for studies and do not represent the occurrence of resistance in oropharyngeal strains in the population from which they were derived.

Studies on clinical isolates in America and Europe (1,2,3) showed similar penicillin results (MICs ≤0,01-8μg/ml), and gentamicin results (MICs 0,06-≥128μg/ml). Viridans streptococci often cause serious infections, notably endocarditis. More rapid killing of these organisms usually result when antimicrobials are used in combination, such as an aminoglycoside in combination with penicillin This was shown in time-kill studies by Wolfe and Johnson (8).

In the present study, using checkerboard techniques, the combination of penicillin plus gentamicin demonstrated synergy against the penicillin-sensitive strain of *Streptococcus sanguis*. The other viridans streptococci (Penicillin MICs 0,25-4μg/ml) produced results varying from synergy to indifference. One strain of *Streptococcus mitis*, resistant to penicillin (MIC 4μg/ml) and gentamicin (MIC 32μg/ml), demonstrated antagonism using this antibiotic combination.

The combination of vancomycin plus gentamicin also produced results varying from synergy to antagonism. Here three strains demonstrated antagonism, and one

strain demonstrated synergy.

The high-level gentamicin resistant strain demonstrated indifference to both antibiotic combinations.

When performing the time-kill procedures, a problem with autolysis was encountered. Cell death was observed after 24h in the control cultures not exposed to antibiotics, even when the broth was supplemented with 5% lysed horse blood. This phenomenon was also experienced in a recent study by Potgieter *et.al.* (9). The problem effectively eliminated the high-level gentamicin *Streptococcus mitis* strain from the time-kill studies.

Time-kill curves on the two isolates of *Streptococcus mitis* both demonstrated indifference when penicillin (MICs 0,5-4µg/ml) was combined with gentamicin (MICs 8-32µg/ml).

However, a study in England by Shanson and co-workers (10) also using time-kill procedures showed synergy when penicillin was combined with gentamicin. Synergy was also demonstrated in studies in America by Lam and Bayer, Sande and Irvin, and Farber *et.al.* (11,12,13). The MIC/MBC results in their studies were similar to those in this study. Lam and Bayer also demonstrated synergy when vancomycin was combined with gentamicin.

The difference in the results reported from the literature and those obtained in this study could be due to strain variation.

No significant differences were observed in MIC/MBC patterns in other studies as opposed to those in this study.

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CHAPTER FIVE

RESULTS: ENTEROCOCCI

5.1 MINIMUM INHIBITORY CONCENTRATIONS / MINIMUM BACTERICIDAL CONCENTRATIONS

The MICs and MBCs of ampicillin, vancomycin and gentamicin against three ampicillin-sensitive and three ampicillin-resistant strains of *Enterococcus faecalis* are demonstrated is Table 5.1.

Ampicillin-sensitive *Enterococcus faecalis* (MICs 1-2μg/ml, MBCs 1-4μg/ml) was also sensitive to vancomycin (MICs 2-4μg/ml, MBCs 2-4μg/ml) but demonstrated low-level resistance to gentamicin (MICs 64μg/ml, MBCs 64μg/ml).

The three strains of ampicillin-resistant *Enterococcus faecalis* (MICs 32-64μg/ml, MBCs 32-64μg/ml) all demonstrated low-level resistance to gentamicin (MICs 64μg/ml, MBCs 64μg/ml) but were susceptible to vancomycin (MICs 2μg/ml, MBCs 2μg/ml).

Table 5.1 **Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations**

Organism	:*Enterococcus faecalis* (Ampicillin-sensitive) (n=3)
Antibiotic	:Ampicillin (Breakpoint 16μg/ml)
MICs	:1 (2), 2 (1)
MBCs	:1 (1), 2 (1), 4 (1)
Antibiotic	:Vancomycin (Breakpoint 4μg/ml)
MICs	:2 (2), 4 (1)
MBCs	:2 (1), 4 (2)
Antibiotic	:Gentamicin (Breakpoint 4μg/ml)
MICs	:64 (3)
MBCs	:64 (3)
Organism	:*Enterococcus faecalis* (Ampicillin-resistant) (n=3)
Antibiotic	:Ampicillin (Breakpoint 16μg/ml)
MICs	:32 (2), 64 (1)
MBCs	:32 (1), 64 (2)
Antibiotic	:Vancomycin (Breakpoint 4μg/ml)
MICs	:2 (3)
MBCs	:2 (3)
Antibiotic	:Gentamicin (Breakpoint 4μg/ml)
MICs	:64 (3)
MBCs	:64 (3)

5.2 CHECKERBOARD SYNERGY STUDIES

Checkerboard studies were performed on selected ampicillin-sensitive and ampicillin-resistant *Enterococcus faecalis* strains using various combinations of antimicrobial agents. The results depicted in the form of isobolograms are shown in Figures 5.1 1 to 5.2.2. (Appendix Figures C.1 - C.3), and ΣFICs in Table 5.2.

The combinations tested were as follows:

Gentamicin plus vancomycin.

In Figure 5.1.1 ampicillin-sensitive *Enterococcus faecalis* demonstrated antagonism (ΣFIC 2,0), in Figures 5.1.2 and 5.1.3 ampicillin-sensitive and -resistant *Enterococcus faecalis* demonstrated indifference (ΣFICs 1,0).

Gentamicin plus ampicillin.

In Figures 5.2.1 and 5.2.2 ampicillin-sensitive *Enterococcus faecalis* demonstrated antagonism and indifference (ΣFICs 2,0 and 1,0).

Table 5.2 Fractional Inhibitory Concentration Indices

Organism	*Enterococcus faecalis* (Ampicillin-sensitive)		
Antimicrobial Combination		ΣFICs	
Vancomycin + Gentamicin		2,0	1,0
Ampicillin + Gentamicin		2,0	1,0

Organism	*Enterococcus faecalis* (Ampicillin-resistant)		
Antimicrobial Combination			ΣFICs
Vancomycin + Gentamicin		1,0	2,0
Ampicillin + Gentamicin		1,0	1,0

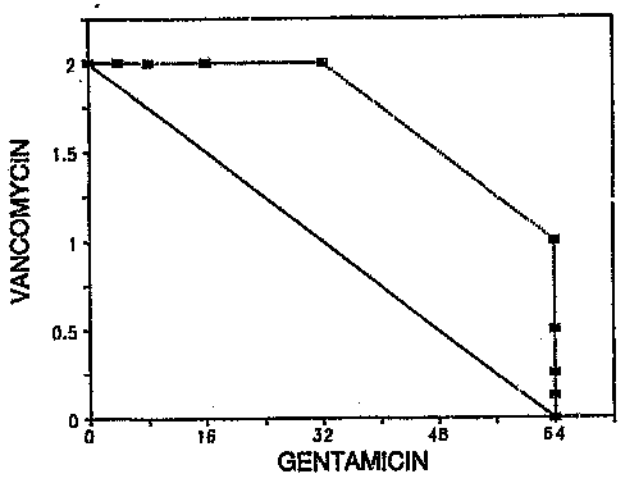

Figure 5.1.1 Activity of Vancomycin (MIC 2µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-sensitive *Enterococcus faecalis*

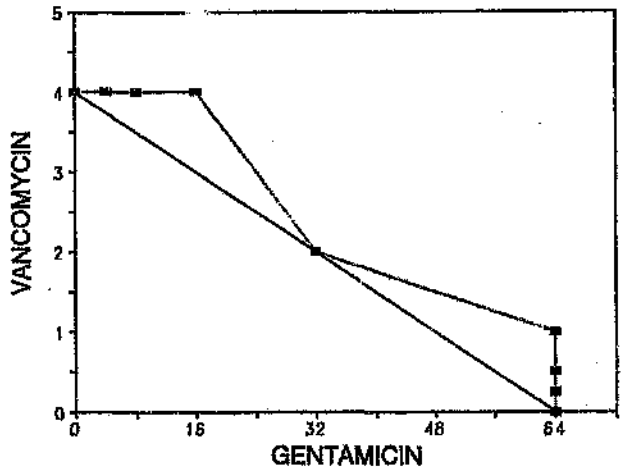

Figure 5.1.2 Activity of Vancomycin (MIC 4µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-sensitive *Enterococcus faecalis*

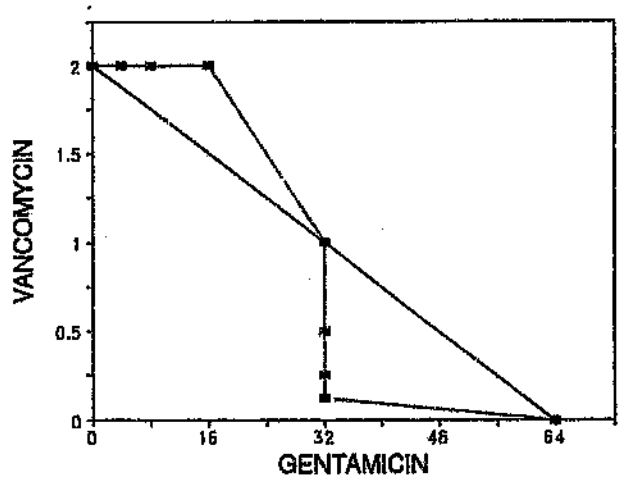

Figure 5.1.3 Activity of Vancomycin (MIC 2μg/ml) plus Gentamicin (MIC 64μg/ml) in combination against ampicillin-resistant *Enterococcus faecalis*

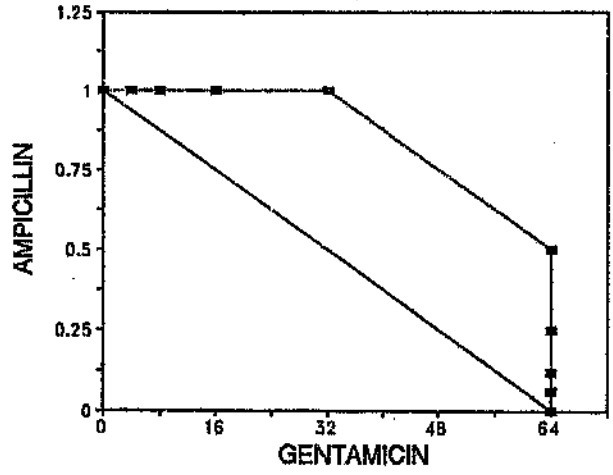

Figure 5.2.1 Activity of Ampicillin (MIC 1μg/ml) plus Gentamicin (MIC 64μg/ml) in combination against ampicillin-sensitive *Enterococcus faecalis*

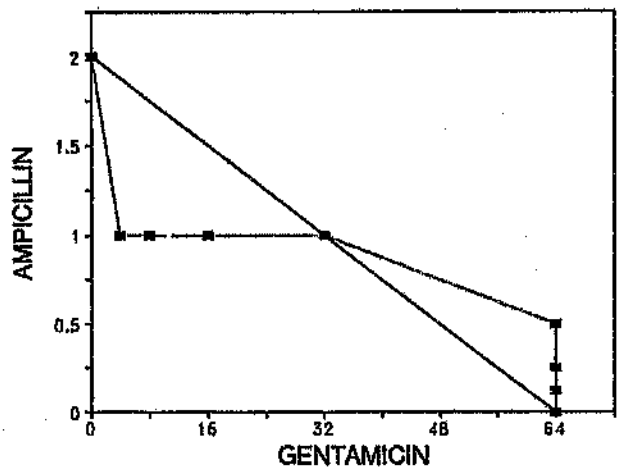

Figure 5.2.2 **Activity of Ampicillin (MIC 2µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-sensitive *Enterococcus faecalis***

5.3 DISCUSSION

All the *Enterococcus faecalis* isolates (ampicillin-sensitive and -resistant) used in this study demonstrated low-level gentamicin resistance (MICs 64µg/ml). At the time this study was initiated, no high-level gentamicin resistant strains were available. Vancomycin demonstrated good activity against all the isolates, all having MICs ≤ the breakpoint of 4µg/ml. Although only vancomycin-sensitive strains were used in this study, vancomycin-resistant strains do occur (1). These results are comparable to other in vitro studies by Eliopoulos *et.al.* (2), Benson *et.al.* (3), Jorgensen *et.al.* (4), Rolston *et.al.* (5), Bartoloni *et.at.* (6), Kaatz and Seo (7), and Hodges *et.al.* (8).

In two studies where an aminoglycoside and a ß-lactam was used in combination, no synergy was demonstrated (9,10). Bush *et.al.* (9) using high-level penicillin resistant isolates of enterococci demonstrated no synergy when penicillin plus gentamicin was used in time-kill studies. In the other study by Mederski-Samoraj and Murray (10), also using time-kill procedures, no synergy was demonstrated when penicillin plus gentamicin was used against high-level gentamicin resistant isolates of enterococci.

In this study the interactions of gentamicin in combination with vancomycin, and gentamicin in combination with ampicillin were compared against selected ampicillin-sensitive and -resistant *Enterococcus faecalis* isolates by checkerboard method. No synergy between any of the combinations of antibiotics was demonstrated. However, in a study by Watanakunakorn and Bakie (11), using time-kill procedures, synergy was demonstrated against enterococci using the combination of vancomycin and gentamicin. In another study by Besnier *et.al.* (12) five patients with enterococcal endocarditis were cured after treatment with a vancomycin-aminoglycoside combination.

The fact that no synergy was demonstrated in this study by the strains of *Enterococcus faecalis* used could once again be due to stain variation, as the strains used were selected.

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CHAPTER SIX

RESULTS: LEUCONOSTOC SPP.

6.1 MINIMUM INHIBITORY CONCENTRATIONS / MINIMUM BACTERICIDAL CONCENTRATIONS

The MICs and MBCs of penicillin, gentamicin, fusidic acid, rifampicin, chloramphenicol, ceftriaxone, oxacillin and cotrimoxazole were determined against five strains of *Leuconostoc* species, and the results are shown in Table 6.1.

While the *Leuconostoc* species were susceptible to gentamicin (MICs 0,25-4µg/ml, MBCs 0,5-8µg/ml), and cotrimoxazole (MICs 0,12-16µg/ml, MBCs 4-64µg/ml), they demonstrated variable resistance to all the other antimicrobials tested. The *Leuconostoc* strains tested had MICs and MBCs that were in the intermediate resistance range based on breakpoints used for viridans streptococci. All the isolates were intermediately resistant to penicillin (MICs 0,25-0,5µg/ml, MBCs 0,5µg/ml), and were fully resistant to fusidic acid (MICs ≥128µg/ml, MBCs ≥128µg/ml). Two of the five isolates was susceptible to rifampicin (MICs 0,12-0,5µg/ml, MBCs 0,25-1µg/ml), while the others were resistant (MICs 4-64µg/ml, MBCs 32-128µg/ml). Two

isolates were susceptible to oxacillin (MICs 1-2μg/ml, MBCs 2μg/ml), the rest being resistant (MICs 8-32μg/ml, MBCs 16-64μg/ml). Three of the isolates were susceptible to ceftriaxone (MICs 1-2μg/ml, MBCs 1-8μg/ml), one was intermediately resistant (MIC 32μg/ml, MBC 32μg/ml), while one was fully resistant (MIC 64μg/ml, MBC 64μg/ml). Three isolates were fully susceptible to chloramphenicol (MICs 4-8μg/ml, MBCs 4-16μg/ml), while the other two were intermediately resistant (MICs 16μg/ml, MBCs 16-128μg/ml).

Table 6.1 **Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations**

Organism	:*Leuconostoc* spp (n=5)
Antibiotic	:Penicillin (Breakpoint 0,12µg/ml)
MICs	:0,25 (1), 0,5 (4)
MBCs	:0,5 (5)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:0,25 (1), 0,5 (1), 2 (2), 4 (1)
MBCs	:0,5 (1), 1 (1), 2 (1), 8 (2)
Antibiotic	:Fusidic acid
MICs	:≥128 (5)
MBCs	:≥128 (5)
Antibiotic	:Rifampicin (Breakpoint 1µg/ml)
MICs	:0,12 (1), 0,5 (1), 4 (1), 32 (1), 64 (1)
MBCs	:0,25 (1), 1 (1), 32 (1), 64 (1), 128 (1)
Antibiotic	:Chloramphenicol (Breakpoint 8µg/ml)
MICs	:4 (1), 8 (2), 16 (2)
MBCs	:4 (1), 8 (1), 16 (2), 128 (1)
Antibiotic	:Ceftriaxone (Breakpoint 8µg/ml)
MICs	:1 (1), 2 (2), 32 (1), 64 (1)
MBCs	:1 (1), 2 (1), 8 (1), 32 (1), 64 (1)
Antibiotic	:Oxacillin (Breakpoint 2µg/ml)
MICs	:1 (1), 2 (1), 8 (2), 32 (1)
MBCs	:2 (2), 16 (1), 32 (1), 64 (1)
Antibiotic	:Cotrimoxazole
MICs	:0,12 (1), 2 (1), 16 (3)
MBCs	:4 (1) 16 (2), 64 (2)

6.2 CHECKERBOARD SYNERGY STUDIES

Checkerboard studies were performed on selective *Leuconostoc* strains using various combinations of antimicrobial agents. The results depicted as isobolograms are shown in Figures 6.1.1 to 6.5.3. (Appendix Figures D.1 - D.9), and ΣFICs given in Table 6.2.

The combinations tested were as follows:

Gentamicin plus penicillin.

Figures 6.1.1 and 6.1.2 both show synergy (ΣFICs 0,1).

Rifampicin plus penicillin.

Here synergy was demonstrated in Figures 6.2.1 to 6.2.3, while Figure 6.2.4 showed indifference (ΣFICs 0,1 and 1,0 respectively).

Ceftriaxone plus gentamicin.

Figures 6.3.1 and 6.3.2 both demonstrate synergy (ΣFICs 0,1).

Oxacillin plus gentamicin.

Figures 6.4.1 to 6.4.3 also demonstrate synergy (ΣFICs 0,1).

Cotrimoxazole plus gentamicin.

Figures 6.5.1 and 6.5.2 show synergy, and Figure 6.5.3 shows indifference (ΣFICs 0,1 and 1,0 respectively).

Table 6.2 Fractional Inhibitory Concentration Indices

Organism *Leuconostoc* species

Antimicrobial Combination			ΣFICs				
Penicillin	+	Gentamicin	0,1	0,1	0,1	0,1	0,1
Penicillin	+	Rifampicin	0,1	1,0	0,1	0,1	0,1
Gentamicin	+	Ceftriaxone	0,1	0,1	0,1	0,1	0,1
Gentamicin	+	Oxacillin	0,1	0,1	0,1	0,1	0,1
Gentamicin	+	Cotrimoxazole	-	-	1,0	0,1	0,1

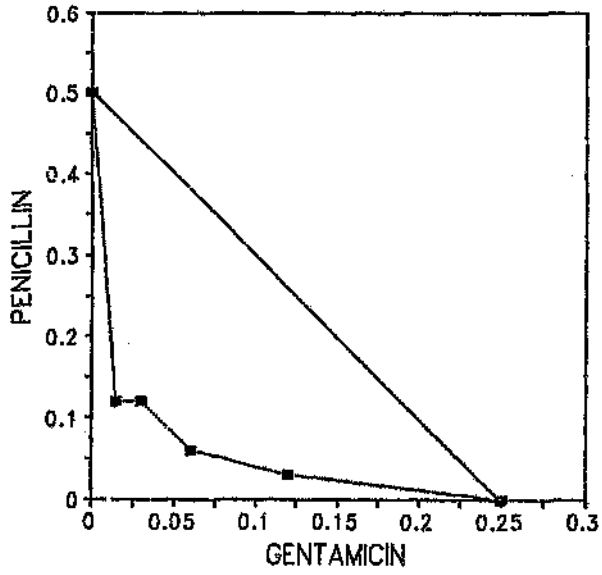

Figure 6.1.1 **Activity of Penicillin (MIC 0,5µg/ml) plus Gentamicin (MIC 0,25µg/ml) in combination against *Leuconostoc* sp.**

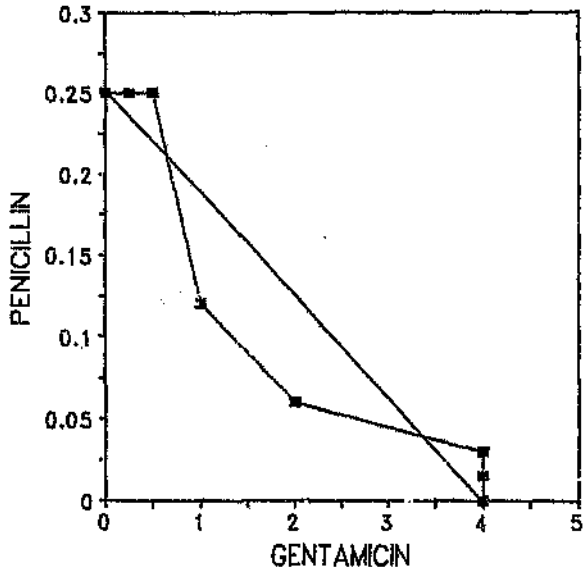

Figure 6.1.2 **Activity of Penicillin (MIC 0,25µg/ml) plus Gentamicin (MIC 4µg/ml) in combination against *Leuconostoc* sp.**

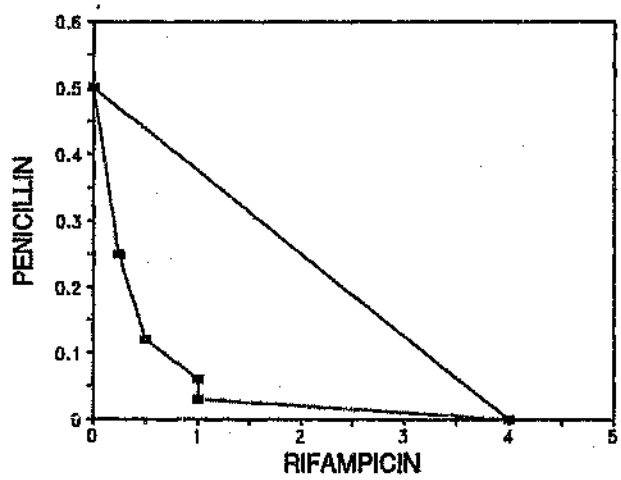

Figure 6.2.1 Activity of Penicillin (MIC 0,5µg/ml) plus Rifampicin (MIC 4µg/ml) in combination against *Leuconostoc* sp.

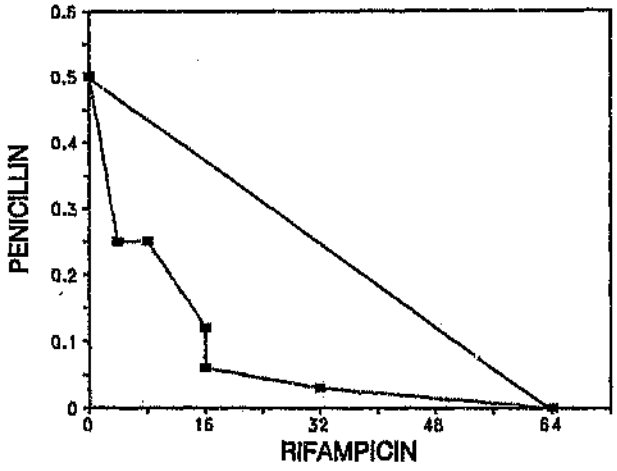

Figure 6.2.2 Activity of Penicillin (MIC 0,5µg/ml) plus Rifampicin (MIC 64µg/ml) in combination against *Leuconostoc* sp.

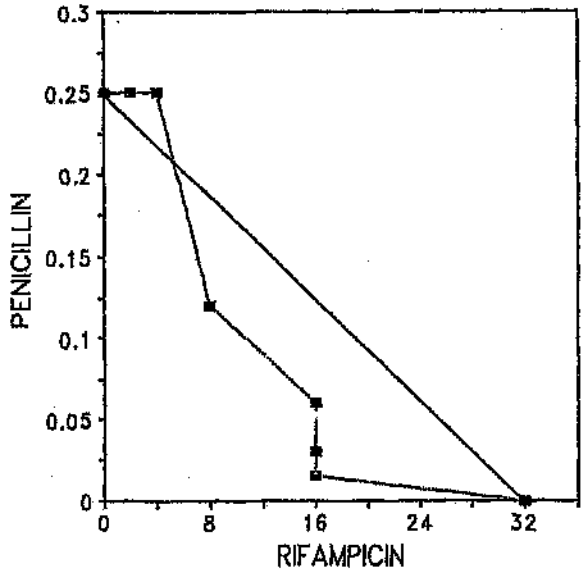

Figure 6.2.3 Activity of Penicillin (MIC 0,25µg/ml) plus Rifampicin (MIC 32µg/ml) in combination against *Leuconostoc* sp.

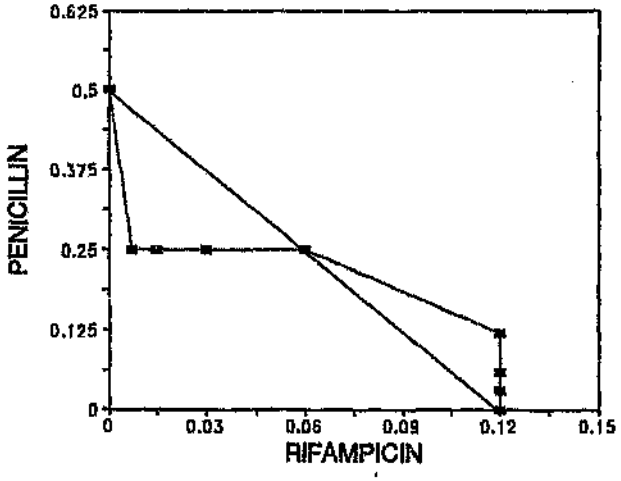

Figure 6.2.4 Activity of Penicillin (MIC 0,5µg/ml) plus Rifampicin (MIC 0,12µg/ml) in combination against *Leuconostoc* sp.

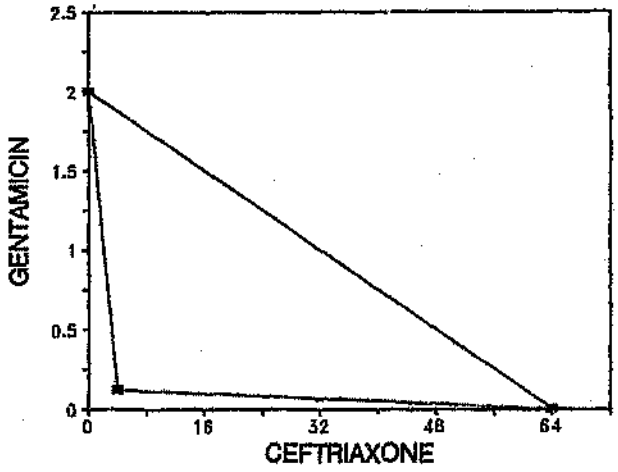

Figure 6.3.1 Activity of Gentamicin (MIC 2μg/ml) plus Ceftriaxone (MIC 64μg/ml) in combination against *Leuconostoc* sp.

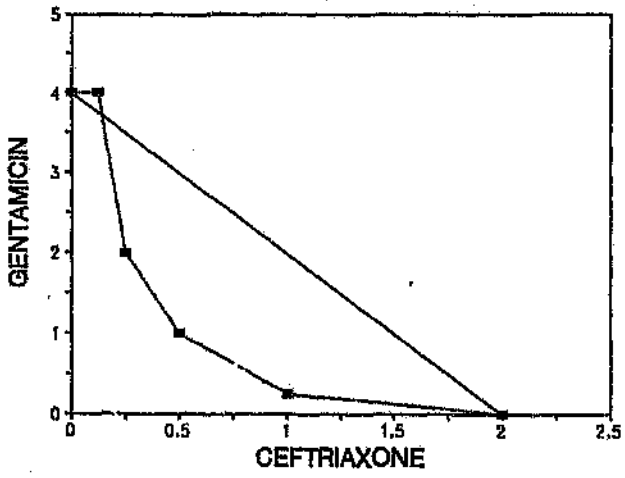

Figure 6.3.2 Activity of Gentamicin (MIC 4μg/ml) plus Ceftriaxone (MIC 2μg/ml) in combination against *Leuconostoc* sp.

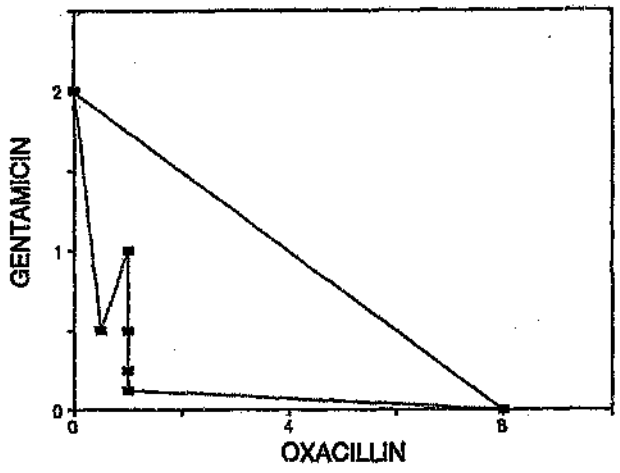

Figure 6.4.1 Activity of Gentamicin (MIC 2µg/ml) plus Oxacillin (MIC 8µg/ml) in combination against *Leuconostoc* sp.

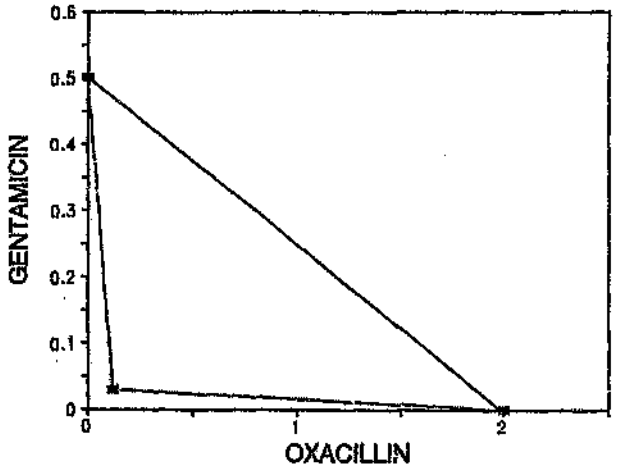

Figure 6.4.2 Activity of Gentamicin (MIC 0,5µg/ml) plus Oxacillin (MIC 2µg/ml) in combination against *Leuconostoc* sp.

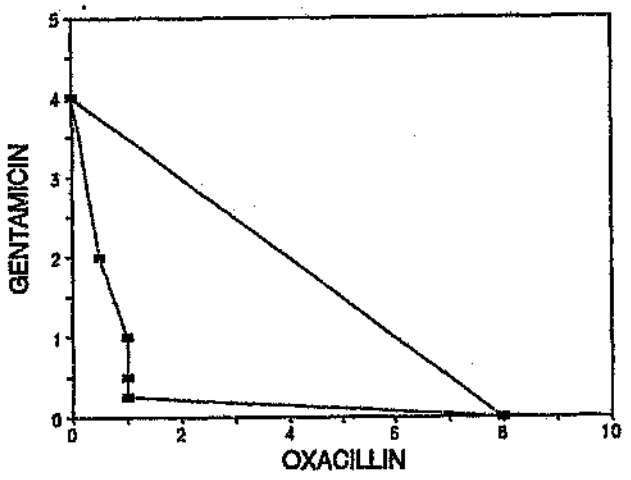

Figure 6.4.3 Activity of Gentamicin (MIC 4µg/ml) plus Oxacillin (MIC 8µg/ml) in combination against *Leuconostoc* sp.

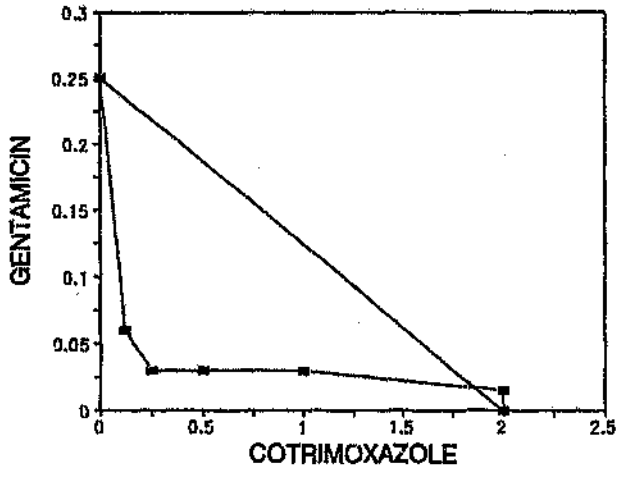

Figure 6.5.1 Activity of Gentamicin (MIC 0,25µg/ml) plus Cotrimoxazole (MIC 2µg/ml) in combination against *Leuconostoc* sp.

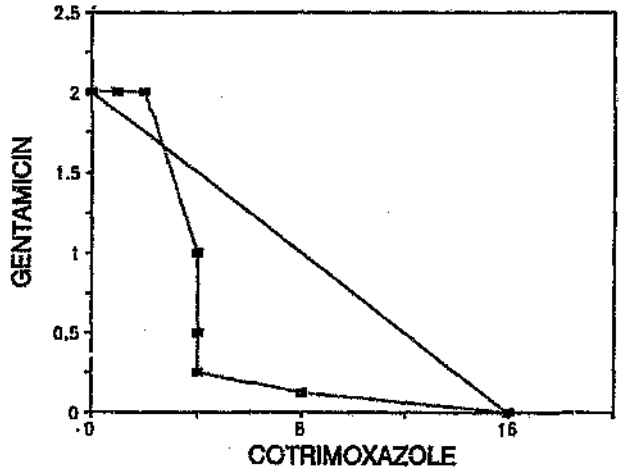

Figure 6.5.2 Activity of Gentamicin (MIC 2µg/ml) plus Cotrimoxazole (MIC 16µg/ml) in combination against *Leuconostoc* sp.

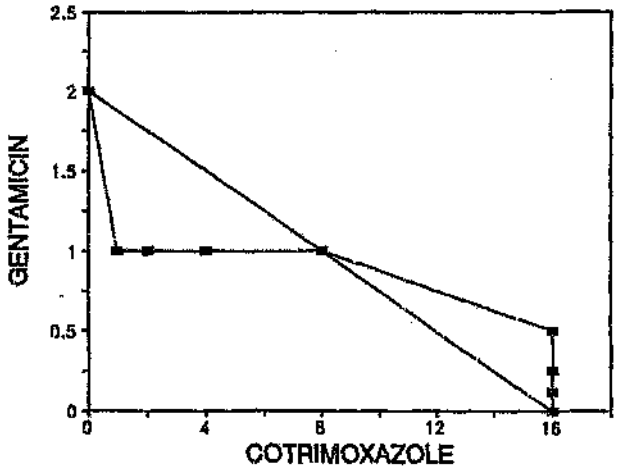

Figure 6.5.3 Activity of Gentamicin (MIC 2µg/ml) plus Cotrimoxazole (MIC 16µg/ml) in combination against *Leuconostoc* sp.

6.3 TIME KILL STUDIES

The in vitro activities of the following antibiotic co. ɔinations were studied by time kill techniques as described in Chapter two:

Penicillin combined with gentamicin against strains of *Leuconostoc* species (strains L4 and L1).

Ceftriaxone combined with gentamicin against *Leuconostoc* species (strain L5).

In Figure 6.6 (strain L4), with penicillin at a bacteriostatic concentration of 0,5µg/ml, (MBC 0,5µg/ml) the combination of penicillin and gentamicin showed indifference. Using gentamicin at the bacteriostatic concentration of 0,25µg/ml, (MBC 1µg/ml), the combination also demonstrated indifference (Figure 6.7). This pattern was duplicated with strain L1 (Figures 6.8 and 6.9).

A ceftriaxone resistant strain L5 (MIC 64µg/ml), was also investigated. However only indifference was demonstrated when gentamicin was combined with ceftriaxone (Figures 6.10 and 6.11).

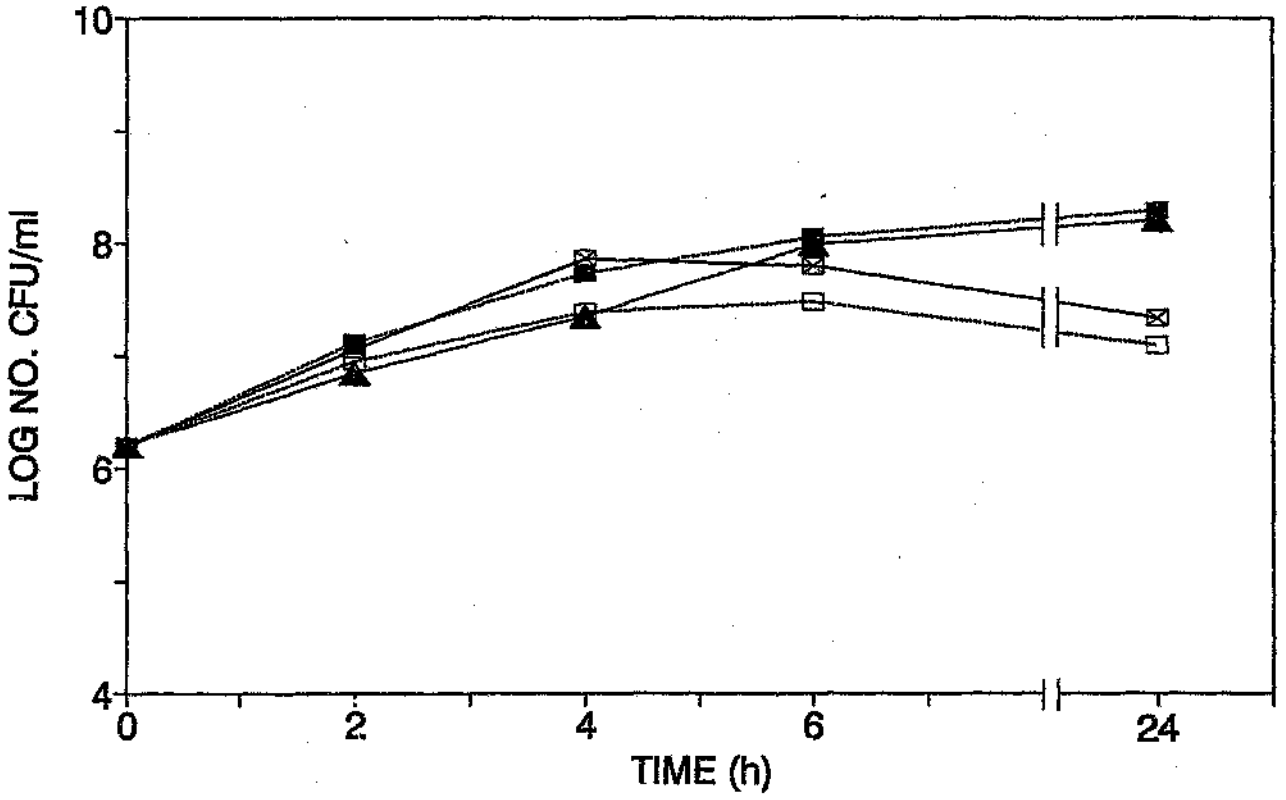

Figure 6.6 Activities of penicillin and gentamicin alone and in combination against *Leuconostoc* strain L4. Symbols: □, penicillin 0,3µg/ml; ■, gentamicin 0,06µg/ml; ⊠, penicillin 0,3µg/ml + gentamicin 0,06µg/ml; ▲, control.

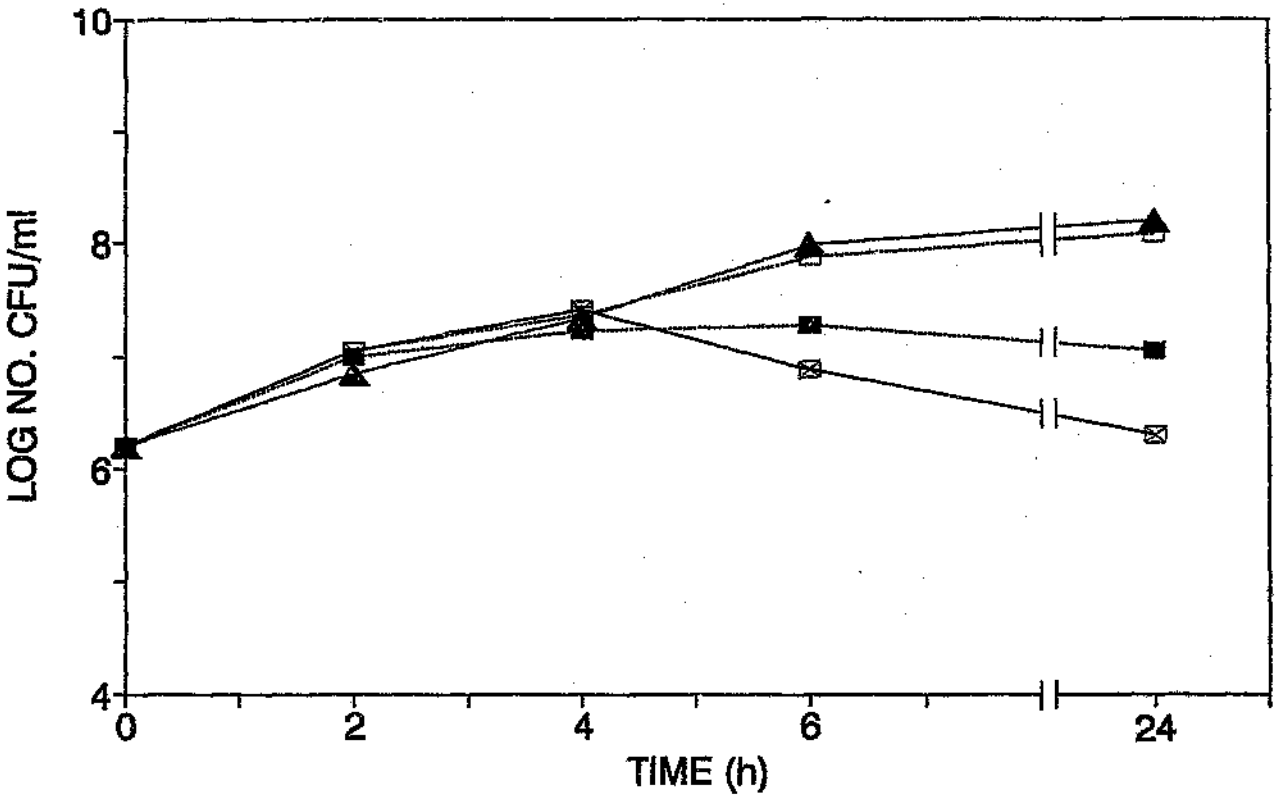

Figure 6.7 Activities of penicillin and gentamicin alone and in combination against *Leuconostoc* strain L4. Symbols: □, penicillin 0,125µg/ml; ■, gentamicin 0,3µg/ml; ⊠, penicillin 0,125µg/ml + gentamicin 0,3µg/ml; ▲, control.

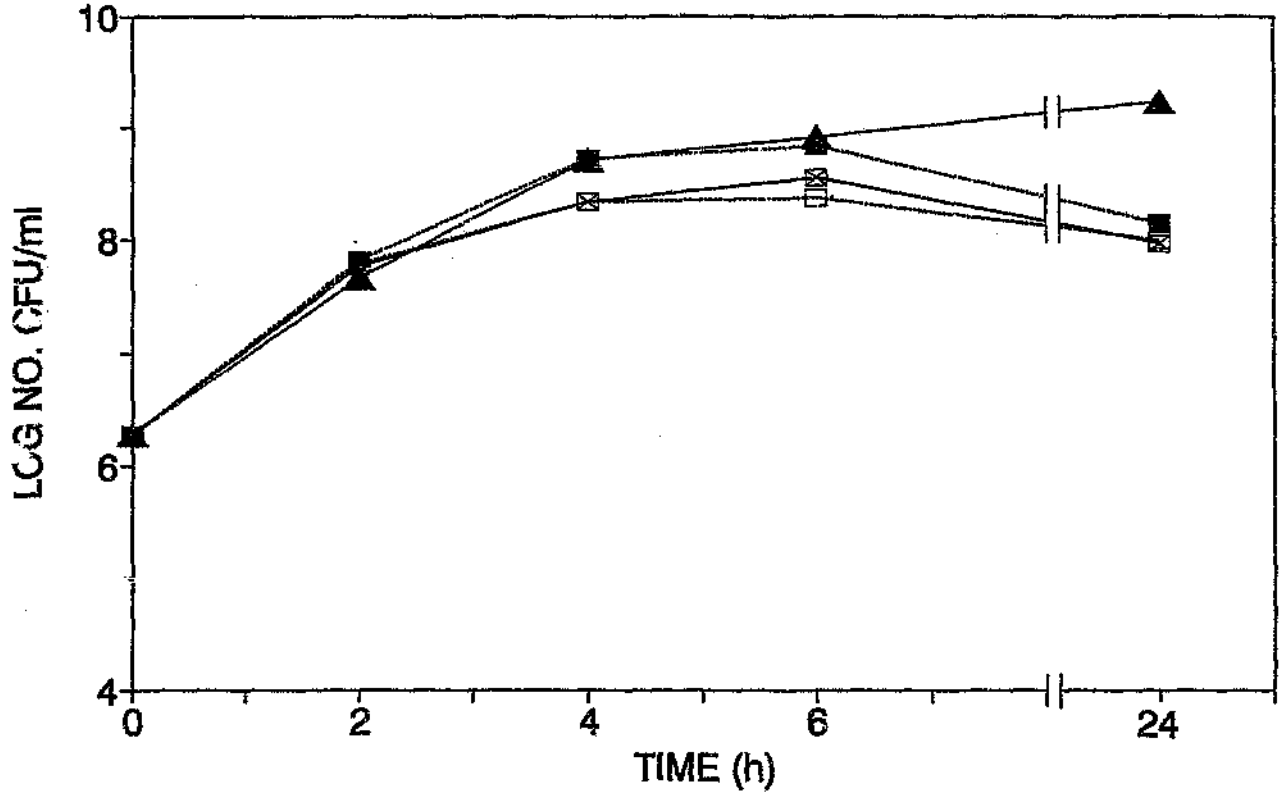

Figure 6.8 Activities of penicillin and gentamicin alone and in combination against *Leuconostoc* strain L1. Symbols: □, penicillin 0,5µg/ml; ■, gentamicin 0.125µg/ml; ⊠, penicillin 0,5µg/ml + gentamicin 0,125µg/ml; ▲, control.

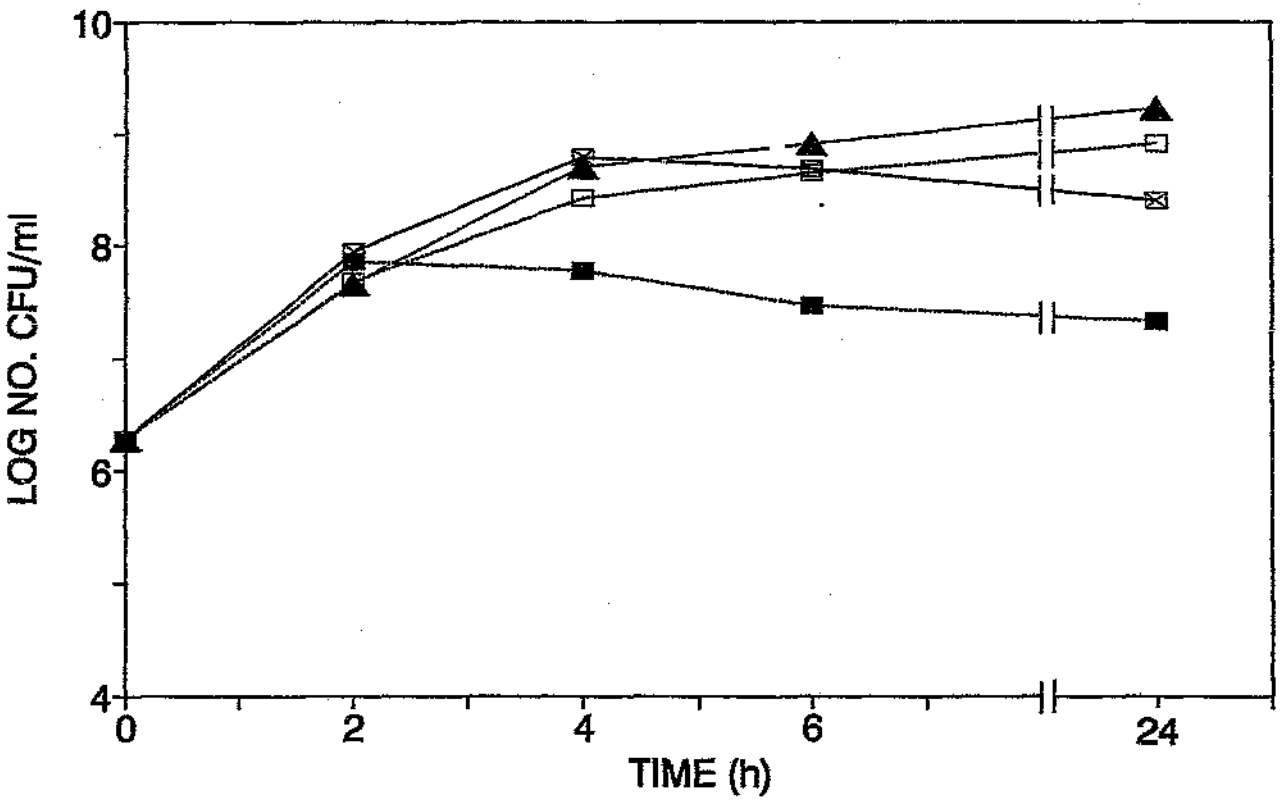

Figure 6.9 Activities of penicillin and gentamicin alone and in combination against *Leuconostoc* strain L1. Symbols: □, penicillin 0,125µg/ml; ■, gentamicin 0,25µg/ml; ⊠, penicillin 0,125µg/ml + gentamicin 0,25µg/ml; ▲, control.

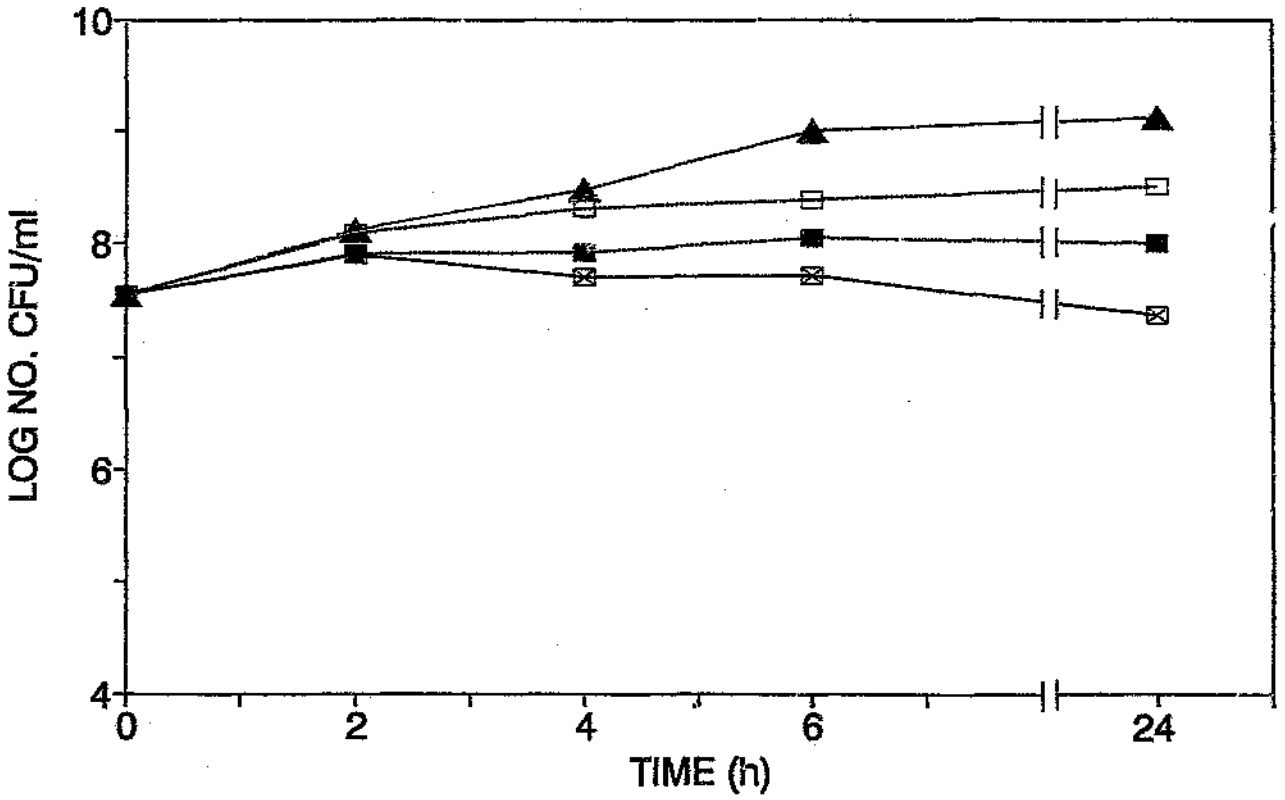

Figure 6.10 Activities of ceftriaxone and gentamicin alone and in combination against *Leuconostoc* strain L5. Symbols: □, ceftriaxone 8,0µg/ml; ■, gentamicin 1,0µg/ml; ⊠, ceftriaxone 8,0µg/ml + gentamicin 1,0µg/ml; ▲, control.

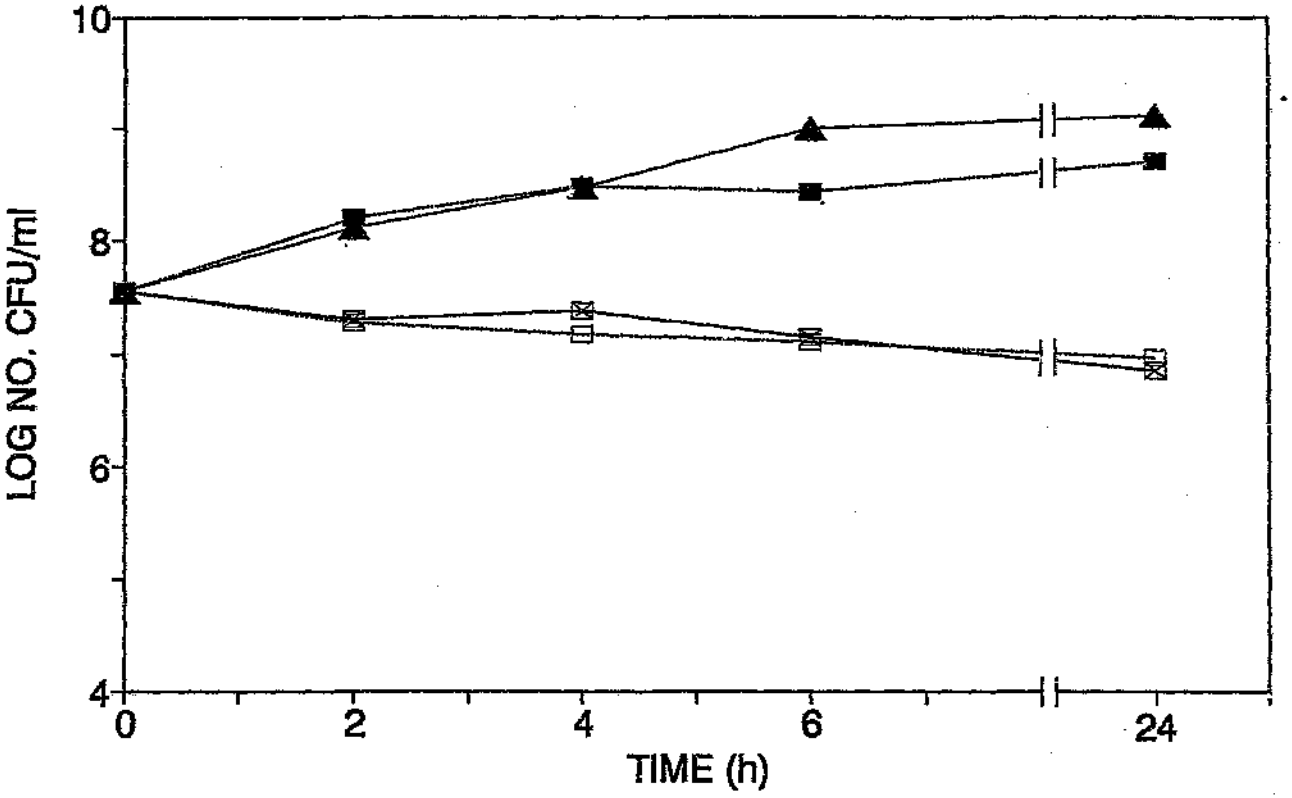

Figure 6.11 Activities of ceftriaxone and gentamicin alone and in combination against *Leuconostoc* strain L5. Symbols: □, ceftriaxone 64,0µg/ml; ■, gentamicin 0,25µg/ml; ⊠, ceftriaxone 64,0µg/ml + gentamicin 0,25µg/ml; ▲, control.

6.4 DISCUSSION

A review of the literature readily relates that *Leuconostoc* species are now recognised as pathogens and that all isolates of *Leuconostoc* species are resistant to vancomycin (1,2).

All five strains in this study were susceptible to gentamicin. In a comprehensive study in 1990 by Swenson *et.al.* (3), 100% of the 79 isolates tested were susceptible to gentamicin, with MICs ranging from 0,25-0,5µg/ml. Earlier in 1985 Buu-Hoï *et.al.* (4) reported MICs of 0,12-2µg/ml in 8 isolates. However, in a study by Handwerger *et.al.* (5) only six out of nine clinical isolates were susceptible to gentamicin. Two isolates were resistant to gentamicin (MICs ≥16µg/ml). Strains investigated in this study had penicillin MICs of 0,25-0,5µg/ml, and were therefore in the intermediate resistant range. Results obtained with the isolates tested in the study by Buu-Hoï *et.al.* showed penicillin MICs of 0,12-1µg/ml. Only 6% of the isolates in the study of Swenson and co-workers were susceptible to penicillin. Swenson reported that 55%, 98%, and 57% of the leuconostoc strains were sensitive to rifampicin, chloramphenicol and ceftriaxone respectively. Although only 5 isolates were examined in this study, the trend was similar.

Highly significant in this study is the fact that virtually all the combinations tested demonstrated synergy with all five *Leuconostoc* isolates using the checkerboard method. The two exceptions were that one strain showed indifference to penicillin plus rifampicin, and one strain showed indifference to gentamicin plus cotrimoxazole.

These same results were, however, not obtained in the time-kill procedures, where only indifference was shown. However, it must be stressed that the concentrations of antimicrobials chosen in the time-kill experiments in this study set very stringent criteria for synergy. The only other literature reporting synergy against *Leuconostoc* isolates was by Buu-Hoï *et.al.* (4) where the activity of penicillin combined with streptomycin or gentamicin was evaluated by killing curves.

Nevertheless it has been established in other studies (6), and also seen in this study, that there are discrepancies when comparing results of checkerboard and time-kill analyses. This study strongly emphasises this fact. However, it is important to continue to determine synergistic interactions using both methods in order to confirm synergy in the event that resistance develops.

6.5 LITERATURE CITED

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CHAPTER SEVEN

RESULTS: CORYNEBACTERIA

7.1 MINIMUM INHIBITORY CONCENTRATIONS / MINIMUM BACTERICIDAL CONCENTRATIONS

The MICs and MBCs of ciprofloxacin, vancomycin and gentamicin were determined against three isolates of *Corynebacterium jeikeium*. These results are tabulated in Table 7.1.

The bacteria were susceptible to both ciprofloxacin (MICs 0,25µg/ml, MBCs 0,25µg/ml) and vancomycin (MICs 1µg/ml, MBCs 1µg/ml). All the isolates were fully resistant to gentamicin (MICs 1024µg/ml, MBCs 1024µg/ml).

Table 7.1 **Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations**

Organism	:*Corynebacterium jeikeium* (n=3)
Antibiotic	:Ciprofloxacin (Breakpoint 1µg/ml)
MICs	:0,25 (3)
MBCs	:0,25 (3)
Antibiotic	:Vancomycin (Breakpoint 4µg/ml)
MICs	:1 (3)
MBCs	:1 (3)
Antibiotic	:Gentamicin (Breakpoint 4µg/ml)
MICs	:1024 (3)
MBCs	:1024 (3)

7.2 CHECKERBOARD SYNERGY STUDIES

Checkerboard studies were performed on selected strains of *Corynebacterium jeikeium*. The results are shown in Figures 7.1 to 7.3, and ΣFICs in Table 7.2.

The combinations tested were as follows:

Gentamicin plus vancomycin: Figure 7.1 showed indifference (ΣFIC 1,0).

Gentamicin plus ciprofloxacin: Figure 7.2 showed synergy (ΣFIC 0,5).

Vancomycin plus ciprofloxacin: Figure 7.3 showed indifference (ΣFIC 1,0).

Table 7.2 Fractional Inhibitory Concentration Indices

Organism *Corynebacterium jeikeium*

Antimicrobial Combination			ΣFICs	
Vancomycin	+	Gentamicin	1,0	1,0
Ciprofloxacin	+	Gentamicin	0,5	0,5
Ciprofloxacin	+	Vancomycin	1,0	1.0

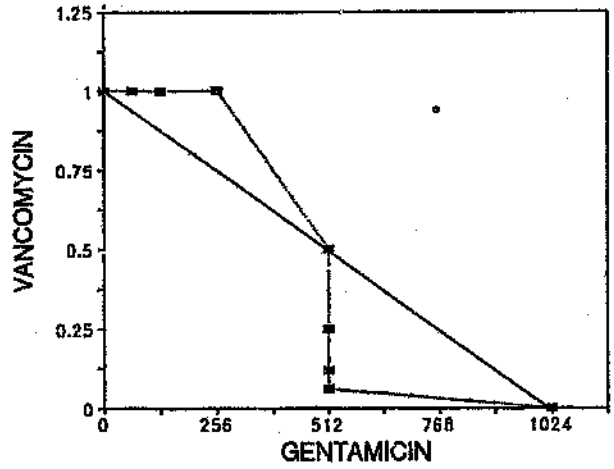

Figure 7.1 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 1024µg/ml) in combination against *Corynebacterium jeikeium*

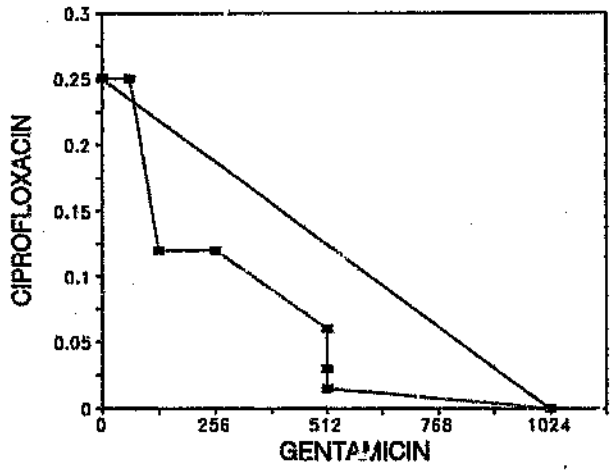

Figure 7.2 Activity of Ciprofloxacin (MIC 0,25µg/ml) plus Gentamicin (MIC 1024µg/ml) in combination against *Corynebacterium jeikeium*

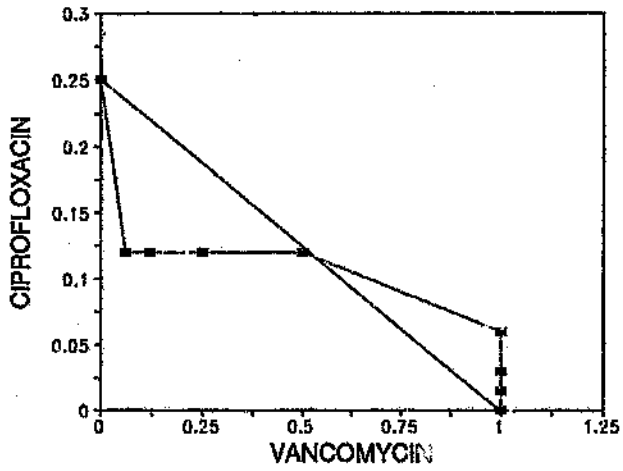

Figure 7.3 Activity of Ciprofloxacin (MIC 0,25µg/ml) plus Vancomycin (MIC 1µg/ml) in combination against *Corynebacterium jeikeium*

7.3 DISCUSSION

The three isolates of *Corynebacterium jeikeium* used in this study were sensitive to ciprofloxacin (MICs 0,25µg/ml), sensitive to vancomycin (MICs 1µg/ml), but showed high-level resistance to gentamicin (MICs 1024µg/ml). Other studies showing vancomycin sensitivity to *Corynebacterium jeikeium* include those of Rolston *et.al.* (1), and Jorgensen *et.al.* (2). Sensitivity to ciprofloxacin (MICs 0,12-0,5µg/ml) was also demonstrated in in vitro studies by Jones and Barry (3). However, resistance to this antibiotic has been shown (4). Of the 44 strains of *Corynebacterium jeikeium* tested in a study by Philippon and Bimet (5), 6 strains demonstrated resistance to ciprofloxacin (MICs 2µg/ml). These strains were also sensitive to vancomycin (MICs ≤1µg/ml), but 28 strains showed low-level resistance to gentamicin (MICs 64µg/ml).

In this study, the only combination to demonstrate synergy was gentamicin plus ciprofloxacin. Note, however, that the strains were ciprofloxacin-sensitive. The other two combinations viz. vancomycin plus gentamicin and vancomycin plus ciprofloxacin both demonstrated indifference.

In a study by Spitzer *et.al.* (6), the combination of

glycopeptide antibiotics and aminoglycosides were evaluated. Synergy could only be detected against an aminoglycoside-susceptible strain. No evidence of synergy was detected against aminoglycoside-resistant strains.

It is imperative that MIC levels be determined when considering treatment of serious infections caused by *Corynebacterium jeikeium* isolates. The usefulness of combination therapy as judged by synergistic affects, except in the case of ciprofloxacin plus gentamicin, could not be established in this study. However, as no antagonism was demonstrated, combination therapy may still be useful even if it will only prevent the emergence of resistance in an organism well known for the development of multiple antibiotic resistance.

7.4 LITERATURE CITED

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CHAPTER EIGHT

FINAL DISCUSSION

Gram-positive bacteria are important causes of hospital-acquired infections, with staphylococcal and enterococcal being the most common. Streptococci, JK corynebacteria and *Leuconostoc* species have also been recognised as important pathogens (1,2,3,4,5,6,7).

Staphylococcus aureus developed the ability to inactivate penicillin, and as other antimicrobial agents were introduced, resistance to them was also acquired (8). One of the major problems to occur is the resistance of staphylococci to methicillin. However, no reports to date have identified vancomycin-resistant isolates.

Other Gram-positive bacteria have also developed resistance to specific antibiotics, such as enterococci to ampicillin and viridans streptococci to penicillin. All *Leuconostoc* species are resistant to vancomycin.

In this study no extraordinary results were obtained when investigating the streptococci. Nevertheless, some conflicting results emerged when compared with those found in the literature. This could to a large extent be

ascribed to the fact that disparity does arise when comparing results between checkerboard and time-kill techniques (9).

Good synergy was demonstrated against *Leuconostoc* species using the combinations of gentamicin plus penicillin; rifampicin plus penicillin; ceftriaxone plus gentamicin; oxacillin plus gentamicin; and cotrimoxazole plus gentamicin. These findings may have important implications as the only other account in the literature reporting combinations of antimicrobial agents being synergistic against *Leuconostoc* isolates was by Buu-Hoï *et.al.*(10). Not enough work has been done in this area, and as *Leuconostoc* species are emerging as important pathogens, it is important to continue to monitor antimicrobial activity against these bacterial strains.

Corynebacterium jeikeium are now acknowledged as causing human infections. A cause for concern is the development of resistance of these bacteria to ciprofloxacin (4). However, a combination that demonstrated synergy in this study by checkerboard was gentamicin plus ciprofloxacin. This could be significant as these bacteria are also multiresistant (11,12,13).

Strain variation in synergy studies make it important to monitor antibiotic combinations continually. It is also

essential to increase information as antibiotic patterns evolve.

There is certainly a need to standardise methodologies, especially with regard to time-kill studies, so that consensus between authors may be established.

Finally, treatment programmes will need to be reviewed continually as resistance mechanisms and patterns develop.

8.1 LITERATURE CITED

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APPENDIX **Supplementary isobolograms**

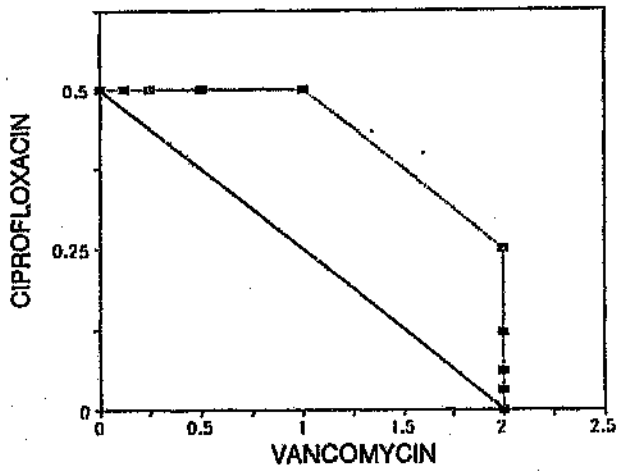

Figure A1 Activity of Ciprofloxacin (MIC 0,5µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

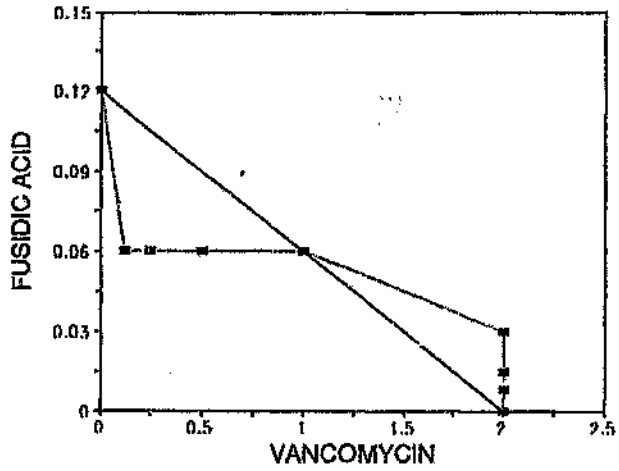

Figure A2 Activity of Fusidic acid (MIC 0,12µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

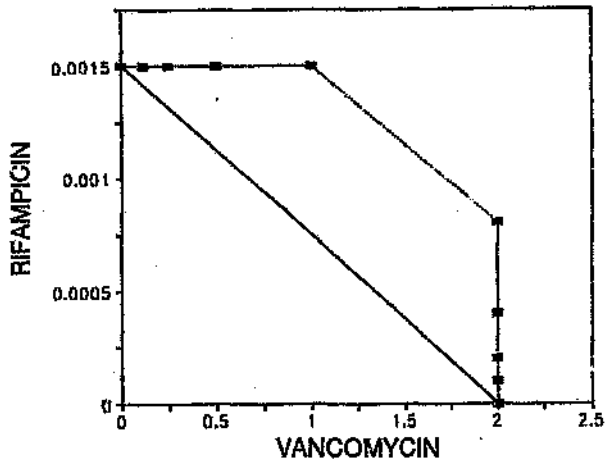

Figure A3 Activity of Rifampicin (MIC 0,0015µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-sensitive *Staphylococcus aureus*

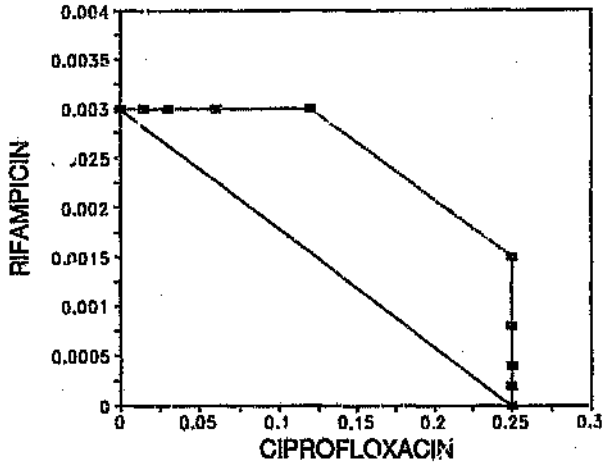

Figure A4 Activity of Rifampicin (MIC 0,003µg/ml) plus Ciprofloxacin (MIC 0,25µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

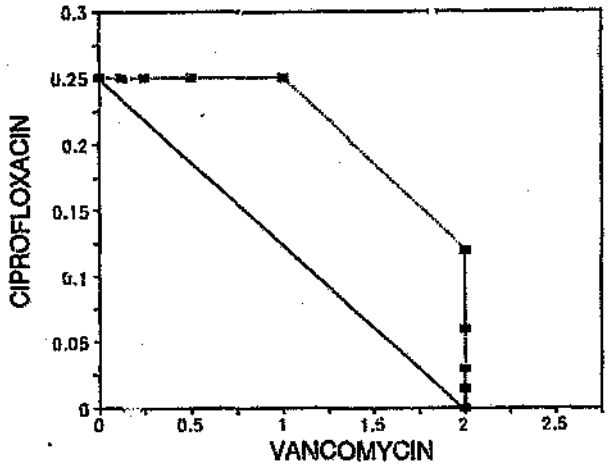

Figure A5 Activity of Ciprofloxacin (MIC 0,25 µg/ml) plus Vancomycin (MIC 2µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

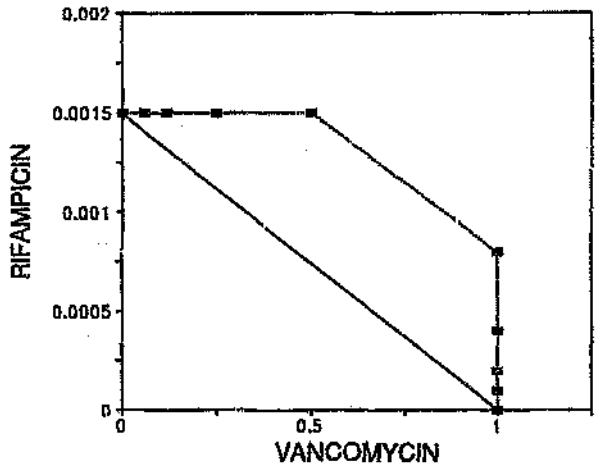

Figure A6 Activity of Rifampicin (MIC 0,0015µg/ml) plus Vancomycin (MIC 1µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

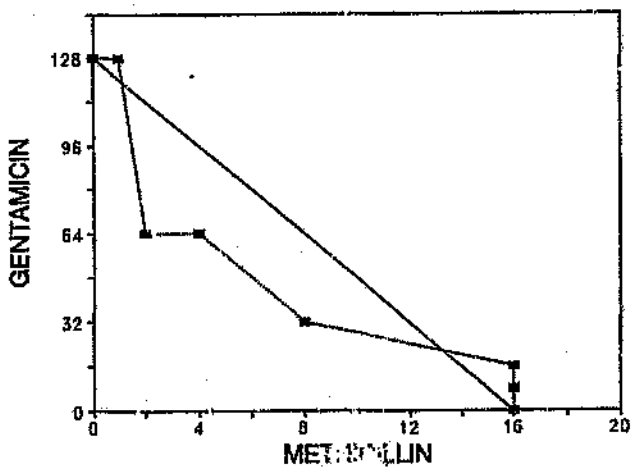

Figure A7 Activity of Gentamicin (MIC 128µg/ml) plus Methicillin (MIC 16µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

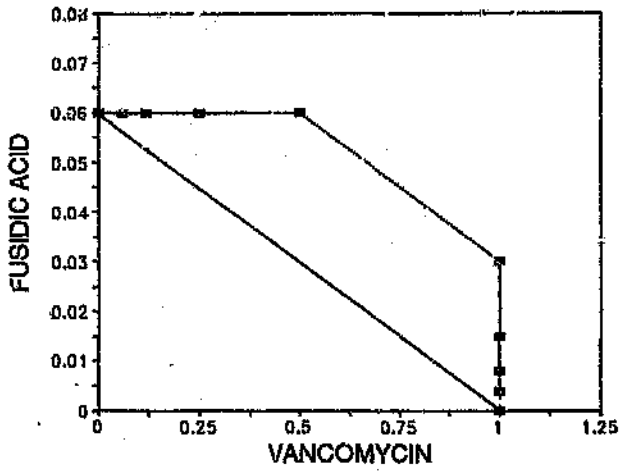

Figure A8 Activity of fusidic acid (MIC 0,06µg/ml) plus Vancomycin (MIC 1µg/ml) in combination against methicillin-resistant *Staphylococcus aureus*

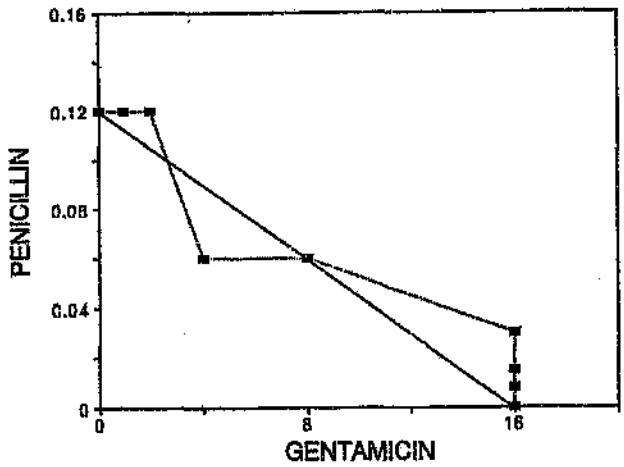

Figure B1 Activity of Penicillin (MIC 0,12µg/ml) plus Gentamicin (MIC 16µg/ml) in combination against *Streptococcus mitis*

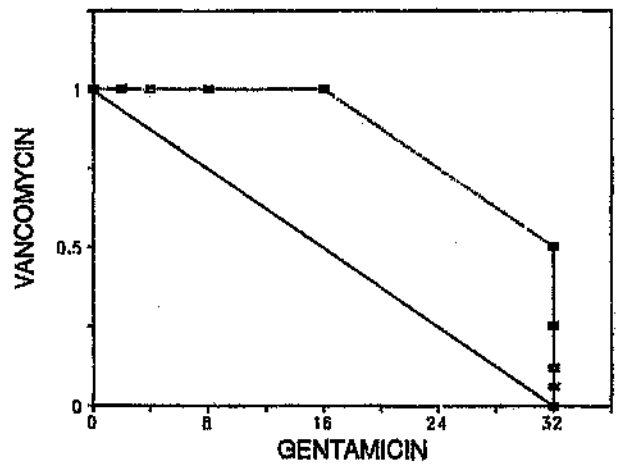

Figure B2 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus mitis*

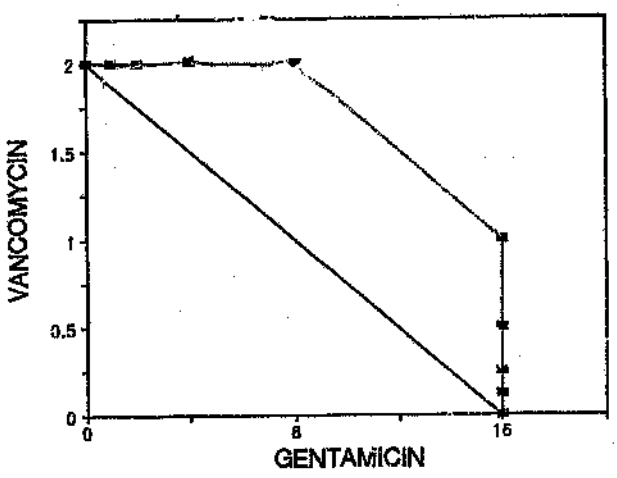

Figure B3 Activity of Vancomycin (MIC 2µg/ml) plus Gentamicin (MIC 16µg/ml) in combination against *Streptococcus mitis*

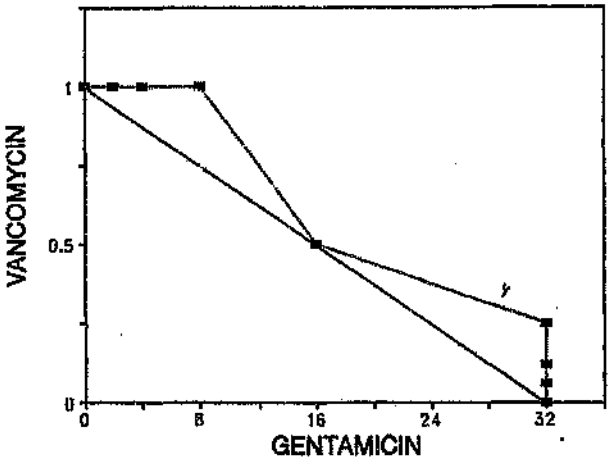

Figure B4 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 32µg/ml) in combination against *Streptococcus sanguis*

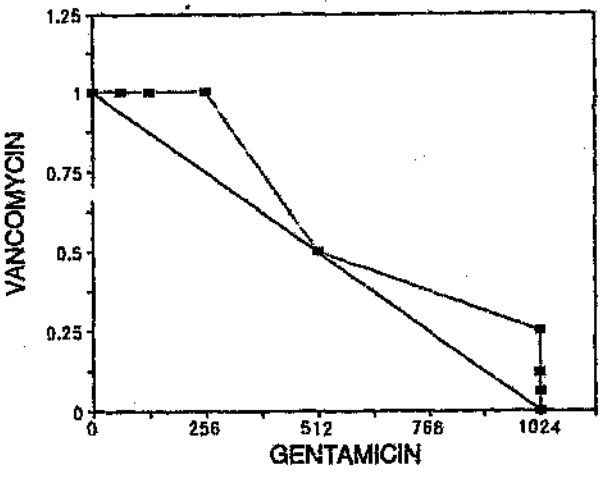

Figure B5 Activity of Vancomycin (MIC 1µg/ml) plus Gentamicin (MIC 1024µg/ml) in combination against *Streptococcus mitis*

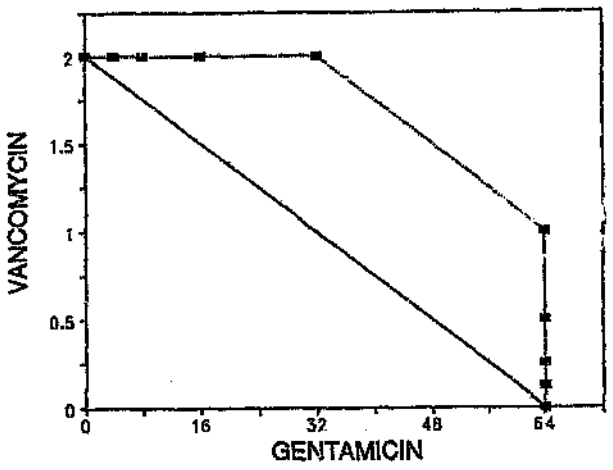

Figure C1 Activity of Vancomycin (MIC 2µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-resistant *Enterococcus faecalis*

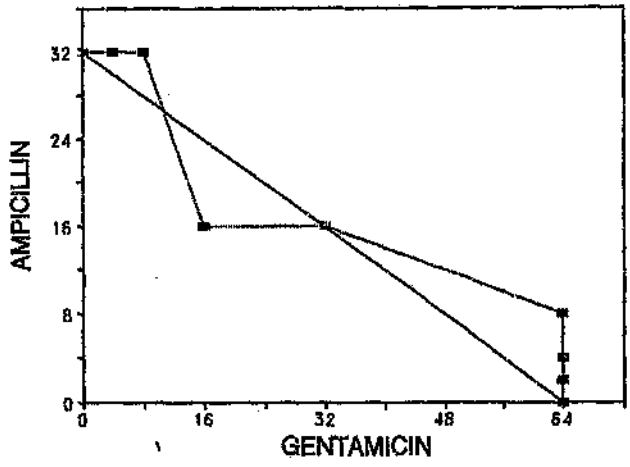

Figure C2 Activity of Ampicillin (MIC 32µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-resistant *Enterococcus faecalis*

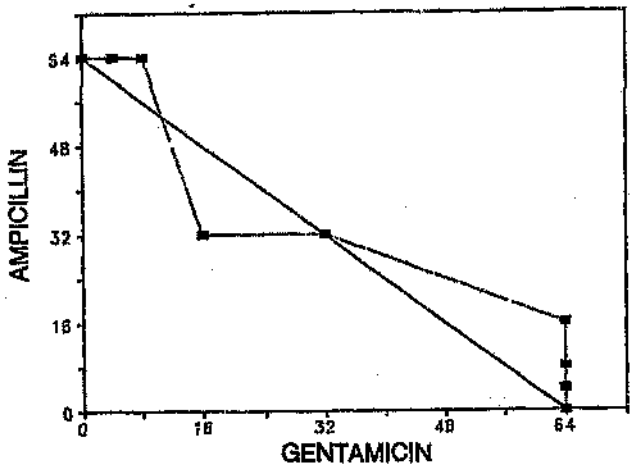

Figure C3 Activity of Ampicillin (MIC 64µg/ml) plus Gentamicin (MIC 64µg/ml) in combination against ampicillin-resistant *Enterococcus faecalis*

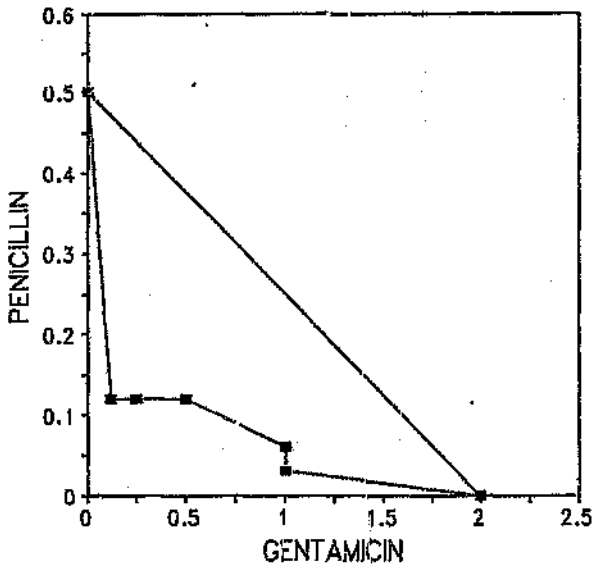

Figure D1 Activity of Penicillin (MIC 0,5µg/ml) plus Gentamicin (MIC 2µg/ml) in combination against *Leuconostoc* sp.

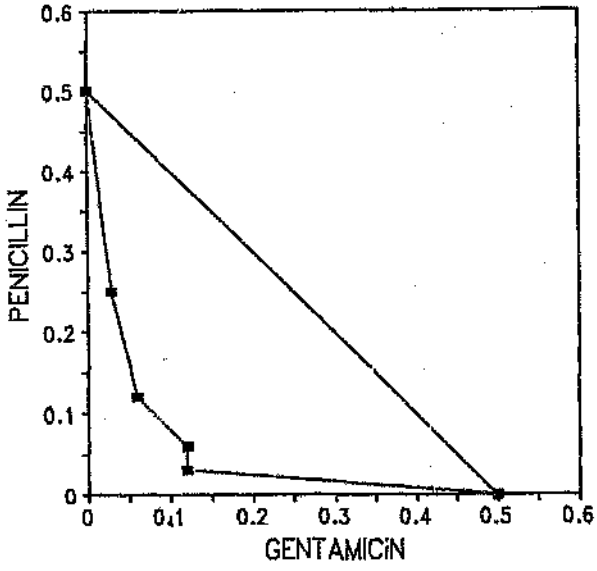

Figure D2 Activity of Penicillin (MIC 0,5µg/ml) plus Gentamicin (MIC 0,5µg/ml) in combination against *Leuconostoc* sp.

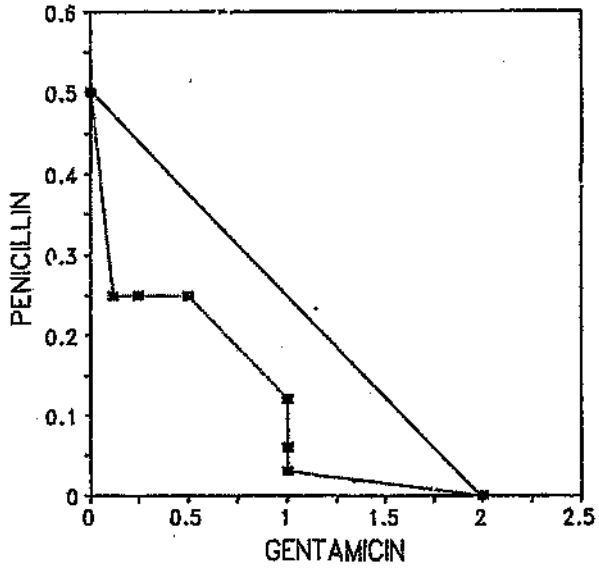

Figure D3 Activity of Penicillin (MIC 0,5µg/ml) plus Gentamicin (MIC 2µg/ml) in combination against *Leuconostoc* sp.

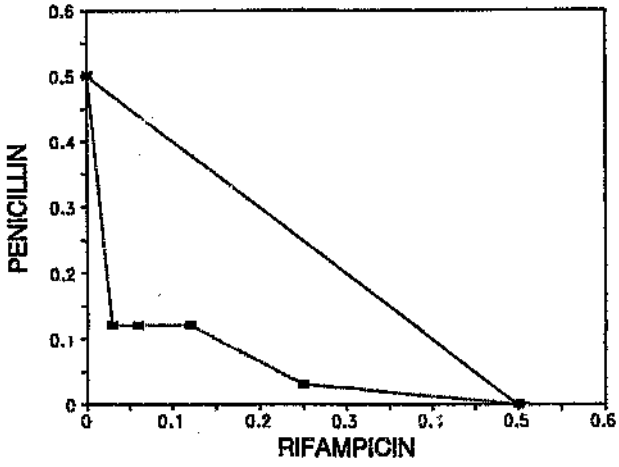

Figure D4 Activity of Penicillin (MIC 0,5µg/ml) plus Rifampicin (MIC 0,5µg/ml) in combination against *Leuconostoc* sp.

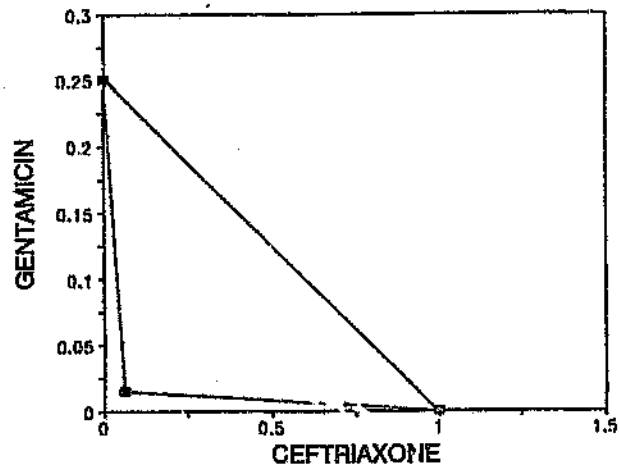

Figure D5 Activity of Gentamicin (MIC 0,25µg/ml) plus Ceftriaxone (MIC 1µg/ml) in combination against *Leuconostoc* sp.

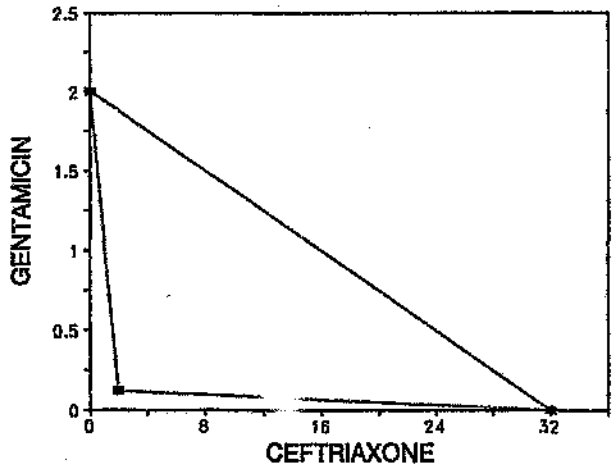

Figure D6 Activity of Gentamicin (MIC 2µg/ml) plus Ceftriaxone (MIC 32µg/ml) in combination against *Leuconostoc* sp.

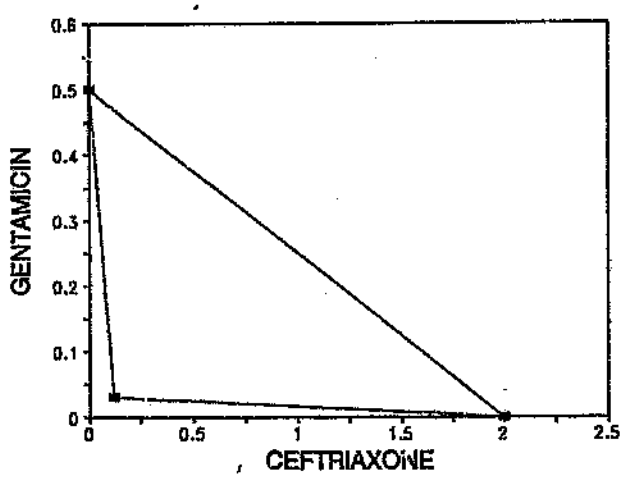

Figure D7 Activity of Gentamicin (MIC 0,5µg/ml) plus Ceftriaxone (MIC 2µg/ml) in combination against *Leuconostoc* sp.

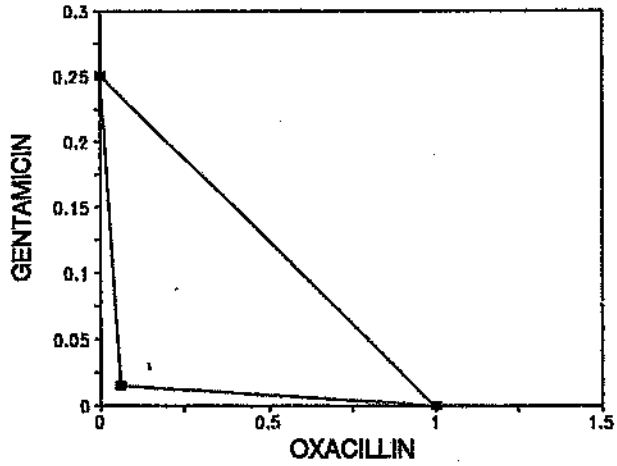

Figure D8 Activity of Gentamicin (MIC 0,25µg/ml) plus Oxacillin (MIC 1µg/ml) in combination against *Leuconostoc* sp.

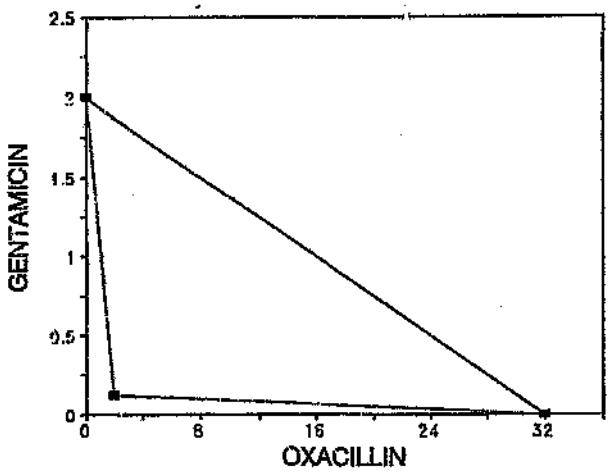

Figure D9 Activity of Gentamicin (MIC 2µg/ml) plus Oxacillin (MIC 32µg/ml) in combination against *Leuconostoc* sp.