MICROBIOLOGICAL ASPECTS OF ENTEROCOCCI ISOLATED AT KING EDWARD VIII HOSPITAL, DURBAN

BY

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SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF MEDICAL SCIENCE

IN THE
DEPARTMENT OF MEDICAL MICROBIOLOGY
UNIVERSITY OF NATAL MEDICAL SCHOOL
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Dedicated to my family and friends for their encouragement,

motivation and support.

ABSTRACT

The increasing frequency of enterococci as a major cause of nosocomial infections and the transmission of these organisms amongst hospital patients demands a greater awareness of the *Enterococcus.* Therapy of enterococcal infections is complicated by the pathogens continually changing resistance patterns to many broad-spectrum antibiotics. In addition, the ability of enterococci to cause serious invasive infections including endocarditis and septicaemia with associated high mortality rates; prompted this study which was aimed at identifying the biological properties of enterococci isolated from blood cultures of patients admitted at King Edward VIII hospital, Durban.

Enterococci were identified to species level by the API 20 Strep system which identified 68% and a conventional biochemical system of Facklam and Collins which identified 100% of the isolates.

The emergence of beta-lactamase producing enterococci in other countries encouraged the testing of all isolates for this enzyme. All were beta-lactamase negative.

The reported false susceptibility for aminoglycosides and cephalosporins with blood enriched media encouraged the testing of these antibiotics with and without the supplementation of 5% lysed blood.

The results showed that an average false susceptibility of 55 % occurred for gentamicin and 35% for tobramycin and netilmicin. The cephalosporins affected, cefotaxime and cefuroxime showed a false susceptibility of 28% and 17% respectively.

The choice of treatment for serious enterococcal infections is a synergistic combination of a beta-lactam antibiotic plus an aminoglycoside for enterococci with intrinsic low-level resistance. The development of high-level aminoglycoside resistance, MIC $\geq 2000 \mu g/ml$ results in loss of synergism. This study showed that 26.4 % of enterococcal isolates displayed high-level aminoglycoside resistance ie. to gentamicin and streptomycin.

Time-kill study showed reduced killing rate for these organisms for the beta-lactams and glycopeptides with low-level gentamicin resistance. The results confirmed that a cell-wall active agent combined with gentamicin can be successfully used for enterococcal therapy if the organism has intrinsic low-level resistance to this aminoglycoside.

Pulsed-field gel electrophoresis (PFGE) carried out on a selected number of *Enterococcus faecalis* and *Enterococcus faecium* with high-level aminoglycoside resistance showed a variability in the restriction endonucelase digestion patterns. This suggests independent development of high-level gentamicin resistance and not clonal expression.
The ease and reliability with which enterococcal isolates may be typed using this technique to compare different strains represent a significant advance.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this thesis was carried out in the Department of Medical Microbiology, University of Natal, under the supervision of Professor A W Sturm.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following individuals :

Professor A W Sturm, supervisor, Head of the Department of Medical Microbiology, University of Natal, for his expert guidance and constructive criticism.

Dr A K C Peer, supervisor, Microbiologist, Pathology Laboratory, Drs N L Pillay, MacIntosh and Partners, for his professional advice and constant encouragement that enabled me to continue with this study.

Dr A Bhamjee, supervisor, Principal Consultant, Department of Medical Microbiology, Univerisity of Natal, for his valuable advice and support throughout this study.

Haffeeza Chenia, Medical Scientist, University of Durban Westville, for her assistance with the technique involving pulsed-field gel electrophoresis and the use of the University's equipment.

Kumari Pillay, my sister, for her understanding and patience in typing this thesis.

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CHAPTER 1

INTRODUCTION

Enterococci have been called the "nosocomial pathogen of the 1990's".
They have been given a genus status distinct from the genus *Streptococcus*, since they differ from them by physiological characteristics and by deoxyribonucleic acid (DNA) contents. It has been a decade since this nomenclature change was proposed by Schleifer and Kilpper-Balz and it is now generally accepted that the genus *Enterococcus* is valid (105). Twelve species have been described namely *E.faecalis, E.faecium, E.durans, E.avium, E.casseliflavus, E.malodoratus, E.gallinarium, E.hirae, E.mundtii, E.raffinosus, E.solitarius, E.pseudoavium* (27).

The genus *Enterococcus* consists of Gram positive facultatively anaerobic organisms that are ovoid in shape. On the Gram stained smear they appear in pairs, singly or in short chains. Optimum growth for this organism occurs at a temperature of 35-37°C but they are able to grow under extreme environmental conditions such as temperatures of 10°C and 45°C. Like streptococci , these organisms do not have cytochrome enzymes and are catalase negative. Enterococci are differentiated from streptococci by their ability to grow in 6.5% salt and at a pH of 9.6. Another distinguishing characteristic is their ability to survive at 60°C for 30 minutes.

Enterococci are able to hydrolyse aesculin and grow in the presence of 40% bile. They also hydrolyse L-pyrrolidonyl-beta-naphthylamide (PYR).
The genus *Enterococcus* is classified within Lancefield group D, based on specific carbohydrate cell wall antigens.

This organism is emerging as a problem pathogen within hospitals because of its transmission amongst hospital patients, and its problematic antibiotic resistance patterns such as , high-level aminoglycoside resistance and multiple resistance to a variety of antimicrobial agents, including vancomycin.

The *Enterococcus*, as the name implies is part of the normal flora of the enteric tract with *E.faecalis* being the most common species. These organisms may be found as colonisers from the genitourinary tract, including the endocervical, vaginal and perineal region in females, as well as the urethra in both males and females (2).
Enterococci are important causes of clinical infections particularly bacterial endocarditis, urinary tract infections, bacteraemia, neonatal sepsis, soft tissue infections and intra-abdominal and pelvic infections.

They have rarely been reported to cause meningitis and pneumonia(6).
The increased awareness of enterococci as a problem pathogen prompted this study which aims at identifying the current biological properties of enterococci in patients admitted to King Edward VIII Hospital, Durban.
107 enterococci, isolated from blood cultures in 1994 at King Edward VIII Hospital, Durban were used in this study.

The enterococci were identified by the Analytical Profile Index (API) Strep System (Bio Mèrieux) and by conventional biochemical tests and classified according to Lancefield grouping using the Streptex Latex Kit (Murex).Electrophoretic analysis of chromosomal DNA patterns by pulse-field gel electrophoresis (PFGE) was performed on a selected number of isolates.

Reports of enterococci producing plasmid-mediated beta-lactamase have further decreased the choice of antibiotics for treatment (74). As with many beta-lactamase producing organisms the amount of beta-lactamase enzyme produced appears to be inoculum dependent.

In a low inoculum of enterococci ie. $<10^6$ CFU/ml, a small amount of enzyme would be produced, thus beta-lactamase susceptible antibiotics such as ampicillin can overwhelm the enzyme resulting in an inhibition of growth around the antibiotic disc. This can result in false susceptible results for penicillin, ampicillin, piperacillin and other ureidopenicillins such as azlocillin and mezlocillin (77). This problem can be overcome by performing a beta-lactamase test routinely or ensuring that an inoculum of at least 10^7-10^8 CFU/ml is used.

Antimicrobial susceptibility testing was performed (modified "Stokes" method, Kirby-Bauer method, minimum inhibitory concentrations and breakpoint method). Isolates were tested for beta-lactamase production by means of the chromogenic cephalosporin method (Nitrocephin). High-level aminoglycoside resistance (MIC $\geq$ 2000μg/ml) was measured for gentamicin and streptomycin.

Synergism studies were performed using time-kill curves on a selected number of isolates.

For the appropriate treatment of infection, rapid, convenient, reproducible, reliable and discriminatory methods for susceptibility testing are relevant aids to the clinician.

The use of the appropriate media for susceptibility testing of enterococci is important since there have been reports of the disc diffusion, minimum inhibitory concentrations (MIC) and breakpoint susceptibility testing methods giving false susceptible results for aminoglycosides and cephalosporins when blood enriched media are used (40,98,109).

This has prompted a comparison of susceptibility test results for the aminoglycosides and cephalosporins with and without the addition of blood to the media. Two disc diffusion methods were compared ie. modified "Stokes" technique and the Kirby-Bauer method.

Minimum inhibitory concentrations (MIC) were determined by the agar dilution method and microdilution technique for the glycopeptides so that minimum bactericidal concentrations (MBC) could be performed for vancomycin and teiçoplanin. This was to determine these antimicrobial agents killing effect (ie. bactericidal or bacteriostatic). Breakpoint susceptibility testing was performed according to National Committee for Clinical Laboratory Standards (NCCLS Document M_{100}-S_4 Vol.12 No.20) values.

Presently, the most disturbing development relates to the reports of vancomycin resistance among enterococci isolated from patients in France, England and the United States of America with MICs ranging from 64 to > 2000μg/ml (50,97).
In view of this observation the universal susceptibility to this antibiotic can no longer be presumed and one should be on the lookout for vancomycin resistant isolates.

The choice of treatment for enterococcal endocarditis is a synergistic combination of a beta-lactam antibiotic plus an aminoglycoside. Enterococci have naturally low-level resistance to aminoglycosides with minimum inhibitory concentrations of $\leq$ 500μg/ml. Their cell wall has limited permeability to aminoglycosides due to the absence of proton motor force (PMF). The synergistic combination of a cell wall antibiotic plus an amino-glycoside, is believed to be caused by the disruption of the bacterial cell wall by penicillin, ampicillin or vancomycin.
This facilitates the penetration and uptake of the aminoglycoside into the bacterial cytoplasm to thus allow the aminoglycoside to act on the bacterial ribosomes, killing the bacterium (67,44).

When high-level aminoglycoside resistance occurs, synergism is lost (99). High-level aminoglycoside resistance is defined as occurring when a drug concentration of ≥ 2000μg/ml is required for inhibition of the organism.

The development of high-level aminoglycoside resistance is believed to occur as a result of selective pressure from the use of broad spectrum antibiotics and the aminoglycosides (57). By acquiring transferable plasmids that encode for various aminoglycoside modifying enzymes, enterococci can exhibit such a phenomenon (19,60).

High-level resistance to gentamicin in enterococci is due to the acquisition of aminoglycoside - modifying enzymes namely 6'-acetyl transferase 2"-phosphotransferase (6'-AAC-2"APH). 3'-aminoglycoside-phosphotransferase enzyme (3'-APH) is responsible for high-level resistance to amikacin.

Streptomycin high-level resistance is caused by aminoglycoside inactivating enzyme 3"-adenyltransferase (3"-ANT) (90).

High-level gentamicin and streptomycin resistance has disseminated among local strains and will undoubtedly continue to spread.

Such resistance is relatively easily detected in the laboratory by high-content discs (120 or 500μg of gentamicin, 300 or 1000μg of streptomycin), by means of using a plate or broth screen method incorporating the appropriate aminoglycoside at a concentration of 500 and/or 2000μg/ml.

More recently, an Etest (AB Biodisk Solva, Sweden) has been developed to predict high-level aminoglycoside resistance.

In this study strains were tested for the detection of high-level resistance to gentamicin, and streptomycin , using the plate screen method.

The range of antimicrobial agents available for treatment in enterococcal infections is limited. With the emergence of resistance to ampicillin as well as vancomycin, susceptibility testing gains importance. The ability of enterococci to develop resistance to a number of useful antimicrobial agents would continue to make this organism a major challenge for the development of new and more effective antibiotics.

Since enterococci have been implicated in hospital acquired infections, typing of isolates is helpful in epidemiological studies. Different patients infected with a single strain suggests that inter-patient transmission has occurred.

Molecular epidemiology of strains of enterococci were determined by pulsed-field gel electrophoresis (PFGE) in *E.faecalis* and *E.faecium* with high-level resistance to gentamicin and / or streptomycin.

This method is able to separate large chromosomal DNA fragments by restriction endonucleases such as *SmaI* in agarose gels by using alternately pulsed, perpendicularly oriented electrical fields from several directions. This method provides consistent reproducibility and enhances resolution of DNA bands, allowing ease and confidence in interpretation of chromosomal bands.

CHAPTER 2

REVIEW OF LITERATURE

2.1 NORMAL DISTIBUTION OF ENTEROCOCCI

The enterococci, as their name implies are part of the normal flora of the enteric tract. *E.faecalis* is the most common isolate, and the concentration increases progressively from the mouth to the large bowel (85). The organism is present in the faeces of most normal adults and carrier isolation rates of as high as 100 percent have been reported in some studies (8).

On the other hand, *E.faecium* has usually been found much less frequently. Given the colonisation of enterococci from the large bowel and the ability of these organisms to survive in high concentrations of bile it is not surprising that enterococci are involved in biliary infection. In addition, these organisms may be isolated in small numbers from the normally colonized part of genito-urinary tract, including the endocervical, vaginal and perineal region in females (2) as well as the urethra of both males and females (46).

2.2 CLINICAL INFECTIONS

Enterococci have been recognised as being potentially pathogenic for humans since 1900. *Enterococcus faecalis* causes disease more frequently than any of the other species, perhaps because it is more commonly a part of the normal human flora and is usually present in higher concentrations.

Infections in which enterococci are most frequently involved are :

2.2.1 Urinary tract infections

Urinary tract infections are the most common type of clinical disease produced by enterococci. This usually presents as uncomplicated cystitis but pyelonephritis and perinephric abscesses and prostatitis may be seen occasionally (75,25).

A large number of enterococcal urinary tract infections are nosocomial and a number of factors may contribute to acquisition of enterococcal urinary infection, including urinary catheterization, frequent instrumentation, recurrent infections, prior antibiotic therapy that select for resistant organisms, debilitated patients and finally transmission of organisms that are resistant to a variety of antimicrobial agents (75,72,112).

Currently, enterococci cause about 10 per cent of all nosocomial infections; this includes approximately 15 per cent of urinary tract infections according to The Centres for Disease Control National Nosocomial Infection Surveillance Survey (86). These numbers are higher as compared to the past. Extensive use of cephalosporins is likely to be responsible for such a phenomenon.

2.2.2 Bacteremia and endocarditis

Although the *Enterococcus* is capable of causing infections of anatomically normal valves, in most cases patients with underlying valvular heart disease or prosthetic valves are involved (75,94). In drug addicts, enterococci are estimated to cause between 5-10 per cent of cases of endocarditis. One study reported 11 out of 20 cases of endocarditis were caused by enterococci (75,92).

2.2.3 Intra-abdominal and pelvic infections

Enterococci are part of the normal intestinal flora and are found in approximately 17 per cent of routine vaginal cultures (7). They are frequently found as part of the mixed aerobic and anaerobic flora involved in intra-abdominal and pelvic infections (24).

Enterococci have been reported to cause salpingitis, endometritis with bacteremia, abscess formation following caesarian section and bacteremia in obstetric and gynecologic patients (75,52,87).
Enterococci can cause peritonitis in patients with nephrotic syndrome or cirrhosis and in patients receiving chronic peritoneal dialysis (34,53).

2.2.4 Neonatal sepsis

Enterococci have been documented to cause neonatal sepsis characterized by fever, lethargy and respiratory distress in the presence of bacteremia and/or meningitis (12).
Neonates with enterococcal sepsis have been reported to have responded well to appropriate antimicrobial therapy (7,5).

2.2.5 Meningitis

Enterococcal infections of the central nervous system are uncommon but have been described in all age groups. The predisposing factors are felt to be underlying long term primary disease, invasive procedures of the central nervous system, head trauma and central nervous system shunts (6,102).

2.3 EPIDEMIOLOGY

Enterococci are part of the normal gut flora of all humans. They are capable of causing infections both in and out of the hospital setting. Previously it was thought that most infections due to enterococci were endogenously acquired (42). The emergence of multi-resistant enterococci causing severe nosocomial infections has focused attention on risk factors for acquisition and spread of these organisms. Recent evidence suggests that most enterococcal infections occurring in hospitalized patients or in patients undergoing therapy such as peritoneal or hemodialysis are exogenously acquired (42). There is evidence that enterococcal strains spread between patients and may even disseminate between institutions (80,81,118). Strains of enterococci causing nosocomial infections have occasionally been found on the hands of medical personnel and have been frequently isolated from environmental sources in hospitals and nursing homes (118,116).

It appears that resistant organisms from patients or hospital personnel first colonize the gastrointestinal tract or occasionally the skin especially the skin folds like the groin before causing infections in patients (93). In the United States, currently enterococci rank second or third in frequency as the cause of nosocomial infections (102).

As yet, no data is available for South Africa. Risk factors for acquiring nosocomial enterococcal infections include serious underlying disease, prolonged hospitalization, prior surgery, renal insufficiency, presence of urinary catheters or bladder instrumentation, stay in an intensive care unit (ICU) and prior antimicrobial therapy (especially aminoglycosides or cephalosporins). This is a major risk factor for acquistion of resistant enterococci (42,116,36,114,15,68).
Other antimicrobial agents that have been associated with enterococcal nosocomial infections include azthreonam, imipenem, ciprofloxacin and vancomycin (36,115,41).

Studies among paediatric patients show that low birth-weight, premature infants with severe underlying conditions, invasive procedure such as intubation, umbilical vessel catheterization, gastrointestinal surgery, use of non-umbilical central catheter, days of central line in place and bowel resection were identified as significant risk factors for enterococcal sepsis in neonates (59,23).

Studies done by Zervous *et al.*(116) have shown that previous antimicrobial treatment particularly with aminoglycosides or cephalosporins, perioperative antibiotic prophylaxis, previous surgery, longer hospitalization and bladder catheterization are risk factors for high-level gentamicin resistance in *E. faecalis*.

Axelrod *et al.*(4) reported that hospitalization for longer than 2 weeks and the administration of five or more antibiotics to patients were two significant risk factors for colonization or infection with *E.faecalis* displaying high-level gentamicin resistance.

Hospitalization and prior antibiotic usage including vancomycin were also identified as risk factors for the acquisition of ampicillin and vancomycin resistant enterococci. (116,10,54,36). Beside longer stay in hospital, female gender, renal dysfunction and preceding imipenem therapy were found to be important factors allowing the organism to become endemic. (10,54,36).

The majority of outbreaks caused by multi-resistant enterococci have been controlled by strict applications of barrier precautions, handwashing and cohorting infected or colonized patients.

Handwashing with soap and water alone may not be effective for the removal of vancomycin resistant *E.faecium* from heavily contaminated hands (111).

Alcoholic chlorohexidine which has rapid bactericidal and residual activity was reported to be effective. Useful control measures suggested by Handwerger *et al.*(36) include daily perineal washing with chlorohexidine in patients with groin or rectal colonization.

For personnel that are colonized with vancomycin resistant enterococci showering with chlorohexidine was found to reduce the level of colonization with enterococci.
In addition, the controlled use of antibiotics has been shown to be effective in the prevention of enterococcal nosocomial infections (36).

Since vancomycin usage has been identified as a major risk factor for nosocomial infection with vancomycin resistant enterococci (36,96), it is important to use this antibiotic only when there is no alternative.

In recent years, selective decontamination of the digestive tract (SDD) has been proposed as a means of reducing nosocomial infections. However SDD in intensive care units lead to emergence of resistance in general and selection of resistant enterococci in the respiratory and gastro-intestinal tract.(21). Studies have shown that SDD does not improve survival among patients receiving mechanical ventilation and its routine use in intensive care units cannot be recommended (31).
Careful monitoring and use of prophylatic and therapeutic antibiotics has the potential to change the current trends in enterococcal nosocomial infections.

2.4 RESISTANCE OF ENTEROCOCCI TO ANTIMICROBIAL AGENTS

Antimicrobial resistance may be either intrinsic or acquired. The term intrinsic resistance is used to indicate resistance which is a natural characteristic of a species and therefore present in most or all strains of that species (67). The genes for intrinsic resistance, like other species characteristics appear to reside on the chromosome. Acquired resistance results from either a mutation in the existing DNA or acquistion of new DNA (75). Table I gives an overview of resistance in enterococci.

Table I **Resistance found in enterococci**

INTRINSIC (Naturally occurring resistance)	ACQUIRED RESISTANCE
Aminoglycoside (Low-level resistance MIC ≤ 500μg/ml)	Aminoglycoside (High-level resistance MIC ≥ 2000μg/ml)
Beta-lactams,particularly semi-synthetic penicillinase resistant penicillins and cephalosporins (manifested by relatively high MICs)	Chloramphenicol
Clindamycin (Low-level)	Erythromycin and high-level clindamycin
	Fluoroquinolones
	Penicillins via penicillinase
	Tetracycline
	Vancomycin

In addition to the relative high MICs with beta-lactams (penicillin, ampicillin, imipenen) enterococci, typically have very high minimum bactericidal concentrations.
(MBCs > 128μg/ml) and are thus a natural example of beta-lactam tolerant organisms (75). The lack of bactericidal activity presumably explains the high failure rate when single drug therapy is used to treat endocarditis caused by susceptible enterococci (73).

In recent years there have been several suggestions that the prevalence of strains of *E.faecium* with higher levels of intrinsic resistance to penicillin and related beta-lactams is increasing and there have been a number of reports of outbreaks of infection with strains of *E.faecium* for which MICs of penicillin ranged from 32 to 256μg/ml(13).

High-level resistance of *E.faecium* to penicillin, not due to beta-lactamase production has been increasingly reported.Most evidence suggests that resistance of this species to penicillin is due to the low overall affinity of its penicillin binding proteins. In Europe and the USA the prevalence rate of this species of enterococci is between 10 and 20 percent (88).
Resistance to ciprofloxacin has also emerged in isolates causing infection in patients on such therapy, presumably due to mutations (115). From 1985 to 1986, ciprofloxacin resistance was only 1,4 percent in the USA.
In 1989 to 1990, ciprofloxacin resistance had increased significantly to 15,2 percent (103). Most ciprofloxacin resistance occurred in strains showing high-level gentamicin resistance, 24 percent of gentamicin-resistant strains were resistant to ciprofloxacin (103).Low-level resistance to aminoglycosides (MIC 16-256 μg/ml) is an inherent trait due to the lack of proton motive force mediated transport.

Aminoglycoside uptake is markedly increased in the presence of cell wall active agents such as penicillin and ampicillin and synergy of killing occurs with the combination of these agents when high-level aminoglycoside resistance is not present.

High-level aminoglycoside resistance is defined as an MIC of ≥ 2000μg/ml; resistance is due to the production of aminoglycoside - modifying enzymes. The clinical significance of this resistance is that it eliminates synergy with beta-lactam antibiotics thereby preventing bactericidal activity. The genes coding for aminoglycoside modifying enzymes responsible for this type of resistance appear to be transferable and are situated on plasmids or transposons.

High-level resistance to erythromycin in enterococci is plasmid mediated and widely distributed. Tetracycline and chloramphenicol high-level resistance has also emerged.

Transferable high-level resistance to trimethoprim/sulfamethoxazole has been reported , and is thought to be due to an altered dehydrofolate reductase of *E.faecium* strains which are intrinsically resistant (88).

In addition to their resistance and tolerance to beta-lactams, enterococci have recently acquired the ability to directly inactivate penicillin and ampicillin by the production of beta-lactamases. The first beta-lactamase producing *Enterococcus* was described in Houston, Texas in the year 1983 (82). Subsequently, beta-lactamase producing enterococci have been isolated from a number of different locations in the United States of America. Fortunately, its spread is limited so far.

The enterococcal beta-lactamases hydrolyse penicillin, ampicillin, piperacillin and other ureidopenicillins which correlates with resistance to these compounds; there is little or no inactivation of penicillinase -resistant semisynthetic penicillins, cephalosporins or imipenem (78,77). With few exceptions, beta-lactamase producing enterococci have also exhibited high-level resistance to gentamicin (89).

The latter 1980's had seen the development of vancomycin resistant enterococci isolated from patients in France, England and the United States of America with MIC ranges from 64-2000μg/ml (50,97). Recently, such isolates have been reported from Johannesburg as well (43). Although the incidence of vancomycin resistance remains low, it threatens an indispensable therapeutic alternative in the penicillin - allergic patient or for enterococcal infections, with penicillin and ampicillin resistance. Three phenotypes of vancomycin resistance have been described for enterococci, Van A, Van B and Van C (76).
Van A isolates, primarily *E.faecium*, are highly resistant to vancomycin (MIC > 1000μg/ml) and are cross resistant to teicoplanin. Van B, in relation to both *E.faecium*, and *E.faecalis,* are moderately resistant to vancomycin (MIC 16-1000μg/ml) but remain susceptible to teicoplanin. Van C also has low-level resistance and affects vancomycin only. Recent studies with Van A, Van B, Van C isolates have shown that the gene for Van A but not Van B is carried on a variety of plasmids and is transmissable to other enterococci, streptococci and listeriae (76,20).

Van A and Van B resistance are both inducable in the presence of antibiotics and induction is accompanied by the synthesis of new membrane-associated proteins (76).
These proteins interfere with vancomycin activity by protecting or modifying the N-acyl-D-alanyl-D-alanine terminal residues in the cell wall (20).

Teicoplanin is an agent that is still available for vancomycin resistant isolates of the Van B and Van C types, however at least three quarters of vancomycin resistant enterococci are resistant to teicoplanin as well (63).

Glycopeptide resistant enterococci are the cause of currently untreatable nosocomial entercoccal infection. The paucity of new antimicrobial agents effective against enterococci suggests that, physicians can expect to encounter such infections with growing frequency.

2.5 PRESUMPTIVE IDENTIFICATION

It has been a decade since Schleifer and Kilpper-Balz proposed that enterococci should be given a genus status distinct from streptococci, since they differ from streptococci by physiological characteristics and DNA contents. It is now generally accepted that the genus *Enterococcus* is valid (104). Twelve species of enterococci have been proposed namely, *E.faecalis, E.faecium, E.durans, E.avium, E.casseliflavus, E.malodoratus, E.gallinarium, E.hirae, E.mundtii, E.raffinosus, E.solitarius, E.pseudoavium* (104,17,18,29,45).

The genus *Enterococcus* consists of Gram positive aerotolerant organisms that are ovoid in shape. On the Gram stained smear they appear in pairs, singly or in short chains. Optimum growth of this organism occurs at a temperature of 35°C but they are able to grow under extreme environmental conditions such as temperatures of 10° and 45°C. Like streptococci, these organisms do not have cytochrome enzymes and are catalase negative. Enterococci are able to grow in 6.5% NaCl and at a pH of 9.6. They are able to survive 30 minutes at 60°C and will grow in the presence of 40% bile. Enterococci hydrolyse aesculin and L-pyrrolidonyl-β-naphthylamide (PYR). Cell walls of most enterococci contain the streptococcal group D antigen.

Although the above mentioned tests appeared to be sufficient in the past for the presumptive identification of enterococci, it has now been recognised that other less commonly encountered gram-positive cocci can also give a positive reaction in some of these tests (28). For example, some cultures of *Lactococcus, Pediococcus, Leuconostoc* and *Aerococcus* species can grow in the presence of 6.5% NaCl and are bile-esculin positive. Some *Lactococcus* , *Aerococcus* as well as *Leuconostoc* species are PYR positive. Certain strains of *Pediococcus* and *Leuconostoc* species possess the group D antigen.

The tests used to differentiate the eight different genera of gram-positive, catalase negative, aerotolerant cocci are listed in table II.

Table II **Basic reactions to place the Gram-positive cocci into eight genera are as follows** (28)

	REACTION (% POSITIVE)							
TEST	*Entero coccus* (200)*	*Strepto-coccus* (645)*	*Lacto-coccus* (16)*	*Aero-coccus* (42)*	*Gemella* (15)*	*Pedio-coccus* (26)*	*Leuco-nostoc* (50)*	*Lacto-bacillus* (42)*
Gas from glucose	<1	0	0	0	0	0	100	50
Vancomycin resistant	<1	0	0	0	0	100	100	90
Reaction to streptococcal grp D antiserum	80	0	0	0	0	95	35	25
Bile aesculin reaction	99	10	75	60	0	100	90	50
PYRase	100	4	69	100	73	0	0	7
Growth : in 6.5% NaCl	100	0	56	100	0	35	60	40
At 45°C	99	35	25	0	0	83	0	60
At 10°C	85	<1	100	0	0	4	75	100

* : No. of strains tested.

These tests identify the bacteria as members of the genus *Enterococcus.* They can be further identified to species level using key phenotypic characteristics to form three groups as shown in table III (28).

Table III **Key tests for identification of enterococci groups**

	REACTION (% POSITIVE)				
SPECIES	GROUP	MANNITOL	SORBITOL	SORBOSE	ARGININE
E.avium					
E.raffinosus	I	+ (100)	+(97)	+(97)	-(0)
E.malodoratus					
E.pseudoavium					
E.faecalis					
E.solitarus					
E.faecium	II	+(99)	V(63)	-(0)	+(94)
E.casseliflavus					
E.mundtii					
E.durans					
E.hirae	III	-(7)	-(0)	-(0)	+(100)
E.faecalis (variant)					

Enterococci may be placed into groups I, II, III (28).

Once grouped, the unknown organism can usually be identified to the species level, by selected tests as described in tables IV, V, VI. Table VII and VIII indicate additional tests used to complete the identification of the enterococcal isolate if the initial identification profile obtained fails to identity the isolate.

Table IV **Identification of group I *Enterococcus* species**

SPECIES	REACTION (% POSITIVE)	
	ARABINOSE	RAFFINOSE
E.avium	+(99)	-(0)
E.raffinosus	+(100)	+(100)
E.malodoratus	-(0)	+(100)
E.pseudoavium	-(0)	-(0)

Table V **Identification of group II *Enterococcus* species**

SPECIES	REACTION (% POSITIVE)				
	ARABINOSE	SORBITOL	LACTOSE	MOTILITY	PIGMENT
E.faecalis	-(0)	+(96)	+(100)	-(0)	-(0)
E.solitarus	-(0)	+(100)	-(0)	-(0)	-(0)
E.gallinarium	+(100)	-(0)	+(100)	+(100)	-(0)
E.faecium	+(100)	V(29)	+(97)	-(0)	-(0)
E.casseliflavus	+(100)	V(50)	+(100)	+(100)	+(100)
E.mundtii	+(100)	V(50)	+(100)	-(0)	+(100)

Table VI **Identification of group III *Enterococcus* species**

SPECIES	REACTION (% POSITIVE)		
	SUCROSE	RAFFINOSE	PYRUVATE
E.durans	-(0)	-(0)	-(0)
E.hirae	+(88)	+(75)	-(0)
E.faecalis (asaccharolytic variant)	-(0)	-(0)	-(0)

Table VII **Further tests used to differentiate *Enterococcus* species**

STRAIN	REACTION (% POSITIVE)				
	ACID FORMATION				HIPPURATE HYDROLYSIS
	Glucose	Inulin	Melibiose	Trehalose	
E.avium	44	0	50	100	0
E.raffinosus	93	0	100	100	33
E.malodoratus	0	0	100	100	0
E.pseudoavium	0	0	0	100	0
E.faecalis	93	0	4	99	24
E.solitarus	100	0	25	100	100
E.gallinarium	0	0	100	100	62
E.faecium	0	0	81	100	23
E.casseliflavus	63	75	100	100	0
E.mundtii	0	0	100	100	0
E.durans	0	0	75	100	33
E.hirae	0	0	88	100	13
E.faecalis (variant)	0	0	0	75	100

Table VIII **Additional tests used to differentiate *Enterococcus* species**

STRAIN	REACTION (% POSITIVE)			
	GROWTH TOLERANCE		VP	GROUP D ANTIGEN
	10°C	0.04% TELLURITE		
E.avium	66	0	62	32
E.raffinosus	27	0	66	40
E.malodoratus	100	0	0	100
E.pseudoavium	100	0	0	0
E.faecalis	99	93	89	91
E.solitarius	75	0	75	100
E.gallinarium	100	8	62	100
E.faecium	97	3	89	68
E.casseliflavus	75	0	25	100
E.mundtii	100	50	100	100
E.durans	100	0	100	75
E.hirae	100	0	100	63
E.faecalis (variant)	100	0	0	100

2.6 LANCEFIELD GROUPING

Grouping of the family Streptococcaceae has been classically done by serotyping using the Lancefield classification. The Lancefield system is based on the immunological detection of antigens, associated with the cell wall of the bacteria. (Groups A,B,C,D,F,G). In the classic Lancefield test these antigens are extracted in soluble form and identified by precipitation with homologous antisera.

Enterococci express the group D antigen. However, group D antigen is not specific for enterococci, but can also be demonstrated on most species of *Pediococcus*, and some *Leuconostoc* and all *Streptococcus bovis*. Thus biochemical tests are necessary to differentiate group D reactive organisms.

2.7 BETA-LACTAMASE PRODUCING ENTEROCOCCI

Beta-lactamase production was first described for *S. aureus* in the early mid 1940s (74). The first published account of a beta-lactamase producing *Enterococcus* was in 1983, when it was found that a strain of *E.faecalis* isolated in Houston, Texas, in 1981 produced beta-lactamase (82). The next report of a beta-lactamase producing enterococci was in 1987 from Philadelphia (39). Since then; a large number of such isolates of *E.faecalis* have been reported from at least 11 cities in four countries (74).

The resistance to penicillin or ampicillin of beta-lactamase producing strains is not always detected by routine disc susceptibility testing because of an inoculum effect (75). When a low inoculum is used ie. $\leq 10^6$ CFU/ml for disc diffusion testing, strains appear to be susceptible but at a high inoculum ie. 10^7-10^8CFU/ml strains appear resistant (MIC > 500μg/ml) (78). An inoculum effect is due to the fact that low numbers of cells do not produce sufficient beta-lactamase to inactivate the drug in the test system. The enterococcal beta-lactamase hydrolyzes penicillin, ampicillin, piperacillin and other ureidopenicillins (azlocillin, mezlocillin) (78,77). There is little or no inactivation of penicillinase resistant semi-synthetic penicillins, cephalosporins or impenem.

In most staphylococci, the amount of beta-lactamase production increases on exposure to penicillin. To date, beta-lactamase production in enterococci is constitutive and remains unchanged on exposure (121). However, even though enzyme production is constitutive, the *Enterococcus* produces much less enzyme than a *Staphylococcus* on exposure.

Evidence supporting the hypothesis that the capability of beta-lactamase production has been recently acquired by enterococci is that it is almost always associated with high-level resistance to gentamicin (74).

Another important concern is that the transferable penicillinase activity may disseminate into other streptococci species such as group-A streptococci or *Streptococcus pneumoniae* for which penicillin is presently the most effective therapy. This is of great concern since it is well known that some R plasmids can transfer between species of the streptococcal family and even to and from *Staphylococcus aureus* (16,26).

Hence, there is a possibility of the occurrence of beta-lactamase production in other species of streptococci in the future.

2.8 ANTIMICROBIAL SUSCEPTIBILITY TESTING

In vitro tests of antimicrobial activity has come a long way and it has been said that it is as old as penicillin itself. These tests have undergone man2y changes resulting in a variety of techniques. This was inevitable in view of the emergence of new pathogens, the frequent introduction of new antibiotics for the treatment of infections and the rapid development of new automated equipment for susceptibility testing.

Many different methods are currently in use, with standardization being achieved by the use of control bacterial organisms. In the end, the correct result should predict the response of the patients treated with the antimicrobial agents. However, there are many confounding factors that determine the relationship between *in-vitro* susceptibility and clinical response. The goal of susceptibility testing in clinical laboratories was defined by a working party of the British Society for Antimicrobial Chemotherapy as follows : " to assist the clinician in his choice of an appropriate therapeutic or prophylactic antibiotic for individuals or groups of patients or to help the clinician to account for failures of response to empirically selected agents"(11).
In addition to these clinical objectives; antimicrobial susceptibility testing in clinical laboratories may also be used to provide information on epidemiology and mechanisms of antimicrobial resistance.

The laboratory provides information that designates organisms as susceptible, intermediate (moderately susceptible) or resistant. Susceptible indicates that the infection is likely to respond to standard doses of the antimicrobial agent, while resistant implies that the infection is unlikely to respond. Infection with an organism designated as intermediate or moderately susceptible may or may not respond to standard doses (which might still be used if the result of failure is of lesser clinical significance) but would more probably respond if the drug is concentrated at the site of infection or if its dosage is increased (11).

Antimicrobial susceptibility testing of bacteria may be determined using a number of different methods. These include disc diffusion methods (Kirby-Bauer, "Stokes" or Comparative), dilution methods (broth or agar), breakpoint method and the Etest.
In a diagnostic laboratory, a convenient method of performing antibiotic susceptibility tests is required and in one form or another, a disc-diffusion method is most commonly used.

A solid culture medium is evenly inoculated with the organism to be tested and blotting paper discs containing the antibiotics are put on the surface.

During incubation at 35-37°C for 18-24 hours antibiotic diffuses radially from the disc into the medium. Interpretation of the susceptibility of the test organisms depends on the type of disc diffusion techniques used. In the Stokes method, the test results are compared to the National Collection Type Culture (NCTC) control organism and by comparing the test zone diameter to the control organism, the test may be interpreted as susceptible intermediate or resistant. In the Kirby-Bauer method, the test zone diameter is compared to the National Committee for Clinical Laboratory Standards (NCCLS) zone diameter interpretive standards and according to the standard zone diameters the tests may be interpreted as susceptible, moderately susceptible or resistant.

To quantitatively measure the in vitro activity of an antimicrobial agent against a bacterial culture a broth or agar dilution technique may be used. Basically a series of two fold dilutions of the antibiotic is prepared in an agar or broth medium.
The tubes or plates are then inoculated with a suitably standardized suspension of the test organism.

After overnight incubation at 35-37°C for 18-24 hours, the tests are examined and the minimum inhibition concentrations (MIC) is determined. MIC is interpreted as the lowest concentration of antibiotic that inhibits bacterial growth. A known control organism (American Type Culture Control - ATCC) or National Collection Type Culture (NCTC) is included with each batch of tests.

The breakpoint method as used in diagnostic laboratories may be regarded as an abbreviated form of MIC determination using one or two chosen concentrations of the antibiotics to be tested. The range of antimicrobials selected for inclusion within a breakpoint testing scheme is related to the species of organisms tested and the clinical site if infection from which they are isolated. The concentrations are selected in order to inhibit susceptible bacteria and allow resistant strains to grow. Test and control organisms are spot inoculated on the surface of an agar plate by use of a replicating apparatus. Inoculated plates are incubated at 35-37°C for a period of 18-24 hours and observed for the presence or absence of bacterial growth.
If one concentration is used, results can be interpreted as susceptible if there is no growth, or resistant if growth is observed. If two concentrations are used, results are interpreted as susceptible if there is no growth on both concentrations, intermediate or moderately susceptible if there is growth on the lower concentration only. Results are interpreted as resistant when both concentrations show growth.

More recently susceptibility testing may also be performed using the Etest [AB Biodisks solva, Sweden]. Briefly, the Etest consists of a impervious strip impregnated with a continuous antimicrobial gradient. It is a new in-vitro susceptibility testing method used for the quantitative determination of susceptibility to antimicrobial agents.

The strip is applied to the surface of an agar plate that has been inoculated evenly with a standardised inoculum of the test organism. This technique involves the diffusion of a continuous antimicrobial gradient from a thin plastic strip, producing an organism inhibition eclipse at which the minimum inhibitory concentration (MIC) is read, after incubation at 35-37°C for 18-24 hours. The MIC is recorded at the intersection of growth on the MIC scale of the strip.

The selection of antibiotics for testing and reporting should be done in a manner that encourages the use of the most effective and least expensive drug whenever possible.

The general drug selection is based on the type of organism been tested, the body site from which the organism was isolated, and the drugs available for use in a particular institution (100).

In the case of enterococci routine determination of susceptibility to penicillin, ampicillin, vancomycin and high-levels of gentamicin and / or streptomycin should be done. Ampicillin along with a test for beta-lactamase production can be used to detect resistance to other beta-lactam antibiotics for enterococci.

The activity of antibiotics is dependant upon inherent characteristics of the organisms and the drugs and is influenced by the environment (media or tube) in which they interact.
Although routine testing of aminoglycosides is generally not recommended for enterococci, some laboratories do include testing of aminoglycoside antibiotics routinely, others may test upon request or in selected clinical situations where an aminoglycoside plus a beta-lactam antibiotic may be used for treatment.

It has been reported that sheep blood enriched Mueller-Hinton agar frequently gave enlarged zone sizes that falsely indicated susceptibility.

For tobramycin and gentamicin, 72% and 70% respectively of isolates changed from resistance to susceptible when 5% sheep blood was used in the media (40) as compared to the same media without blood.

There was less of a problem with amikacin and kanamycin, as false susceptibility only occurred with 4 and 2% of the isolates respectively (40) for disc diffusion susceptibility testing.

In comparison, agar dilution MICs demonstrated uniform resistance with or without the presence of blood; amikacin and kanamycin showed a false susceptibility rate of 4 - 6 % with sheep blood agar plates (40).

False susceptibility to aminoglycosides was shown to be caused by heme in concentrations as low as 0.03μg/ml (40). It has been postulated that the heme effect is related to a catalytic cleavage of intracellular H_2O_2 which results in lipid peroxidation (40).

In an attempt to find other organisms demonstrating false susceptibility to aminoglycosides, Jenkins *et al.* tested 14 non enterococcal isolates by disc diffusion for susceptibility to gentamicin, tobramycin, amikacin, and kanamycin to compare media with and without the addition of 5% sheep blood.

The isolates tested included the following : *Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus equinus and Streptococcus bovis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Providencia stuartii, Morganella morgani, Salmonella typhi, Citrobacter freundii* and *Enterobacter cloacae* (40).

None of these organisms demonstrated false susceptibility with the aminoglycosides tested.
Similar effects have been reported for enterococci when tested against second and third generation cephalosporins (98,109).
Disc diffusion results obtained with the addition of blood showed discrepancies of 4% for cephalothin, cefamandole and cefoperazone .

In contrast, there was a much greater discrepancy for cefotaxime [18%] and for cefuroxime, ceftizoxime and cefmenoxime [15%] (109).
Such variations was also observed when different brands of media were used.

Since this phenomenon has not been observed with other bacterial species, it seems likely that intrinsic characteristics of enterococci are contributing factors to the mechanisms involved.

Sahm *et al.* (98), have postulated that the reason for false susceptibility to second and third generation cephalosporins in enterococcal isolates may be due to interactions between medium components and the heterocyclic ring at the 7-acyl position. This influences their affinity for penicillin-binding proteins. This is supported by the fact that this structure is not present in those cephalosporins that were not affected.

2.9 HIGH-LEVEL AMINOGLYCOSIDE RESISTANCE

High-level aminoglycoside resistance is defined as occurring when a drug concentration of $\geq 2000 \mu g/ml$ is required for inhibition of the organism (68,14).

The development of high-level resistance is believed to occur as a result of selective pressure from the use of aminoglycosides and broad spectrum antibiotics (57).

The genetic determinants encoding the aminoglycoside-modifying enzymes responsible for high-level resistance were found to be plasmid-mediated. The importance of these observations is that high-level aminoglycoside resistance confers resistance to bactericidal synergism between the aminoglycoside and the cell wall active antibiotics (62).

Ubukata *et al.* (110) isolated a bi-functional resistance enzyme from *S.aureus* that possess both 6'-acetyltransferase and 2"-phosphotransferase activities. This bi-functional enzyme is also found in strains of enterococci.

Data from Ferreti *et al.* (30) suggest that the gene specifying this enzyme is plasmid conferred and arose as a result of gene function.

The enzymatic modification catalyzed by these enzymes make the organisms resistant to all aminoglycosides (viz. gentamicin, kanamycin, tobramycin, amikacin and streptomycin).

The aminoglycoside-modifying enzymes are located on the following genetic elements (90) as seen in the table below.

Table IX **Activity of aminoglycoside-inactivating enzymes**

	AMINOGLYCOSIDE				
ENZYME	GENTA	AMIK	STREPTO	TOBRA	KANA
APH 3"			+		
APH 3'-1					+
APH3'-2					+
AAC 2'	+			+	+
AAC 6'	±	±		+	+
AAC 3-1	+			±	±
AAC 3-2	+			+	+
ANT 2"	+			+	+
AAD 3"			+		
APH 2"-AAC 6'		+		+	+
APH 3'-5"		+			+
ANT 3'-APH 5"			+		
AAC 6'-APH 2"	+	+	+	+	+

+ : Inactivation

± : Aminoglycoside is poor substrate , poor inactivation.

APH : describes a phosphorylating enzyme

ANT : describes an adenylating enzyme

AAC : describes an acetylating enzyme

The number following relates to the carbon atom of the molecule carrying the side chain which is attacked by the enzyme.
In some cases there is more than one enzyme of the same class which can react at a single site; these are further designated -1, -2, *etc.*

High-level aminoglycoside resistance is relatively easily detected in the laboratory. This involves the supplementation of an agar or broth medium with a particular aminoglycoside to a final concentration of 500 and/or 2000μg/ml and the inoculation of the enterococci onto the agar surface or broth as described by Moellering *et al* (68). The inoculum of the organisms adjusted to match a 0.5 McFarland Standard (approximately 10^8 CFU/ml) and further diluted to achieve a final inoculum of approximately 10^4-10^5 CFU/ml. Inoculated plates incubated at 35-37°C and observed for the presence or absence of growth after 24 and 48 hours. Any growth on the agar surface or any turbidity in the broth method is interpreted as resistance.

In 1992, Leclercq *et al.* (51), described an agar disc diffusion method involving the surface inoculation of a susceptibility test agar medium such as Mueller-Hinton agar with enterococci onto which high content aminoglycoside discs are placed.

The high content disc concentrations are as follows : gentamicin 500μg, streptomycin 1000μg, kanamycin 1000μg. It has been established that kanamycin more accurately predicts high-level amikacin resistance than does amikacin (14,62).
A suspension of the test organism is prepared in Mueller-Hinton broth to a density of approximately 10^6-10^7 CFU/ml. The surface of the medium is inoculated so that an even distribution of the organism is obtained.

This has to be done within 15 minutes of its preparation to avoid changes in the inoculum density. High-level aminoglycoside discs are applied to the dried inoculated plates using a sterile needle or forceps, ensuring even contact of the discs to the medium. Plates are incubated at 37°C for 18 hours and the diameter of any zones of inhibition produced around the discs is measured. The zone size break points for the detection of high-level aminoglycoside resistance are shown in table X.

Table X **Interpretation of high-level aminoglycoside resistance using high-level discs**

		INHIBITION ZONE DIAMETER(mm)	
AMINOGLYCOSIDE	DISC CONTENT(μg)	High-level RESISTANCE	Low-level RESISTANCE
Gentamicin	500	< 11	≥ 17
Kanamycin	1000	< 10	≥ 14
Streptomycin	1000	< 12	≥ 14

Lower concentration of aminoglycoside discs have also been used : 120μg of gentamicin, 300μg of streptomycin and 300μg of kanamycin. Zone diameters of ≥ 10mm indicates the presence of synergy.

In 1992, it had been established that the Etest may be used to predict high-level resistance to aminoglycoside among enterococci (101). Gentamicin, and streptomycin Etest MIC range from 0.06 to 1024μg/ml and 0.125 to 2048μg/ml . A value of greater than 1000μg/ml for each of these drugs indicates high-level resistance.

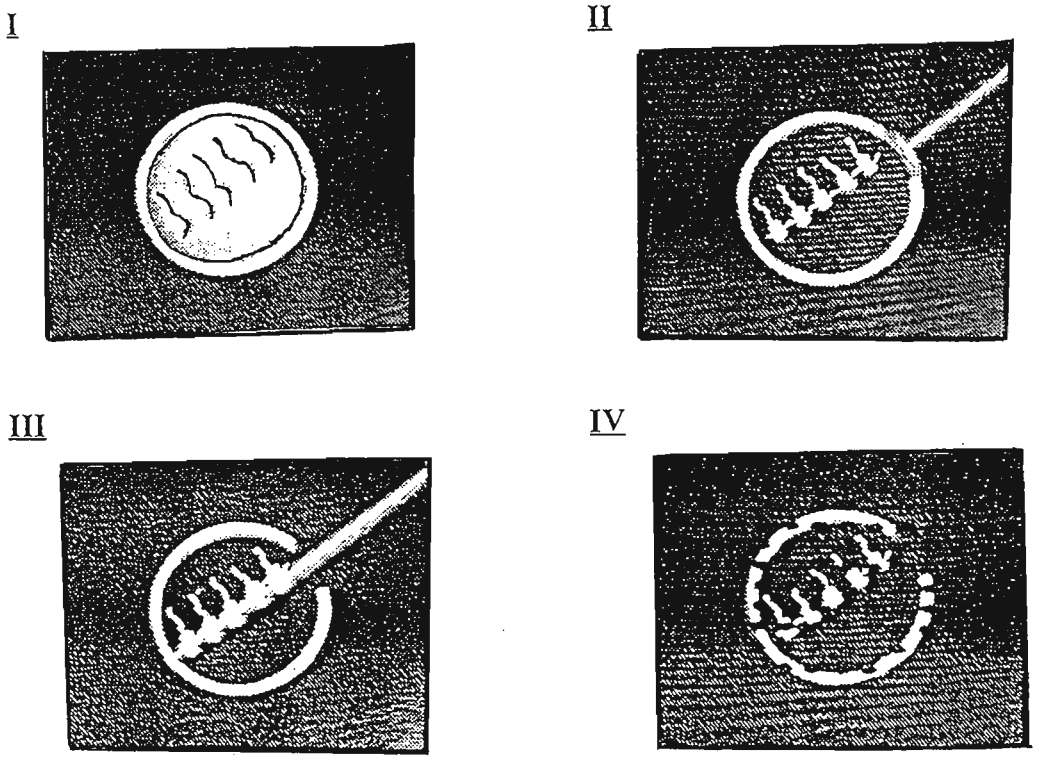


Fig. 1 Diagrams showing synergistic action with an aminoglycoside and penicillin (65)

I - Enterococcal cell walls are impermeable to aminoglycosides

II - The disruption of bacterial cell walls by a penicillin

III - Allows the aminoglycoside to penetrate, bind to ribosome

IV- And kill the *Enterococcus*

2.10 THE STUDY OF SYNERGISM

Tests for synergism include killing curves, disc diffusion synergism tests and checkerboard titrations.

Killing curves have been widely applied to the evaluation and comparison of combinations of drugs. Usually, only one concentration of each antibiotic is tested, compatible with blood levels during therapy. This method provides a dynamic picture of antimicrobial action and interaction over time. At periodic intervals, ie. 0, 4 and 24 hours of incubation, colony counts are performed and plotted on semilog graph paper with the survivor colony count on the ordinate (y-axis) in logarithmic scale and the time interval on the abscissa (x-axis) in the arithmetic scale. Performed with various organisms against one drug or with several drugs against the same organism, such rate determination then allows one to compare the rates of decline in the in vitro survivor count and thus to find the most rapidly bactericidal agent.

In synergy studies the effect of combinations of drugs is measured. Synergism is defined as a $\geq$ 100 fold increase in killing at 24 hours with the combination, in comparison with the most active single drug.

Antagonism is defined as $\geq$ 100 fold decrease in killing with the combination of antibiotics, in comparison with the most active single antibiotic. Additive effect or indifference on the other hand is described as no increase in killing with the drug combination, in comparison with the most active single drug.

The disc diffusion method can also be used to assess interactions of antimicrobials. This technique uses a standardized inoculum of approximately 10^8CFU/ml on a suitable agar plate.

To assess possible interactions between two drugs discs containing the different drugs are placed on the plate that has been inoculated with the test organism.
The distance by which the discs are separated is generally equal to or slightly less than the sum of the radii of their zones of inhibition when examined alone. After overnight incubations, 16-18 hours at 35 to 37°C the plates are examined.

The pattern observed with additive or indifferent combinations is that of two independent circles. With synergistic combinations, enhancement or bridging is observed at or near the junction of the two zones of inhibition. With the antagonistic combinations, truncation is observed near the junction of the two zones of inhibition as seen in fig. 2.

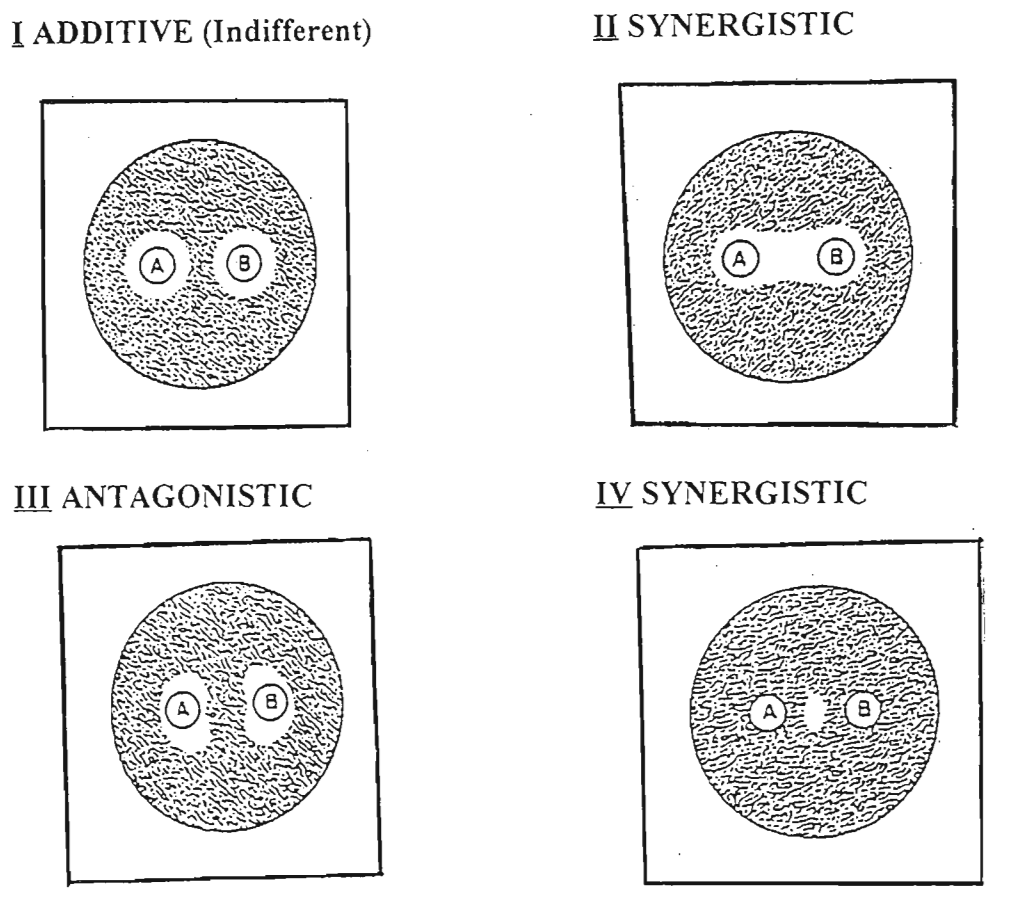


Fig. 2 Assessment of antimicrobial combinations with the disc diffusion technique(55)

A & B	:	Discs containing antibiotics placed on Mueller-Hinton agar plates inoculated with a bacterial isolate
I	:	Indicates an additive or autonomous result.
II & IV	:	Indicates synergism
III	:	Indicates antagonism

(**Shading** : Indicates bacterial growth.

Clear areas : Indicates zones of growth inhibition)

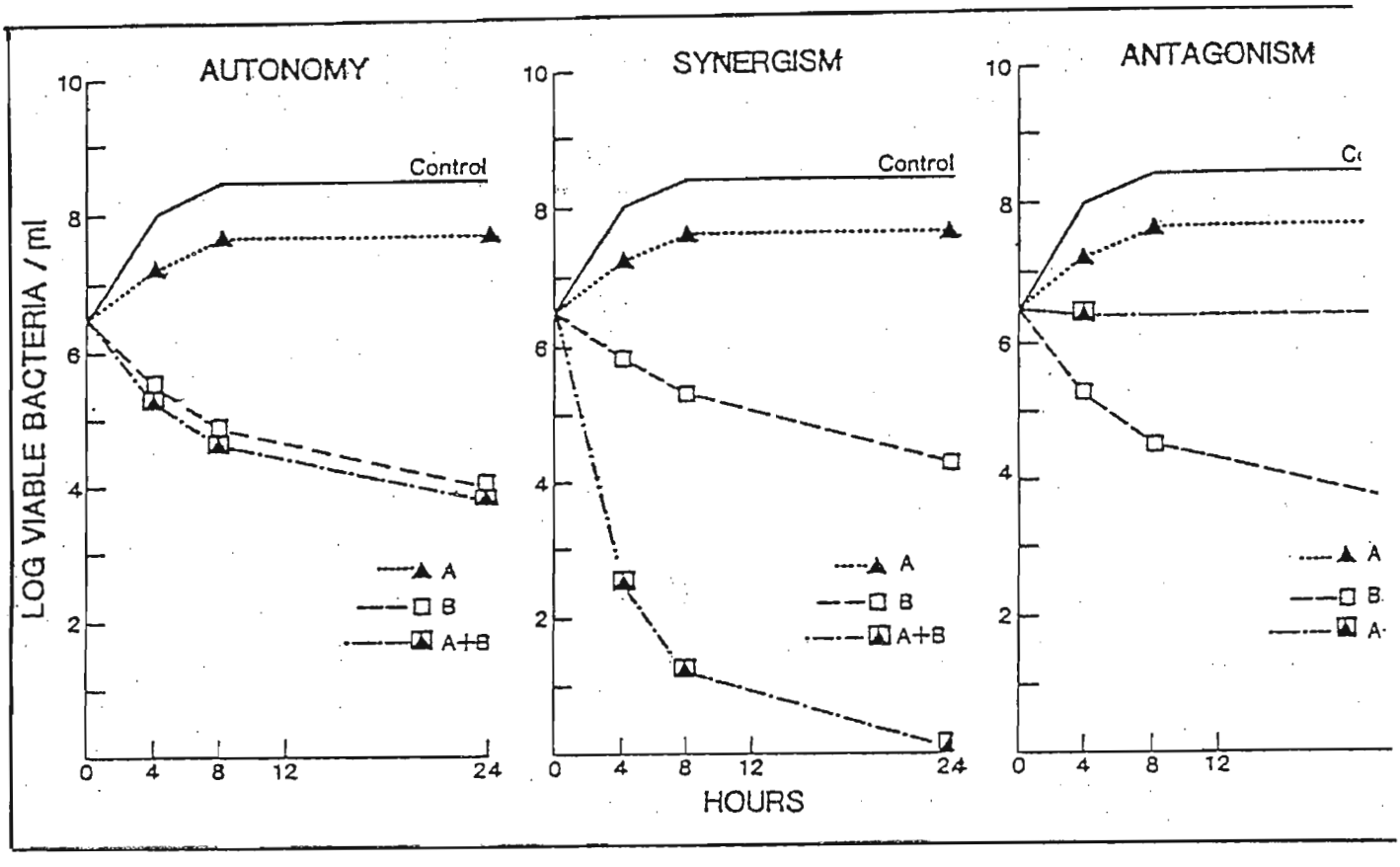


Fig. 3 Time-kill curve showing bactericidal effect and antimicrobial synergy (55)

C : Control (No Antibiotic)

A : Antibiotic A

B : Antibiotic B

A + B : Both Antibiotics

PANEL I : Additive, Indifferent or Autonomous effect

PANEL II : Synergistic effect

PANEL III : Antagonism is illustrated

The combined antibacterial action of two drugs can be precisely determined by performing a checkerboard titration. In this method a suitable range of dilution of both drugs is added to broth (or agar) so that every concentration of each drug is present alone and in every possible combination with the other. The range of concentrations should include that which is inhibitory for each drug acting alone. The test is inoculated with a standard inoculum of approximately 10^6 CFU/ml.

The test is incubated at 35 - 37°C for 16-24 hours and if the control tube shows good growth, the presence or absence of growth is recorded.

Synergy is only considered significant if the concentration inhibiting growth in the combination is at least 4-fold lower than that of one drug acting alone.
Cidal synergy occurs only when both drugs tested singly are bactericidal. Such synergy is for eg. observed with the beta-lactam aminoglycoside combinations. If in such combinations the beta-lactam is present in a bactericidal concentration, the concentration of aminoglycoside needed to give cidal effect with the combination can be well below the MIC.

Table XI **Combined action of penicillin and streptomycin against *E. faecalis* illustrating synergy**

	PENICILLIN(μg/ml)								
STREPTOMYCIN (μg/ml)	16	8	4	2	1	0.5	0.25	0.12	N
256	-	-	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	G
16	-	-	-	-	-	-	-	-	G
8	-	-	-	-	-	G	G	G	G
4	-	-	-	-	-	G	G	G	G
2	-	-	-	-	G	G	G	G	G
NIL	G	G	G	G	-	-	-	-	-

G = Growth in tube/ agar.

- = No growth

The bottom horizontal row contains only streptomycin.

The right-hand vertical row contains only penicillin.

The remainder of the square will have every possible combination of both drugs, indicated in the top horizontal and left hand vertical row.

The tube in the top right-hand corner is the drug-free control marked N.

Cidal antagonism is seen when the cidal action of one antibiotic (eg. penicillin) is eliminated by the presence of the second drug (eg. tetracycline).

Although resistance to antimicrobials may be the result of drug-inactivating enzymes (9,22), or an insensitive target site [eg. a ribosome resistance to aminoglycoside (120,119)], it may also be due to a permeability barrier. In these situations, a drug could become active, if another agent altered the permeability of the bacterial cell in order to permit its entry.

It has been postulated that beta-lactams may enhance the entry of aminoglycosides in this manner in a number of bacterial species (55).

Work on enterococci (*E.faecalis*) has shown that the uptake of ^{14}C- labelled streptomycin is significantly increased in the presence of penicillin (69). Furthermore, this effect is not specific for penicillin only, it is also seen with other agents acting on the cell wall such as ampicillin and vancomycin, all of which presumably act similarly to permit increased entry of aminoglycoside (69).

In *E.faecalis*, it has been stated that sequential exposure to ampicillin, followed by an aminoglycoside (amikacin) results in a greater bactericidal effect than when the drugs are applied in the reverse order providing support for the primary effect on the cell wall in the initiation of bactericidal synergism (56).

In the case of enterococci, the first observation of penicillin, streptomycin synergism was probably made in 1947 (37).

Subsequently, many other investigators have demonstrated a synergistic effect of penicillin with various aminoglycosides against enterococci (38,47,68,70,71).

It gradually became apparent that not all enterococci were synergistically killed by these combinations. The explanation for this discrepant behaviour among clinical isolates of enterococci was not clear until 1970, when two groups of investigators pointed out that enterococcal isolates with high-level streptomycin resistance (MIC $\geq$ 2000μg/ml) were resistant to the penicillin, streptomycin combinations (68,107).
Subsequent work showed that penicillin enhances the uptake of radiolabelled [^{14}C] streptomycin in strains with and without high-level streptomycin resistance (69).

Therefore, it seemed unlikely that the resistance to synergism observed in strains with high-level streptomycin resistance was due to maintaining a permeability barrier.
However streptomycin failed to inhibit the incorporation of radiolabelled phenylalanine into trichloroacetic acid - precipitable material and also failed to cause misreading (120,119). This affinity of the ribosomal target site suggests that the resistance is the result of decreased aminoglycoside concentration.

Additional studies showed that strains with high-level streptomycin and kanamycin resistance contained a 45 megadalton (MDa) plasmid that was transferable by conjugation. Transfer of the plasmid resulted in high-level resistance and resistance to synergy in the recipient strain (49).

Thus, it seems clear that at least in some clinical isolates of enterococci a conjugative plasmid is responsible for the observed resistance to penicillin plus aminoglycoside synergism. This made an altered ribosome as the mechanism of resistance unlikely.
Subsequently, aminoglycoside - inactivating enzymes were found, which explain the observed resistance to synergism. In resistant strains phosphotransferase inactivates amikacin and kanamycin by phosphorylating the 3'-hydroxyl group, and an adenyltransferase inactivates streptomycin (48).

Fig. 4 shows the different mechanisms of resistance for beta-lactam aminoglycoside synergy. The mechanism of high-level aminoglycoside resistance in clinical isolates of *E.faecalis* studied to date is usually plasmid-mediated synthesis of aminoglycoside - inactivating enzymes, but ribosomal resistance and other mechanisms play a role in some strains, especially those with high-level streptomycin resistance alone. In vivo resistance to synergism is also observed in combinations that are synergistic in vitro.

Mechanisms of such resistance are multiple. The most important factors appear to be protein binding of one or both drugs and the relationship of the MIC of the β-lactam antibiotic to achievable serum and tissue levels (55). The recent demonstration of transferable beta-lactamase production raises the possibility that this trait could spread widely among enterococci and this would then obviate the utility of penicillin plus aminoglycoside synergism.

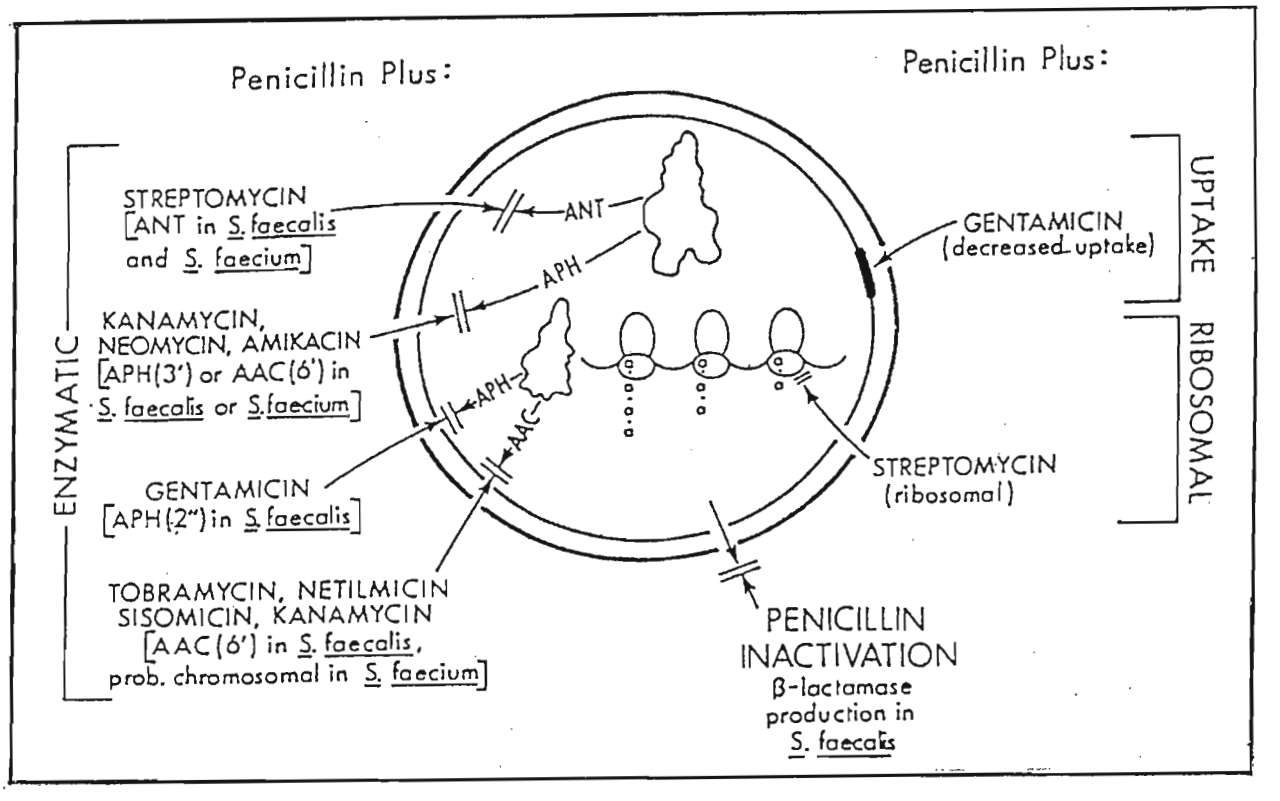


Fig. 4 Mechanisms of resistance to penicillin + aminoglycoside synergism against enterococci (55)

Of these resistance mechanisms, the most prevalent are enzymatic inactivation. Ribosomal resistance to streptomycin also occurs. Enzymatic resistance is also plasmid-mediated. although the AAC(6') in *E.faecium* appears to be chromosomal (ANT = adenyltransferase, APH(3') = 3'phosphotransferase, AAC(6')= 6'acetyltransferase).

2.11 TYPING OF ENTEROCOCCI USING PULSED-FIELD GEL ELECTROPHORESIS

The principle theoretical advantage of these new electrophoretic approaches over biotyping of isolates is that since these methods samples the chromosome, it is independent of phenotypic changes (35). Furthermore, unlike plasmid analysis, it is the stable part of the genome which is sampled. The approach is useful in practice because it is highly discriminatory, stable and may be used to type different types of bacteria (35).

The epidemiological analyses of nosocomial pathogens is a problem of long-standing interest. Epidemiological assessment of enterococci has been limited by the lack of a convenient, reproducible and discriminatory method for comparing isolates. Recently, different methods for genotyping have been used for the comparison of such isolates (ie. molecular epidemiology). For example, restriction fragment chromosomal patterns (RFCP - analysed by agarose gel electrophoresis) have been utilized in the epidemiologic analysis of a variety of nosocomial pathogens (32). However, restriction enzymes used generate a large number of DNA fragments thus making it difficult to accurately compare different isolates (32).

Restriction endonucelases have been identified that rarely cut the bacterial chromosome eg. < 20 restriction sites (32). However, most of the resulting DNA fragments are too large to be resolved by conventional gel electrophoresis.
Until recently, large fragments were not possible to separate. A number of "alternative" electrophoretic methods such as contour clamped homogeneous electric (CHEF), pulsed-field gel electrophoresis (PFGE) and field-inversion gel electrophoresis. (FIGE) have been shown to be capable of separating the mega-base sized chromosomal DNA fragments produced by rare cutting enzymes (32).

These methods provide the advantage that fragments of DNA over a much wider size range (ie. 10 Kb- 1.5Mb) can be resolved on a single gel, thus generating interpretable patterns. These techniques yield restriction endonucleases digestion patterns which consist of relatively few, generally well separated fragment bands and which are much less ambiguous than the patterns generated by conventional electrophoresis (1).

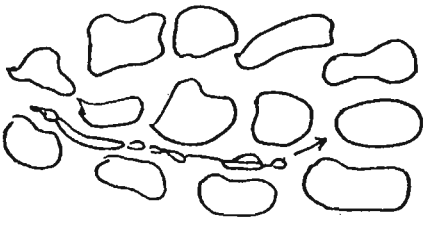
Conventional agarose gel electrophoresis utilizes unidirectional electric fields to separate DNA molecules. Their movement is forced through an agarose matrix as they are attracted toward the positive electrophoretic charge. Under these conditions DNA molecules < 40Kb in size migrate through the agarose gel depending on their size.
DNA molecules with a molecular weight of > 40Kb display aberrant migration through the agarose matrix which is possibly due to a longitudinal orientation of the molecules. This causes them to migrate through the gel in a manner that is unrelated to size (32).

In standard gel electrophoresis the electric field is applied constantly in one direction as shown in figs. 5 and 6. Alternative electrophoretic methods such as CHEF, PFGE, and FIGE are able to separate large DNA fragments by continually changing the direction of the electric field which is applied alternatively in two directions (ie. the location of the positive charge). The time spent in each direction is called the pulse time. If the pulse time is too long the DNA molecule will effectively reorient itself rapidly to the new field and move by ordinary electrophoresis; no size fractionation will occur. At intermediate pulse time (ie. about 30s) molecules spend most of their time reorienting and as a result effective fractionation occurs. If the pulse time is too short the molecule will not have time to reorient at all, thus no size fractionation will occur.

By varying both the direction and the duration of the electric field, these alternative electrophoretic methods force the DNA molecules to continually reorient in the agarose matrix.

The overall result is that mega-base-sized DNA fragments move in the desired direction and are electrophoretically separated in the agarose gel (32,107).

NORMAL GEL (A)



DNA

PULSED-FIELD GEL (B)

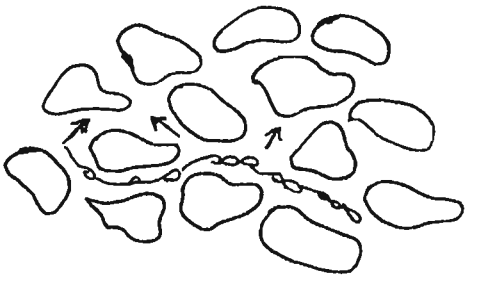
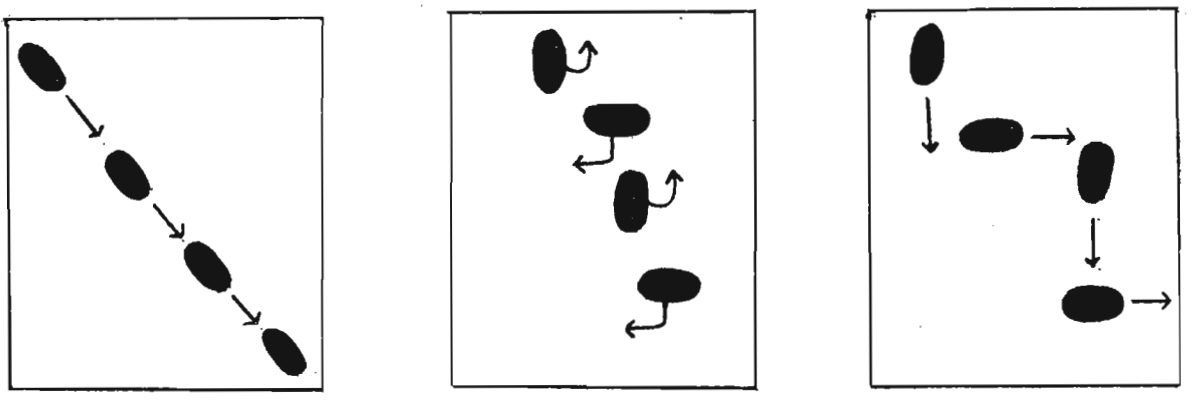


Fig. 5 Separation of DNA by ordinary and pulsed-field gel electrophoresis

(A) DNA travelling in a matix during standard electrophoresis is oriented parallel to the field and is sieved through the matrix.

(B) DNA travelling in the alternating fields used during pulsed -field gel electrophoresis is forced to spend most of its time reorienting itself perpendicular to its long axis.



DNA molecules completely reorient themselves along their long axis and no separation occurs.

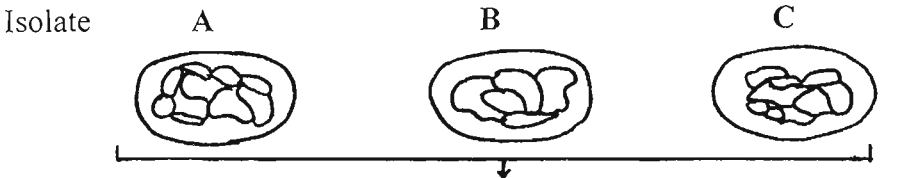
Molecules spend most of their time trying to reorient.

Molecules only seen along the average electric field.

Fig. 6 Separation of DNA molecules by pulsed-field gel electrophoresis (PFGE) depends on the pulse time (106)

From the three alternative electrophoretic methods described, the most commonly used technique for the separation of chromosomal DNA in enterococci is pulsed-field gel electrophoresis (79,1,80,35).

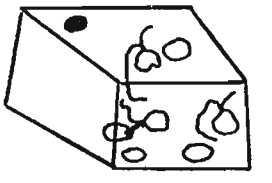
Pulsed-field gel electrophoresis (PFGE) involves the embedding of the organisms in agarose, lysing the organisms *in situ* and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently such as *Sma* I.. *Sma* I was chosen because enterococci have a low guanine plus cytosine (G+C) content and thus have relatively few *Sma* I recognition sites (79). Slices of agarose containing the chromosomal DNA fragments are inserted into the walls of an agarose gel. The restriction fragments are resolved into a pattern of discrete bands in the gel by an apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of the isolates are then compared with one another to determine how similar or different they are.



Cells grown overnight.

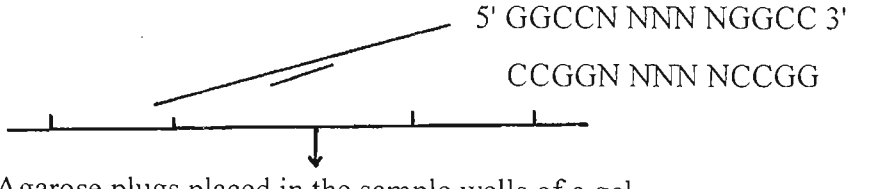
Each culture embedded in an agarose plug to prevent shearing of DNA.

Cells lysed in situ and treated to remove inhibitors.



DNA cut with an infrequently cutting restriction enzyme.

DNA recognition site : (example)



Agarose plugs placed in the sample wells of a gel.

Pulsed-field electrophoresis (alternating electrical pulses and direction) used to separate the large DNA fragments

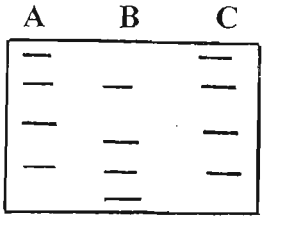


Fig. 7 Steps involved in performing pulsed-field gel electrophoresis [PFGE] (64)

The first report of typing of enterococci by a chromosomal DNA based typing method ie. by pulsed-field gel electrophoresis was by Murray *et al.* in 1990 (79).

Their study compared chromosomal restriction endonuclease digestion patterns of 27 isolates of *E.faecalis* from 3 different locations ie. Chile, Texas and Thailand by using pulsed-field gel electrophoresis. Most isolates tested gave unique restriction patterns. However, one pattern was observed for 5 of 14 isolates from Thailand and patterns were also shared by more than one isolate in Chile and Texas.

In a study by Abraham *et* al., pulsed-field gel electrophoresis was used to compare 34 isolates of *E.faecium* from 6 different geographic locations. Their results revealed quite heterogeneous patterns. 27 of the 34 isolates were differentiated from each other, as defined by one or more mismatched fragment bands. Five patterns were shared by 2 or more isolates, and each set of isolates with a shared pattern originated in the same medical centre, suggesting a common epidemiologic background (1).

Pulsed-field gel electrophoresis was used to compare 14 beta-lactamase producing *E.faecalis* isolated from hospitalized patients in 7 states and 3 continents, in a study by Murray *et al.*(80). The restriction endonuclease digestion patterns of isolates from Connecticut, Massachusetts, Lebanon and Argentina were all markedly different, indicating these were different strains. However, isolates from Delaware, Texas, Pennsylvania, Florida and Virginia were similar, indicating that these isolates were derivatives of a single strain. The spread of beta-lactamase producing enterococci within the hospital setting was also demonstrated.

In comparing alternative electrophoretic methods, it should be noted that chromosomal restriction fragments do tend to appear somewhat more diffuse with field-inversion gel electrophoresis (FIGE) than with the contour clamped homogeneous electric field (CHEF) or pulsed-field gel electrophoresis (PFGE).
In addition CHEF and PFGE is capable of resolving larger DNA molecules than is FIGE. FIGE is potentially faster than the other 2 methods and is less expensive since it utilizes a conventional agarose gel electrophoresis apparatus (32).

The major goal of strain typing studies is to provide laboratory evidence that epidemiologically related isolates collected during an outbreak of disease are also genetically related and thus represent the same strain. This information is helpful for understanding and controlling the spread of disease in both hospitals and communities.

Pulsed-field gel electrophoresis demonstrates great promise as an epidemiological and investigational tool since it is not difficult to perform and is reproducible. The utilization of this technique, should add a new dimension to our knowledge of the epidemiology of antibiotic resistance in enterococci.

CHAPTER 3

MATERIALS AND METHODS

107 enterococci, isolated from blood cultures in 1994 at King Edward VIII Hospital, Durban were identified by the API 20 Strep System (Bio Mèrieux) which was compared to conventional biochemical tests. All the isolates were classified according to Lancefield grouping and tested for the production of beta-lactamase.

Antimicrobial susceptibility testing was performed, comparing two disc diffusion methods ie. modified "Stokes" and the Kirby-Bauer method.

Breakpoint susceptibility testing and minimum inhibitory concentration (MIC) were also performed on all of the 107 enterococcal isolates.

High-level aminoglycoside resistance was measured for gentamicin and streptomycin on all the isolates.

Synergism studies were performed using time-kill curves on a selected number of isolates.

Electrophoretic analysis of chromosomal DNA patterns by pulse-field gel electrophoresis (PFGE) was performed on a selected number of *E.faecalis* and *E.faecium* isolates with high-level aminoglycoside resistance.

3.1 IDENTIFICATION OF ENTEROCOCCI

3.1.1 API identification

This is a standardised method consisting of a strip of 20 microtubes containing dehydrated substrates that demonstrates enzymatic activity or the fermentation of sugars.

The metabolic end-products during the incubation period are either revealed through spontaneous coloured reactions or by the addition of reagents. The fermentation tests were inoculated with an enriched medium which reconstitutes the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator. These reactions are read by referring to the interpretation table and the identification is obtained using the identification table API 20 Strep analytical profile index or the API laboratory computer program.

3.1.2 Identification by conventional biochemical tests

3.1.2.1 Salt tolerance test

Growth in broth and an agar plate containing 6.5% NaCl was determined in brain-heart infusion broth base and nutrient agar base respectively. Turbidity in broth and growth on the agar surface indicated that the isolates were salt tolerant. A negative reaction is indicated by a lack of turbidity and no growth on the agar surface.

3.1.2.2 Bile aesculin test

The bile-aesculin test is based on the ability of certain bacteria notably the enterococci to hydrolyse aesculin (1%) in the presence of 40% bile. Such bacteria produce glucose and aglycone aesculetin. Aesculin reacts with an iron salt to form a dark brown or black complex resulting in a diffuse blackening of the bile-aesculin medium which contains ferric citrate as the source of ferric irons.

3.1.2.3 Arginine deamination

Degradation of arginine results in an increase in pH by the development of a purple colour which indicates a positive reaction. A yellow colour indicates a negative result which is due to acid accumulation from metabolism of glucose only.

3.1.2.4 Pyruvate utilization

Pyruvate is oxidatively decarboxylated by an enzyme system termed the pyruvate dehydrogenase complex, producing acetyl-coenzyme A (acetyl CoA). A positive reaction was recorded when the indicator changed from green (which is a negative result) to yellow.

3.1.2.5 Tellurite tolerance

Tolerance to tellurite was determined on an agar medium containing 0.04% potassium tellurite. Tellurite is reduced to tellurite salts, forming greyish or black colonies after 24-48 hours of incubation, indicating a positive reaction. A negative reaction is indicated by no growth on the agar surface, or growth without blackening of colonies.

3.1.2.6 Hippurate hydrolysis

Hydrolysis of 1% sodium hippurate tested in broth, results in the production of glycine and sodium benzoate. Glycine is deaminated by the oxidising agent ninhydrin, which becomes reduced during the process and turns purple, indicating a positive reaction. No colour change (colourless) is considered a negative reaction.

3.1.2.7 Starch hydrolysis

Hydrolysis of starch was determined by inoculating a brain- heart infusion agar plate containing 2% soluble starch and flooding the surface of the inoculated agar with Gram's iodine.

Complete clearing indicates hydrolysis of starch which is indicative of a positive reaction. Non hydrolysed starch turns a dark blue colour when complexed with iodine, indicating a negative reaction.

3.1.2.8 Voges-Proskauer (VP) test (COBLENTZ)

The VP test detects the production of acetylmethylcarbinol (acetoin), an intermediate in the formation of butylene glycol. In the presence of oxygen and 40% potassium hydroxide (KOH) acetoin is oxidised to diacetyl, and α-naphthol which serves as a catalyst to produce a red colour complex, which is a positive reaction. A negative reaction is indicated by no colour development (colourless).

3.1.2.9 Carbohydrate fermentation tests (1%)

The glycolytic pathway, gives rise to pyruvic acid which uses the enzyme lactate dehydrogenase to produce lactic acid. A positive reaction appears yellow. The medium remains purple in the absence of carbohydrate utilization indicating a negative reaction.

The following carbohydrates were tested in broth :

(i) mannitol
(ii) sorbose
(iii) sorbitol
(iv) inulin
(v) arabinose
(vi) melibiose
(vii) sucrose

(viii) trehalose
(ix) lactose
(x) glycerol
(xi) salicin
(xii) maltose
(xiii) raffinose
(xiv) glucose

3.1.2.10 Test for growth at 10°C and 45°C

Growth at 10°C and 45°C was determined in brain-heart infusion broth base medium. Growth at these temperatures indicated the organisms ability to survive these adverse conditions.

3.1.2.11 Survival at 60°C for 30 minutes

Ability of the organism to survive in a water bath at a temperature of 60°C for a period of 30 minutes. The presence of bacterial growth indicates the organisms ability to survive at this extreme temperature for that period.

3.1.2.12 Type of haemolysis

The haemolytic reaction (alpha, beta or gamma) was determined on an agar plate media containing 5% horse blood.

Alpha haemolysis : A zone of partial haemolysis or green discoloration occurs in the agar immediately surrounding the colony. The red cells membrane remains intact.

Beta-haemolysis : A distinct zone of total haemolysis of red cells occurs, creating a clear, colourless ring surrounding and/or under the colony. The red cells membrane is destroyed.

No haemolysis (γ) : No lysis of red blood cells in the medium occurs, and there is no change in colour.

3.1.2.13 Motility

Motility which is brought about by rotation of the bacterial flagella was determined in modified Difco motility medium. Motile strains grow outward to the edge of the tube and downward towards the bottom of the tube (a diffuse zone of growth flaring out of the line of inoculation), indicating a motile strain. Non motile strains grow on the line of inoculum only.

3.1.2.14 Detection of L-pyrrolidonylpeptidase (PYR)

PYR test is a rapid colourimetric test for use in the differentiation of enterococci from Lancefield group D streptococci. The hydrolysis of L-pyrrolidony-β-naphthylamide, liberates free L-pyrrolydone carboxylic acid and β-naphthylamide. Discs impregnated with PYR serve as a substrate for the detection of pyrrolidonylpeptidase. Following inoculation, enzymatic hydrolysis of the substrate occurs. The resulting beta-naphylamine forms a red colour complex on addition of a colourimetric developer, indicating a positive reaction. No colour change (colourless) is indicative of a negative reaction.

3.1.2.15 Determination of Lancefield group antigen

The majority of *Streptococcus* species possess group specific antigens which are usually carbohydrate structural components of the cell wall. Lancefield showed that these antigens can be extracted in soluble form and identified by precipitation reactions with homologous antisera. The Streptex system employs a simple enzyme extraction procedure. Antigen in the resulting extract is identified by using polystyrene latex particles coated with group-specific antibodies. The latex particles agglutinate strongly in the presence of homologous antigen, and remain in a smooth suspension when the homologous antigen is absent.

[NB : Methods, positive reaction, negative reaction, controls :
See APPENDIX - Section A]

3.2 TEST FOR BETA-LACTAMASE PRODUCTION

3.2.1 Chromogenic Cephalosporin Method

The rapid chromogenic cephalosporin method can be used with nitrocefin (Glaxo Ltd). The inactivation of beta-lactam antibiotics is by enzymes termed beta-lactamases which are extra-cellular enzymes produced by many strains of bacteria that specifically hydrolyse the amide bond in the beta-lactam ring of penicillin analogues. The molecule undergoes a distinct colour change from yellow to red indicating a positive reaction. A negative reaction is shown by no colour change (remains yellow).

[NB: Method, positive reaction, negative reaction, controls :
See APPENDIX - Section B]

3.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING

3.3.1 Disc diffusion testing

A standardized inoculum of the organism (10^8 CFU/ml) was swabbed onto the surface of a Mueller-Hinton agar plate. Filter paper discs impregnated with antimicrobial agents were placed onto the agar. After overnight incubation, the diameter of the zone of inhibition around each disc was measured. The size of the zone is inversely proportional to the minimum inhibitory concentration of the organism. By referring to the NCCLS recommendations for the Kirby-Bauer method, or to the "Stokes" criteria for the "Stokes" method, a qualitative report of susceptible, moderately susceptible /intermediate or resistant can be obtained.

Two disc diffusion methods were compared ie. Modified "Stokes" technique and the Kirby-Bauer in accordance with standard methods. (91,84).

Table XII **Concentration of antibiotics used for the disc diffusion methods**

	MODIFIED "STOKES"	KIRBY-BAUER
ANTIBIOTICS TESTED	CONCENTRATION	CONCENTRATION
Penicillin	1μg	10 units
Ampicillin	10μg	10μg
Erythromycin	5μg and 10 μg	5μg
Tetracycline	10μg	30μg
Chloramphenicol	10μg	30μg
Clindamycin	2μg	2μg
Fusidic acid	10μg	10μg

	MODIFIED "STOKES"	KIRBY-BAUER
ANTIBIOTICS TESTED	CONCENTRATION	CONCENTRATION
Cotrimoxazole	25μg	25μg
Rifampicin	5μg	5μg
Ciprofloxacin	5μg	5μg
Cephalothin	30μg	30μg
Cefamandole	30μg	30μg
Cefuroxime	30μg	30μg
Cefoxitin	30μg	30μg
Cefotaxime	30μg	30μg
Gentamicin	10μg	10μg
Tobramycin	10μg	10μg
Netilmicin	10μg	10μg
Amikacin	10μg	30μg
Streptomycin	30μg	30μg
Vancomycin	30μg	30μg
Teicoplanin	30μg	30μg

Table XIII **Comparison of disc diffusion susceptibility testing methods**

	MODIFIED "STOKES"	KIRBY-BAUER
MEDIA	Mueller-Hinton agar supplemented with 5% lysed horse blood	Mueller-Hinton agar supplemented with 5% lysed horse blood
INOCULUM	Semi-confluent growth	Inoculum standardized to match the turbidity of a 0.5 McFarland standard (approx. 10^8CFU/ml)
INOCULATION OF MEDIA	Using a rotary plate method, control organism *S.aureus* NCTC 6571, was inoculated on half of the agar plate and the test organism on the other half. A central band of 3-4mm was left uninoculated between the test and control organism to which the antibiotic discs were applied.	The medium was inoculated evenly over the entire surface of the agar plate in three directions to ensure even distribution of the inoculum. Drug impregnated discs were applied to the agar surface.

	MODIFIED "STOKES"	KIRBY-BAUER
INCUBATION	35-37°C for 16-18 hours in an atmosphere of 5-10% CO_2.	35-37°C for 16-18 hours in an atmosphere of 5-10% CO_2.
CONTROL ORGANISMS	*S.aureus* NCTC 6571.	*S.aureus* ATCC 25923. The control results were compared to the NCCLS control limit table for monitoring antimicrobial disc susceptibility zone diameter (mm) limits.

[NB: Detailed methods for disc diffusion susceptibility testing :

See APPENDIX - Section C]

3.3.2 Minimum inhibitory concentration (MIC)

3.3.2.1 Agar dilution technique

MICs were measured by means of the agar dilution method in accordance with NCCLS Document M2-A4 (83). The agar dilution procedure is a standard reference that quantitatively measures the in-vitro activity of an antimicrobial agent against the bacterial isolate. Serial two-fold dilutions of each antibiotic was made in Mueller-Hinton agar supplemented with 5% lysed horse blood. In addition, for the aminoglycosides and cephalosporins Mueller-Hinton agar only was used. Each of several concentrations of an antimicrobial agent was incorporated into a tube of molten agar, which was then mixed, poured into a petri dish and allowed to solidify. Final plate concentrations ranged from 256 to 0.03μg/ml. The replicator device of Steers, Foltz and Graves provided standardized inoculation of approximately 10^4CFUs of the test and control strains. Inoculated plates were read after overnight incubation at 35-37°C. The MIC was determined by observing the lowest concentration of antibiotic that inhibited visible growth of the bacterium. Reference strain *S.aureus* ATCC 29213 was used as the control organism.

3.3.2.2. Broth microdilution method

The broth microdilution method was used to quantitatively measure the in-vitro activity of an antimicrobial agent against a bacterial isolate. A sterile plastic microdilution tray containing various concentrations of antimicrobial agents were inoculated with a standardized suspension of the test organism. Following overnight incubation at 35-37°C, the MIC was determined by observing the lowest concentration of antibiotic that inhibited visible growth of the bacterium.

[NB: Detailed methods for agar dilution & broth microdilution MIC :
See APPENDIX - Section D]

3.3.3 Minimum bactericidal concentration (MBC)

Following performance of the microdilution susceptibility test, wells containing concentrations of antimicrobial agent equal to and greater than the MIC were subcultured to determine whether the initial inoculum was inhibited from multiplying (bacteriostatic action) or was killed (bactericidal action). The MBC test endpoint was determined as the lowest concentration of antimicrobial agent that killed ≥99.9% of the test inoculum, within a defined period of time. The reduction is usually expressed as the proportion of the inoculum (number of viable colony forming units introduced) that is rendered incapable of reproduction on subculture.

[Detailed method for minimum bactericidal concentration (MBC) :
See APPENDIX : Section E]

3.3.4 Breakpoint susceptibility testing

Breakpoint susceptibility testing refers to a method by which antimicrobial agents are tested only at the specific concentrations necessary for differentiating between the interpretive categories of susceptible, intermediate and resistant; rather than in the full range of doubling-dilution concentrations used to determine MICs. If two appropriate drug concentrations are selected, any one of the interpretive categories may be determined. Growth at both concentrations indicates resistance, growth only at the lower concentration signifies an intermediate result, and no growth at either concentration is interpreted as susceptible. In the breakpoint method of susceptibility testing, fixed concentrations of antimicrobial agents are incorporated into agar. The concentrations are chosen according to the cut-off points of bacterial susceptibility or resistance. The following concentrations for antibiotics to be tested were chosen according to NCCLS Document M_7-A_2 (83).

Penicillin : 16μg/ml and 256μg/ml
Ampicillin : 8μg/ml and 256μg/ml
Imipenem : 4μg/ml
Piperacillin : 4μg/ml
Ciprofloxacin : 1μg/ml and 8μg/ml
Vancomycin : 4μg/ml
Teicoplanin : 4μg/ml

Cephalothin : 16μg/ml
Cefuroxime : 16μg/ml
Cefamandole : 16μg/ml
Cefotaxime : 32μg/ml
Gentamicin : 8μg/ml
Amikacin : 32μg/ml

Media used was Mueller-Hinton agar supplemented with 5% lysed horse blood. In addition, Mueller-Hinton agar only was used for the aminoglycosides and cephalosporins tested. Control organisms *S.aureus* ATCC 29213, *E.coli* ATCC 25922 and test organisms were spot inoculated with a standardized inoculum of approximately 10^4CFU on the surface of the agar plate using a replicating apparatus.

Plates were incubated at 35-37°C in an atmosphere of 5-10% CO_2 for 18-24 hours. Results were recorded as susceptible, moderately susceptible / intermediate or resistant depending on the presence or absence of growth for each inoculum spot.

[NB : Detailed method for breakpoint susceptibility testing :
See APPENDIX - Section F]

3.3.5 Detection of high-level aminoglycoside resistance

3.3.5.1 Agar screen technique

High-level aminoglycoside resistance was detected by assessing growth at high concentrations of gentamicin (500 and 2000μg/ml) and streptomycin (2000μg/ml) by the standard agar screen method.

Brain-heart infusion agar plate was divided into 4 quadrants. 3 quadrants were supplemented with an aminoglycoside, 500μg/ml of gentamicin, 2000μg/ml of gentamicin and 2000μg/ml of streptomycin and the 4th quadrant served as a positive growth control (*E.faecalis* - MIC 2048μg/ml). Each quadrant was inoculated with 10μl of a standardized suspension of 10^8CFU/ml to achieve a final inoculum of 10^6CFU/ml. Inoculated medium was incubated at 35-37°C in an ambient atmosphere for 18-24 hours. Each quadrant was examined for the presence or absence of bacterial growth. Bacterial growth indicated the presence of high-level resistance to either gentamicin, streptomycin or both the aminoglycosides. No growth in the presence of the aminoglycosides indicated the absence of high-level resistance.

[NB: Detailed method for high-level resistance to aminoglycosides :
See APPENDIX - Section F]

3.3.6 The study of synergism using time-kill curves

The time-kill curve can be used to study the dynamics of synergism or antagonism for a combination of antimicrobial agents by determining the number of viable bacteria remaining over time after exposure to each individual antibiotic and various combinations. Time-kill assay data can assess both the rate an extent of killing.

Drug concentrations selected for testing are based on clinical achievable levels in blood. Killing curves were performed using the following antibiotics at the stated concentrations. The concentration of antibiotics for the beta-lactams were chosen according to the minimum inhibitory concentration (MIC) of the isolates.

Gentamicin : 5μg/ml (Fixed)
Vancomycin : 10μg/ml (Fixed)
Teicoplanin : 5μg/ml (Fixed)
Penicillin : 6μg/ml (MIC < 32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC > 256μg/ml)
Ampicillin : 10μg/ml (MIC <32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC > 256μg/ml)
Imipenem : 10μg/ml (MIC < 32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC >256μg/ml)

For inoculum preparation colonies of each isolate was grown in brain-heart infusion (BHI) broth at 35-37°C overnight. Turbidity of an overnight broth culture was adjusted by adding fresh sterile broth to match a 0.5 McFarland standard (approx. 10^8CFU/ml). Inoculum was further diluted to achieve a final concentration of 10^8CFU/ml.

NB: Prior to inoculation of inoculum, each tube of fresh BHI broth was supplemented with the appropriate aminoglycoside, beta-lactam or glycopeptide antibiotic either alone or in combination.

At 0,4,24 hour intervals after inoculation, the number of viable CFUs were determined by performing serial dilution bacterial colony counts (10^1-10^8).

By using the viable colony count determined at each time interval, a 24 hour time-kill curve was established for each isolate tested.

Results for the control and each antimicrobial agent concentration, were plotted against time.

Susceptibility to an aminoglycoside, beta-lactam or glycopeptide synergy is defined as a >100 fold increase in killing by the drug combination over the killing accompanied by the most active of the two drugs when tested separately. Resistance to synergy is a <100 fold increase in killing.

[NB: Detailed method for the study of synergism :
See APPENDIX - Section H]

3.4 TYPING OF ENTEROCOCCI USING PULSED-FIELD GEL ELECTROPHORESIS

PFGE was performed on 11 enterococcal isolates. Lambda phage DNA concatemers ("lambda ladder") was used as a molecular size standard, with the lowest DNA size of 48.5 and the highest of 532.5 kilobases.

Seven *E.faecalis* and 4 *E.faecium* isolates with different patterns of high-level aminoglycoside resistance (gentamicin and / or streptomycin) were chosen. All the *E.faecalis* strains were susceptible to ampicillin and penicillin. PFGE was performed on 3 *E.faecium* strains that were susceptible to ampicillin and penicillin and 1 *E.faecium* isolate that was resistant to ampicillin and penicillin.

Restriction fragment analysis of genomic DNA was prepared from *E.faecalis* and *E.faecium* isolates by previously identified methods (79).Briefly, overnight cultures were grown in brain-heart infusion (BHI) broth and packed cells were suspended in 5ml PIV solution. 2.5ml of this suspension was mixed with 2.5ml of 1.6% low melting temperature agarose gel and pipetted into small plug molds to solidify. For lysis plugs were placed in 10ml lysis solution.
The lysis solution was then replaced with ESP. Plugs were incubated overnight at 50°C with gentle shaking, then washed three times with TE buffer. Enterococcal DNA's were digested with restriction enzyme *Sma* I. Digestion was performed by placing 1-2mm thick slice of each plug into restriction buffer and enzyme, incubated and washed. Plugs were melted at 55-60°C and loaded into wells with 1.2% agarose gels in 0.5X TBE buffer.

Electrophoresis was performed with the Contour Clamped Homogenous Electric Field apparatus (CHEF-DRII) by using pulse times beginning with 5 seconds and ending with 35 seconds at 200V for 32 hours. Gels were stained with ethidium bromide for 30 minutes, destained in distilled water for 12 hours and photographed with UV radiation.

[NB. Detailed method for pulsed-field gel electrophoresis :
See APPENDIX :Section I]

CHAPTER 4

RESULTS

4.1 IDENTIFICATION

All 107 isolates were confirmed to belong to the genus *Enterococcus.* All the isolates tested positive for the group D antigen. A comparison between the API 20 Strep system, with and without the aid of the computer software program, and the Facklam identification scheme is shown in the table XIV

Table XIV **Comparison of the API 20 Strep system and the Facklam scheme**

API 20 STREP SYSTEM			FACKLAM SCHEME
ORGANISM	ANALYTICAL PROFILE INDEX	SOFTWARE, PROFILE RECOGNITION PROGRAM	CONVENTIONAL BIOCHEMICAL TESTS
E.faecalis	65	78	70
E.faecium	16	16	16
E.gallinarium	2	2	2
E.raffinosus			13
E.casseliflavus			6
E.faecium /casseliflavus *		6	
Isolates not speciated	24	5	

* Strains identified as *E.faecium* with a possibility of being *E.casseliflavus.*

The results were identical with all three systems for 83 (77.5%) of the enterococci.

The following discrepancies were noted :

Thirteen (12.1%) of the isolates that were identified as *E.faecalis* by the API 20 Strep system with the computer software program were identified as *E.raffinosus* by the conventional biochemical tests (Facklam).

Six (5.6%) of the strains identified as *E.faecium/casseliflavus* by means of the the computer software program were identified as *E.casseliflavus* by the Facklam system.

Five (4.7%) of the isolates that the API 20 Strep system with and without the computer identification profile failed to identify, were beta-haemolytic enterococci.

These 5 strains were identified as *E.faecalis* by the Facklam scheme. In addition, of the 24 (22%) that were not specified by the analytical profile index, 13(12%) were identified as *E.raffinosus*, 6(5.6%) were identified as *E.casseliflavus*.

4.2 LANCEFIELD GROUPING

All isolates expressed the group D antigen.

4.3 BETA-LACTAMASE PRODUCTION

All enterococcal strains were beta-lactamase negative

4.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Table XV **Summary of the two disc diffusion methods**

	MODIFIED "STOKES"			KIRBY-BAUER		
ANTIBIOTIC	S	I	R	S	I	R
Penicillin	nil	22 20.6%	85 79.4%	nil	93 87%	14 13.1%
Ampicillin	nil	93 87%	14 13%	nil	93 87%	14 13.1%
Imipenem	nil	93 87%	14 13%	93 87%	nil	14 13.1%
Piperacillin (Pipril)	nil	93 87%	14 13%	85 79.5%	8 7.5%	14 13.1%
Cotrimoxazole	23 21.5%	55 51.4%	29 27%	9 8.4%	nil	98 91.6%
Rifampicin	nil	65 60.7%	42 39.3%	20 18.7%	12 11.2%	75 70.1%

	MODIFIED "STOKES"			KIRBY-BAUER		
ANTIBIOTIC	S	I	R	S	I	R
Chloramphenicol	19 17.8%	65 60.7%	40 37.4%	75 70%	11 10%	21 19.6%
Clindamycin	nil	2 1.9%	105 98.1%	nil	8 7.5%	99 92.5%
Tetracycline	43 40.2%	2 1.9%	62 57.9%	43 40.2%	1 0.9%	63 59%
Ciprofloxacin	nil	73 68.2%	34 31.8%	nil	55 51.4%	52 48.6%
Teicoplanin	107 100%	nil	nil	nil	107 100%	nil
Vancomycin	107 100%	nil	nil	107 100%	nil	nil

S : Susceptible I : Intermediate R: Resistant

Table XV compares the results for both disc diffusion methods.

The dicrepancies between the two methods for penicillin and chloramphenicol reflect the higher concentrations used by the Kirby-Bauer method as compared to the Stokes method.

Although the same concentration of antibiotic was used for cotrimoxazole, rifampicin and ciprofloxacin for both methods, the Kirby-Bauer method showed a higher percent of resistance than the Stokes method.

Table XVI **Breakpoint susceptibility test results**

ANTIBIOTIC	BREAKPOINTS		
	S	LLR	HLR
Penicillin	≤ 16μg/ml 93 (86.9%)	> 16μg/ml 14(13.1%)	> 256μg/ml (0%)
Ampicillin	≤ 8μg/ml 93(86.9%)	> 8μg/ml 14(13.1%)	> 256μg/ml (0%)
Imipenem	≤ 4μg/ml 93(86.9%)	> 4μg/ml 14(13.1%)	> 256μg/ml (0%)
Piperacillin	≤ 4μg/ml 93(86.9%)	> 4μg/ml 14(13.1%)	> 256μg/ml (0%)
Ciprofloxacin	≤ 1μg/ml (0%)	> 1μg/ml 107 (100%)	> 8μg/ml (0%)
Vancomycin	≤ 4μg/ml 107 (100%)	> 4μg/ml (0%)	
Teicoplanin	≤ 4μg/ml 107(100%)	> 4μg/ml (0%)	

S : Susceptible LLR : Low-level resisitance HLR : High-level resistance

Table XVI shows the susceptibility of all strains as obtained with the agar incorporated breakpoint methodology. Penicillin, ampicillin and piperacillin showed identical results with 86.9% being interpreted as susceptible and 13.1% as resistant. None of the isolates grew on the plate containing >256μg/ml. This indicates that the MICs were between 32-256μg/ml. Ciprofloxacin MICs were in the range of 2-8μg/ml. All isolates were susceptible to vancomycin and teicoplanin.

Tables XVII, XVIII and XIX show the range, geometric mean and the MIC_{50} and MIC_{90} (μg/ml) of all enterococcal species, *E. faecalis*, and *E. faecium* isolates tested respectively.

Table XVII **Minimum inhibitory concentration (MIC) for all 107 enterococcal species**

	MIC (μg/ml)			
ANTIBIOTIC	RANGE	GEOMETRIC MEAN	50%	90%
Penicillin	0.25 - 128	6.76	1	16
Ampicillin	0.25 - 64	8.35	0.5	16
Imipenem	0.25 - 128	6.66	1	32
Vancomycin	0.5 - 4	9.92	1	2
Teicoplanin	0.25 - 1	8.85	0.5	1

Table XVIII **Minimum inhibitory concentration (MIC) for *E.faecalis* (70 isolates)**

	MIC (μg/ml)			
ANTIBIOTIC	RANGE	GEOMETRIC MEAN	50%	90%
Penicillin	0.25 - 16	5.96	1	1
Ampicillin	0.25 - 1	7.61	0.5	0.5
Imipenem	0.25 - 1	9.34	1	1
Vancomycin	0.5 - 4	8.56	1	2
Teicoplanin	0.25 - 1	9.56	0.5	1

Table XIX **Minimum inhibitory concentration (MIC) for *E.faecium* (16 isolates)**

	MIC (μg/ml)			
ANTIBIOTIC	RANGE	GEOMETRIC MEAN	50%	90%
Penicillin	0.5 - 256	5.02	16	64
Ampicillin	0.5 - 64	5.78	16	32
Imipenem	0.5 - 128	5.78	16	32
Vancomycin	0.5 - 2	7.10	1	2
Teicoplanin	0.5 - 2	7.25	0.5	2

All 3 beta-lactams showed similar results. When one compared the E.*faecalis* and *E.faecium* isolates, the *E.faecalis* were fully susceptible but many of the *E.faecium* strains were resistant. The glycopeptides showed similar results for both the species.

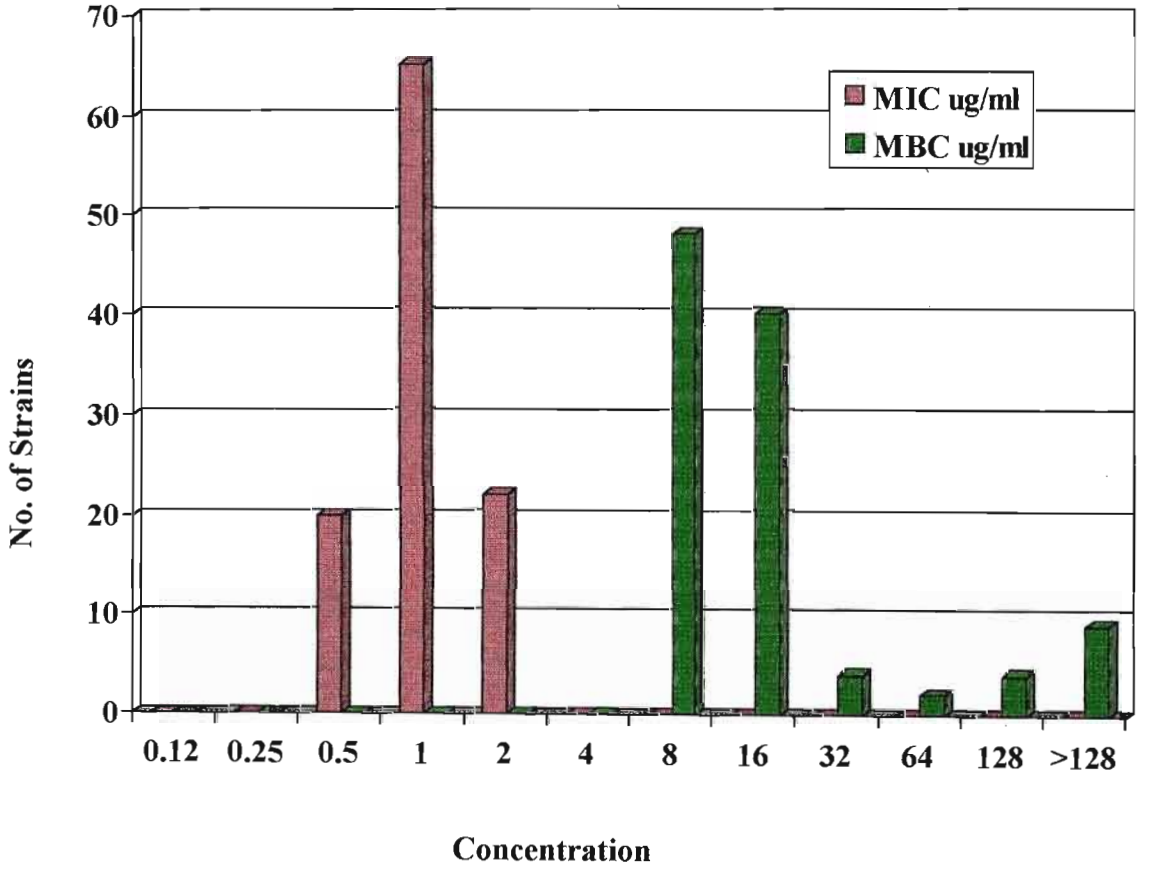


Fig.8 Comparison of the distribution of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)for vancomycin.

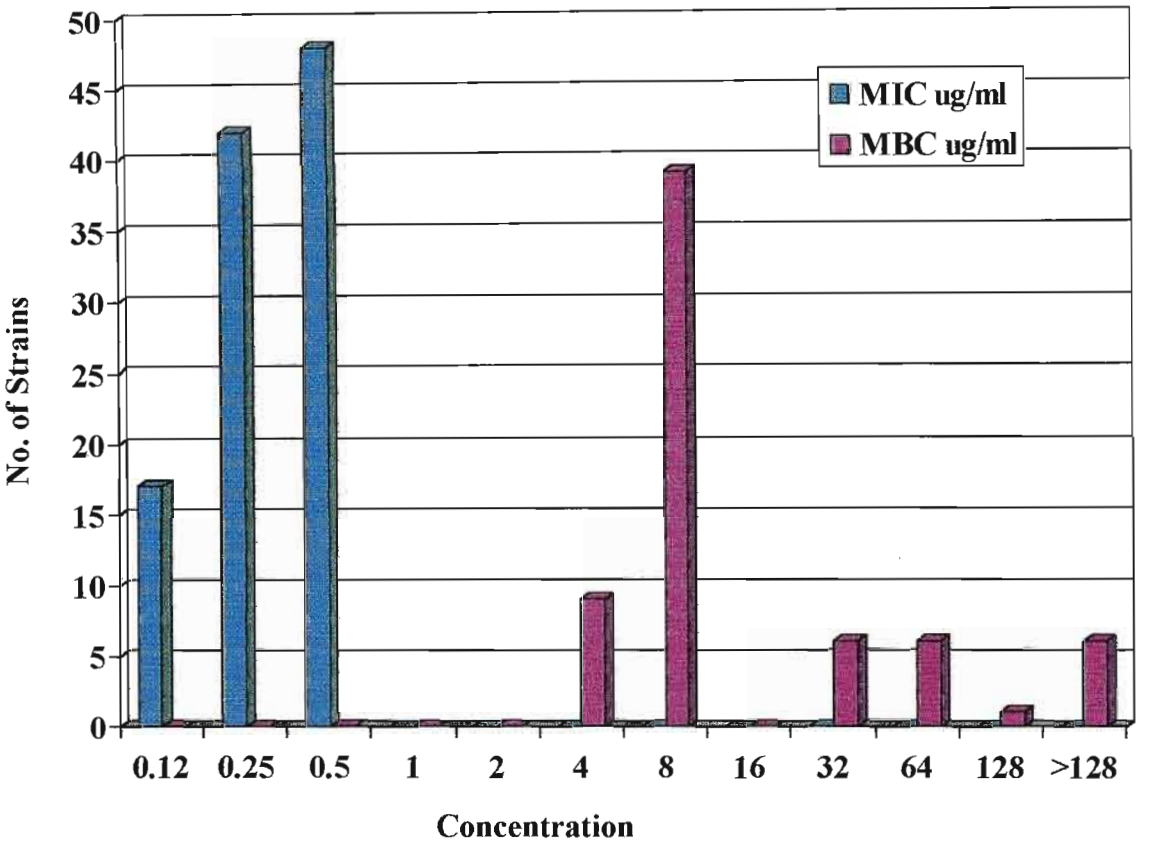


Fig.9 Comparison of the distribution of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for teicoplanin

Figs. 8 and 9 show there was a substantial difference between the MIC/MBC ratio of the glycopeptides.

Tables XX, XX1, XXII and XXIII show the influence of lysed blood on the susceptibility test results of aminoglycosides and cephalosporins. Gentamicin, tobramycin and netilmicin showed a high percent of false susceptibility for the Stokes, Kirby-Bauer, Breakpoint and MIC methods. The aminoglycoside that was unaffected by the presence of blood in all methods was amikacin. Cephalothin, cefamandole and cefuroxime were not significantly affected by the addition of blood to the medium. The cephalosporin that showed a high percent of false susceptibility in all four methods in the presence of lysed blood was cefotaxime.

Table XX **Comparison of susceptibility testing of aminoglycosides and cephalosporins with Mueller-Hinton agar with and without the presence of 5% lysed blood (Stokes)**

ANTIBIOTIC	MUELLER-HINTON AGAR +5% LYSED BLOOD			MUELLER-HINTON AGAR ONLY		
	S	I	R	S	I	R
Gentamicin	52 (48.6%)	8 (5.6%)	47 (43.9%)	nil	6 (5.6%)	101 (94.4%)
Tobramycin	38 (35.5%)	2 (4.7%)	67 (62.9%)	nil	nil	107 (100%)
Netilmicin	36 (33.6%)	6 (5.6%)	65 (60.7%)	nil	4 (3.7%)	103 (96.3%)
Amikacin	nil	nil	107 (100%)	nil	nil	107 (100%)
Cefotaxime	32 (29.9%)	3 (2.8%)	72 (67.3%)	nil	5 (4.7%)	102 (95.3%)
Cefamandole	nil	50 (46.7%)	57 (53.3%)	nil	45 (48.6%)	63 (60.7%)
Cefuroxime	20 (18.7%)	nil	87 (81.3%)	nil	3 (2.8%)	104 (97%)
Cephalothin	nil	60 (56.1%)	47 (43.9%)	nil	56 (52.3%)	61 (48.7%)

Table XXI **Comparison of susceptibility testing with Mueller-Hinton agar (MHA) supplemented with 5% lysed horse blood and Mueller-Hinton agar only for aminoglycosides and cephalosporins (Kirby-Bauer)**

	MUELLER-HINTON AGAR +5% LYSED BLOOD			MUELLER-HINTON AGAR ONLY		
ANTIBIOTIC	S	I	R	S	I	R
Gentamicin	55 (51.4%)	20 (18.7%)	32 (29.9%)	nil	26 (24.3%)	81 (75.7%)
Tobramycin	35 (32.7%)	9 (8.4%)	63 (58.9%)	nil	7 (6.5%)	100 (93.4%)
Netilmicin	33 (30.8%)	7 (6.5%)	67 (62.6%)	nil	9 (8.4%)	98 (91.6%)
Amikacin	nil	nil	107 (100%)	nil	nil	107 (100%)
Cefotaxime	30 (28%)	1 (0.9%)	76 (71%)	nil	1 (0.9%)	106 (99.1%)
Cefamandole	5 (4.7%)	5 (4.7%)	97 (90.6%)	nil	2 (1.9%)	105 (98.1%)
Cefuroxime	18 (16.8%)	nil	89 (83.2%)	nil	2 (1.9%)	105 (98.1%)
Cephalothin	5 (4.7%)	45 (42%)	57 (53.2%)	nil	48 (44.8%)	59 (55.1%)

Table XXII **Comparison of aminoglycoside and cephalosporin results with Muller-Hinton agar (MHA) supplemented with 5% lysed horse blood and Mueller-Hinton agar only (Breakpoints)**

	MUELLER-HINTON AGAR +5% LYSED BLOOD		MUELLER-HINTON AGAR ONLY	
ANTIBIOTIC	S	R	S	R
Gentamicin	≤16μg/ml 57(53.3%)	≥32μg/ml 50(46.7%)	≤16μg/ml 4(3.7%)	≥32μg/ml 103(96.3%)
Tobramycin	≤4μg/ml 44(41%)	≥8μg/ml 63(58.9%)	≤4μg/ml (0%)	≥8μg/ml 107(100%)
Netilmicin	≤8μg/ml 40(37.4%)	≥16μg/ml 67(62.6%)	≤8μg/ml (0%)	≥16μg/ml 107(100%)
Amikacin	≤16μg/ml (0%)	≥32μg/ml 107(100%)	≤16μg/ml (0%)	≥32μg/ml 107(100%)
Cefotaxime	≤8μg/ml 30(28%)	≥32μg/ml 77(72%)	≤8μg/ml 2(4.7%)	≥32μg/ml 105(98.1%)
Cefamandole	≤8μg/ml 5(4.7%)	≥16μg/ml 102(95.3%)	≤8μg/ml 5(4.7%)	≥16μg/ml 102(95.3%)
Cefuroxime	≤8μg/ml 18 (16.8%)	≥16μg/ ml 89(83.2%)	≤8μg/ml 2(4.7%)	≥16μg/ml 105(98.1%)
Cephalothin	≤8μg/ml 55(51.4%)	≥16μg/ml 52(48.6%)	≤8μg/ml 52(48.6%)	≥16μg/ml 55(51.4%)

Table XXIII **Comparison of aminoglycosides and cephalosporins on Mueller-Hinton agar (MHA) only and Mueller-Hinton agar supplemented with 5% lysed horse blood (MIC)**

	MUELLER-HINTON AGAR +5% LYSED BLOOD			MUELLER-HINTON AGAR ONLY		
	MIC (μg/ml)			MIC (μg/ml)		
ANTIBIOTIC	RANGE	50%	90%	RANGE	50%	90%
Gentamicin	4 - >256	4	>256	8 - >256	16	>256
Tobramycin	4- >256	8	>256	8 - >256	32	>256
Netilmicin	4- >256	8	>256	8 - >256	32	>256
Amikacin	32 - >256	64	>256	32 - >256	64	>256
Cefotaxime	8 - >256	8	>256	16 - >256	64	>256
Cefamandole	8 - >256	64	>256	16 - >256	64	>256
Cefuroxime	8 - >256	16	>256	16- >256	64	>256
Cephalothin	8 - >256	32	>256	16 - >256	32	>256

4.5 HIGH-LEVEL AMINOGLYCOSIDE RESISTANCE

Table XXIV **Results of high-level aminoglycoside resistance**

ORGANISM	GENTA (500 and 2000μg/ml)	STREPTO (2000μg/ml)	GENTA AND STREPTO	TOTAL RESISTANCE
E.faecalis (n=70)	2 (2.8%)	5 (7.1%)	15 (21.4%)	22 (31.4%)
E.faecium (n=16)		4 (25%)	5 (31.2%)	9 (37.5%)
E.casseliflavus(n=6)	1 (16.7%)	1 (16.7%)	3 (50%)	5 (83%)
E.raffinosus (n=13)		1 (7.7%)	1(7.7%)	2 (15.4%)
E.gallinarium (n=2)			1 (50%)	1 (50%)

n = total number of isolates tested.

The results of the detection for high-level aminoglycoside resistance are shown in Table XXIV Isolates that were resistant to 500μg/ml of gentamicin were also resistant to 2000μg/ml. Thirty-nine (26.4%) of the 107 enterococci tested displayed high-level aminoglycoside resistance. Three (2.8%) of the isolates showed high-level resistance to gentamicin only, 11 (10.3%) to streptomycin only and 25 (23.4%) to both gentamicin and streptomycin.

4.6 THE STUDY OF SYNERGISM

Figs. 10.1 to 17.3 show time-kill synergy studies of ampicillin, penicillin, imipenem, vancomycin, teicoplanin and gentamicin alone and in combination.

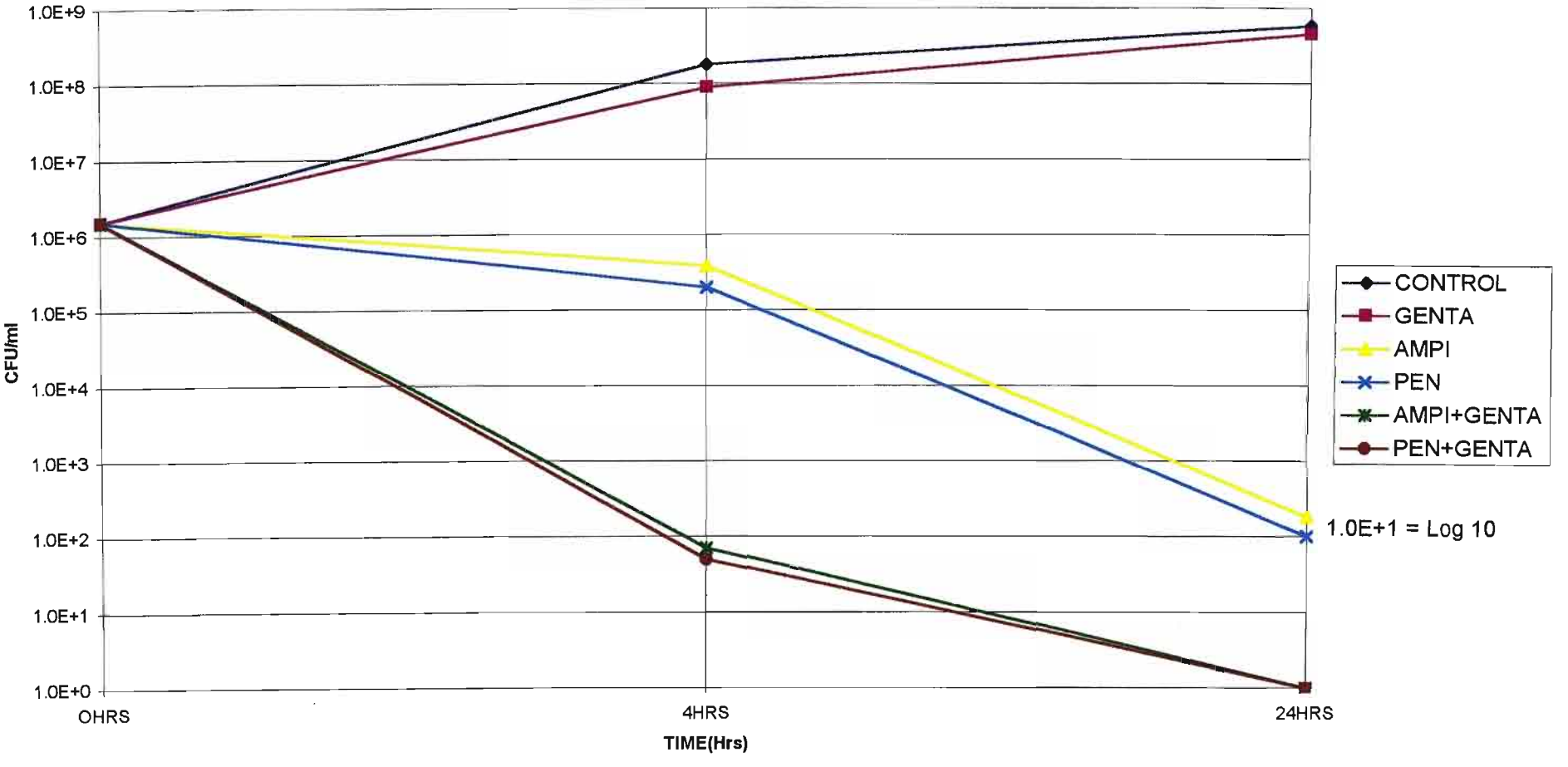


Fig 10.1 Time kill study showing synergism against *E.faecalis* displaying low-level resistance to gentamicin when combined with penicillin or ampicillin

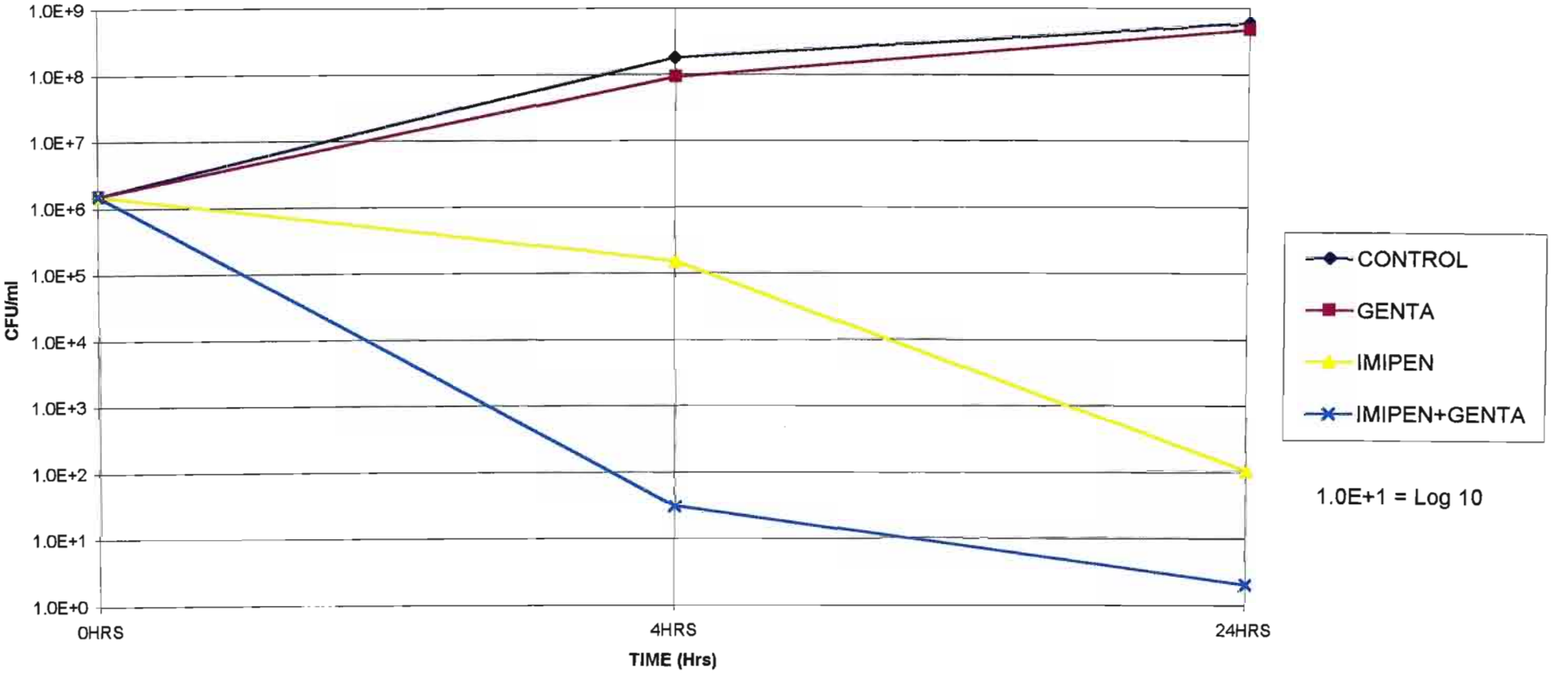


Fig. 10.2 Time-kill curves showing imipenem plus gentamicin displaying synergism against *E.faecalis.* This isolate displayed low-level gentamicin resistanance

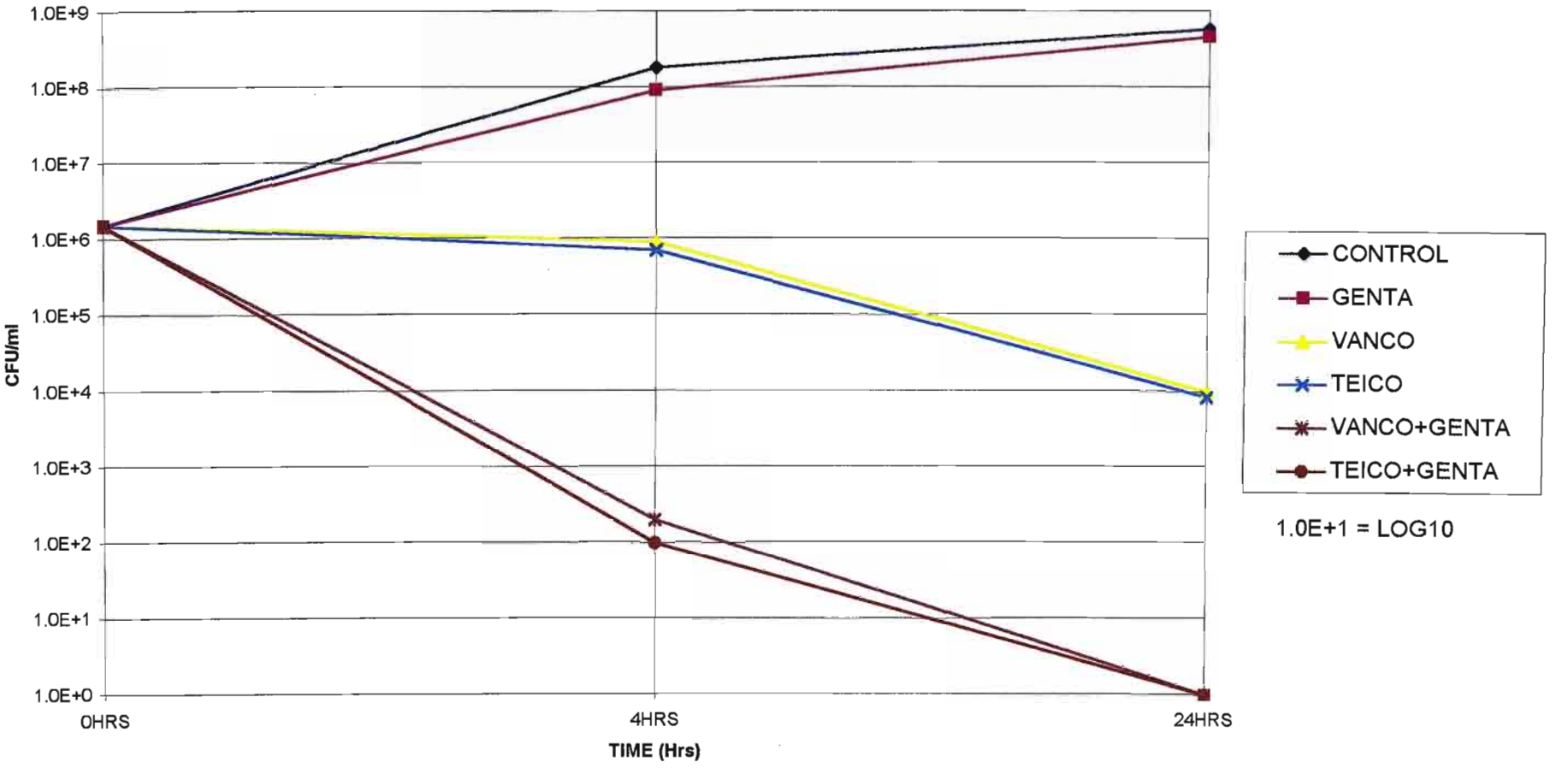


Fig. 10.3 Time kill study showing synergism with vancomycin or teicoplanin in combination with gentamicin. This *E.faecalis* isolate displayed low-level resistance to gentamicin

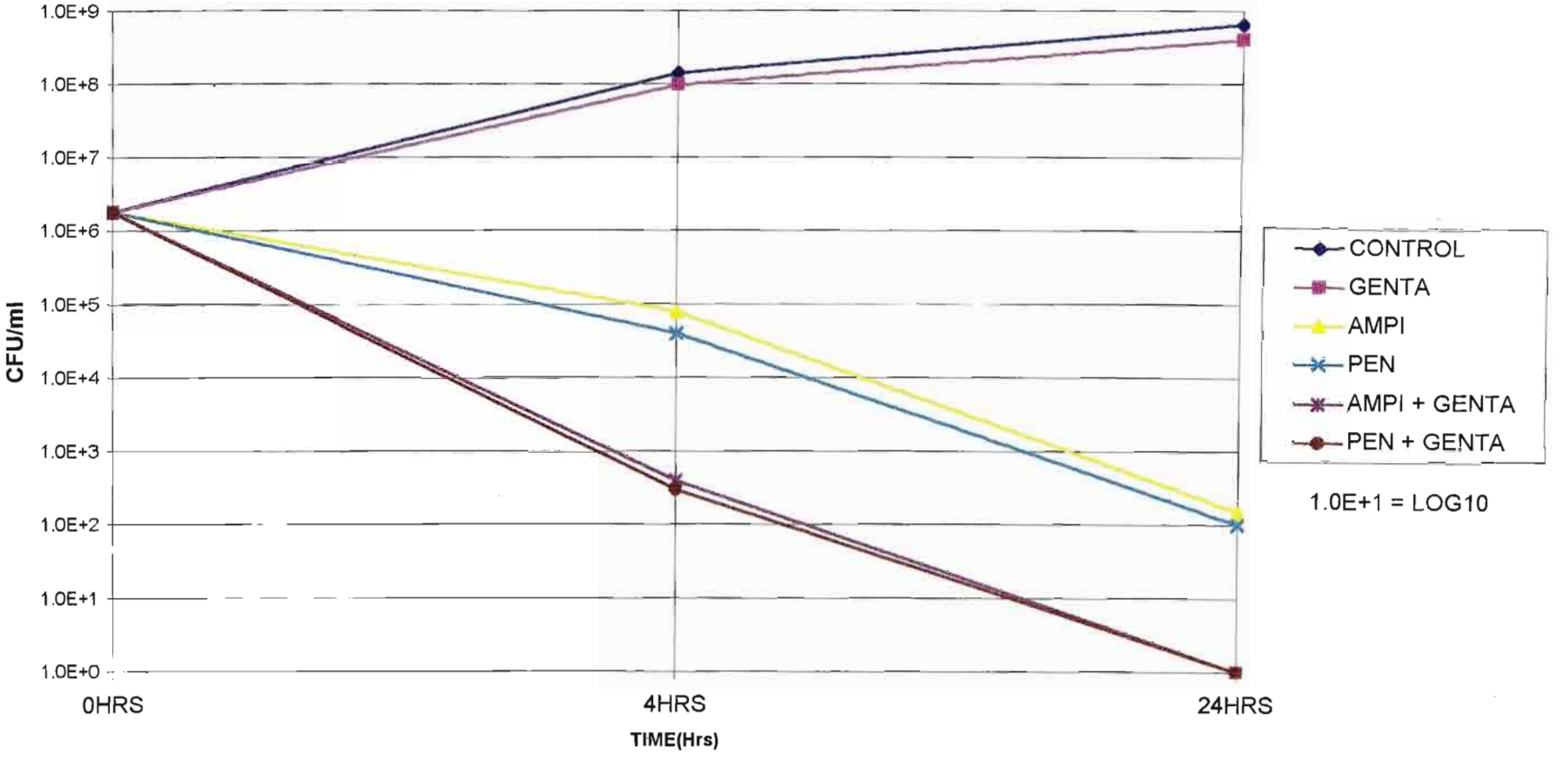


Fig. 11.1 Time-kill study curves showing synergism of ampicillin and penicillin in combination with gentamicin. This *E.faecalis* isolate showed low-level resistance to gentamicin

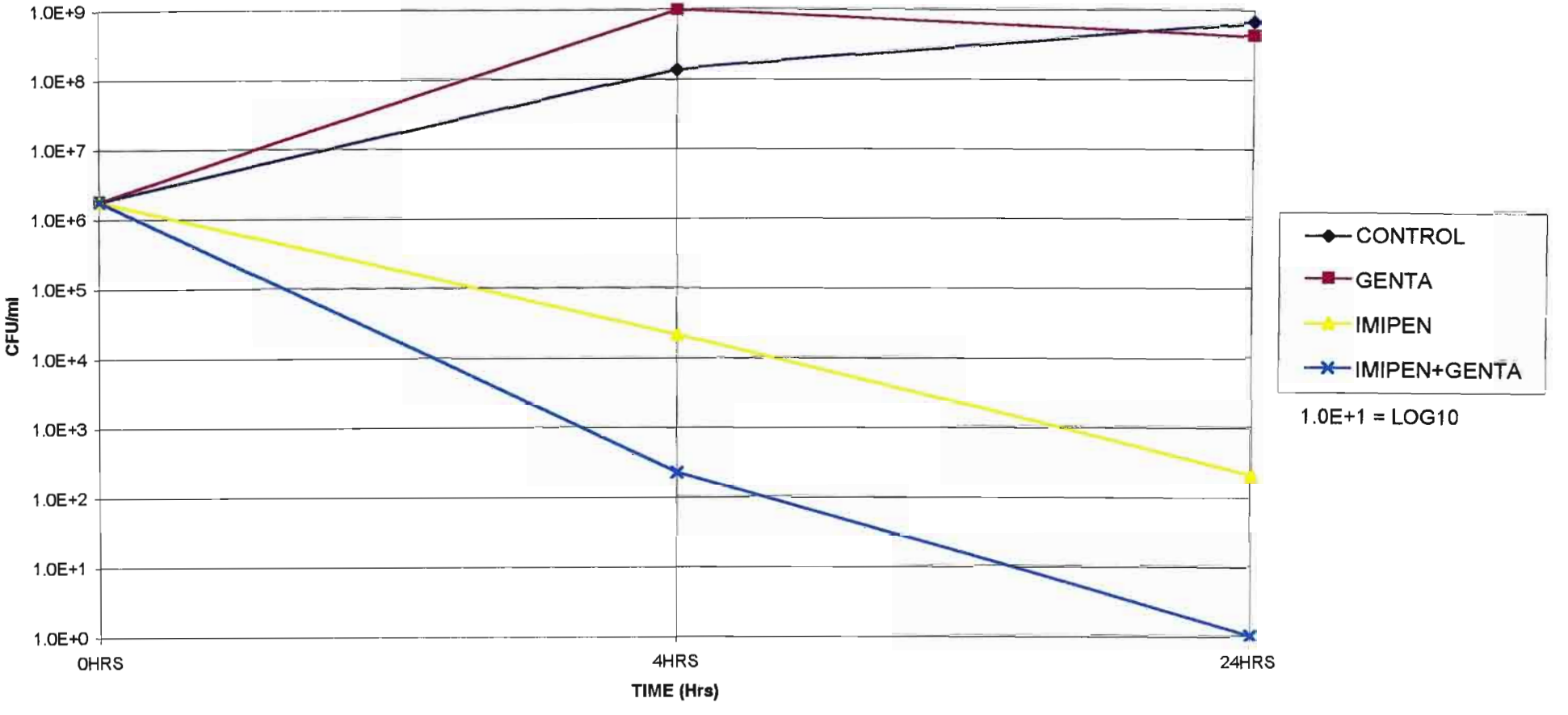


Fig. 11.2 Time-kill study showing synergism against *E.faecalis* with imipenem in combination with gentamicin. This isolate displayed low-level resisatance to gentamicin

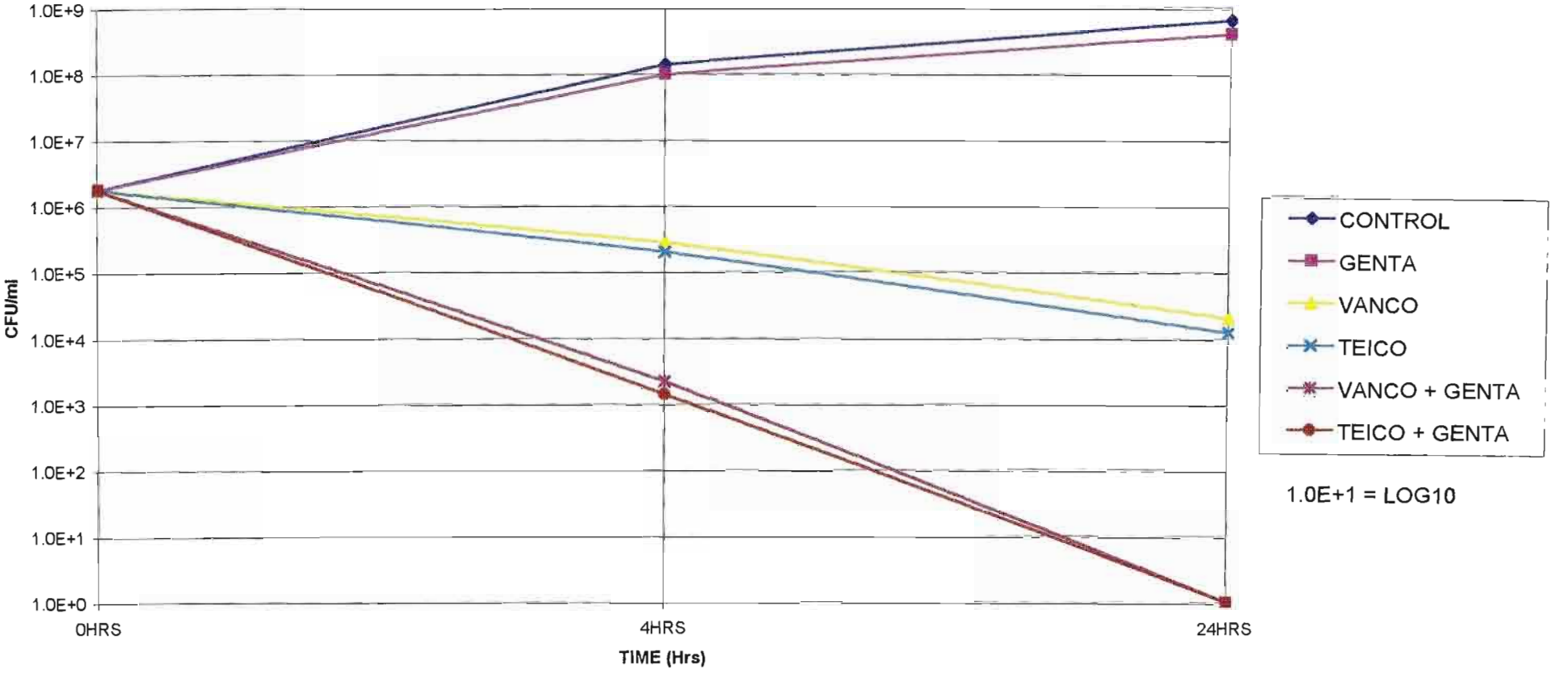


Fig.11.3 Vancomycin or teicoplanin displaying synergism in the time-kill study when combined with gentamicin against *E.faecalis* with low-level resistance to gentamicin

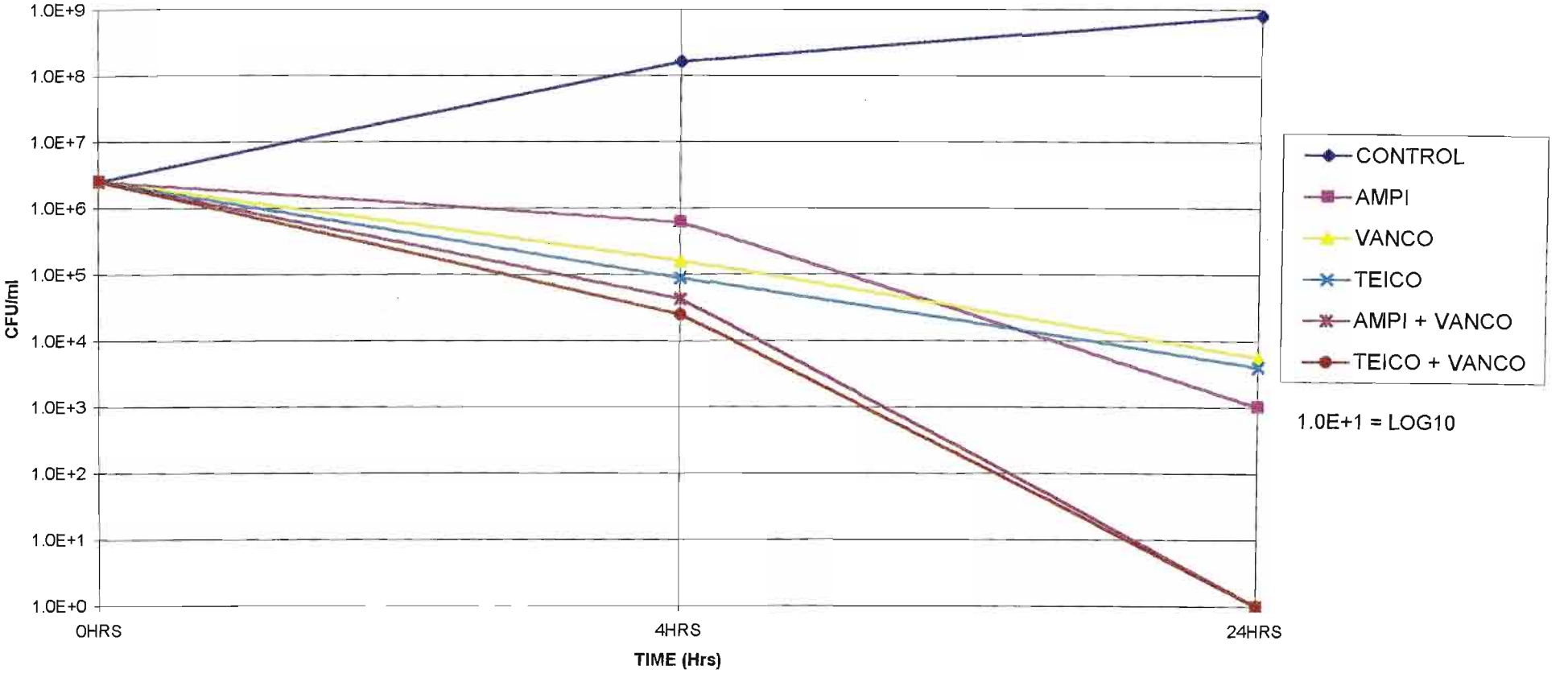


Fig.12.1 Time-kill study of *E.faecalis* in the presence of high-level gentamicin resistance shows ampicillin combined with either vancomycin or teicoplanin displaying synergism

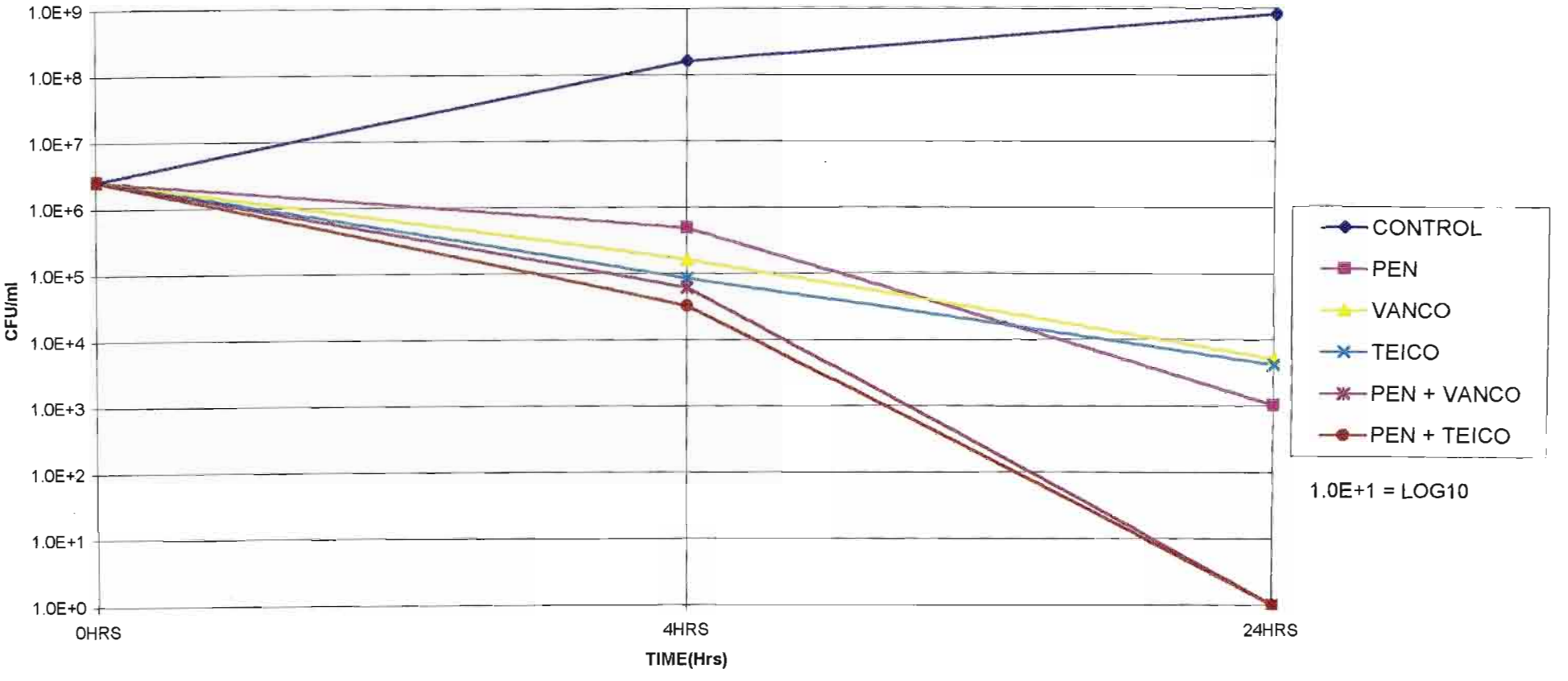


Fig.12.2 Time kill study showing synergism when vancomycin or teicoplanin were combined with penicillin against *E.faecalis* with high-level gentamicin resistance

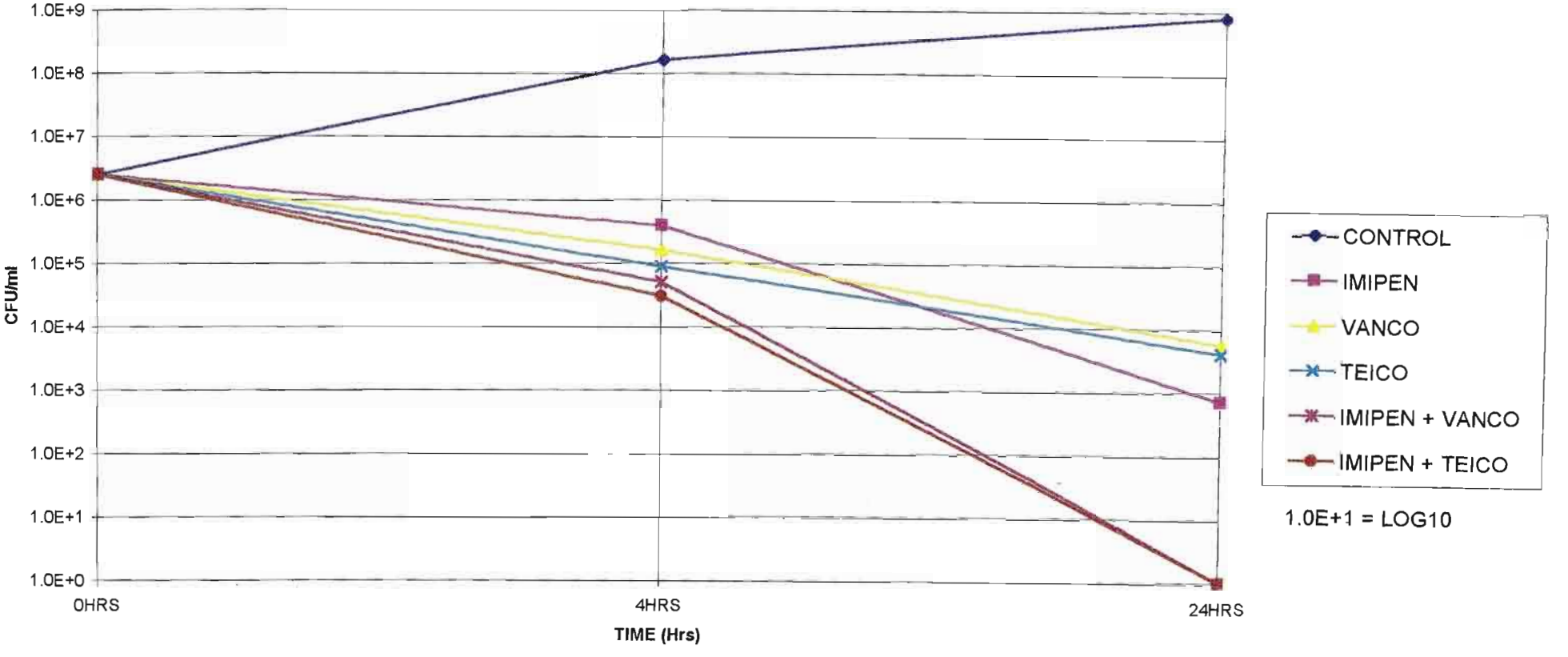


Fig.12.3 Imipenem and vancomycin or teicoplanin showing synergism against *E.faecalis* in the presence of high-level gentamicin resistance in the time-kill study

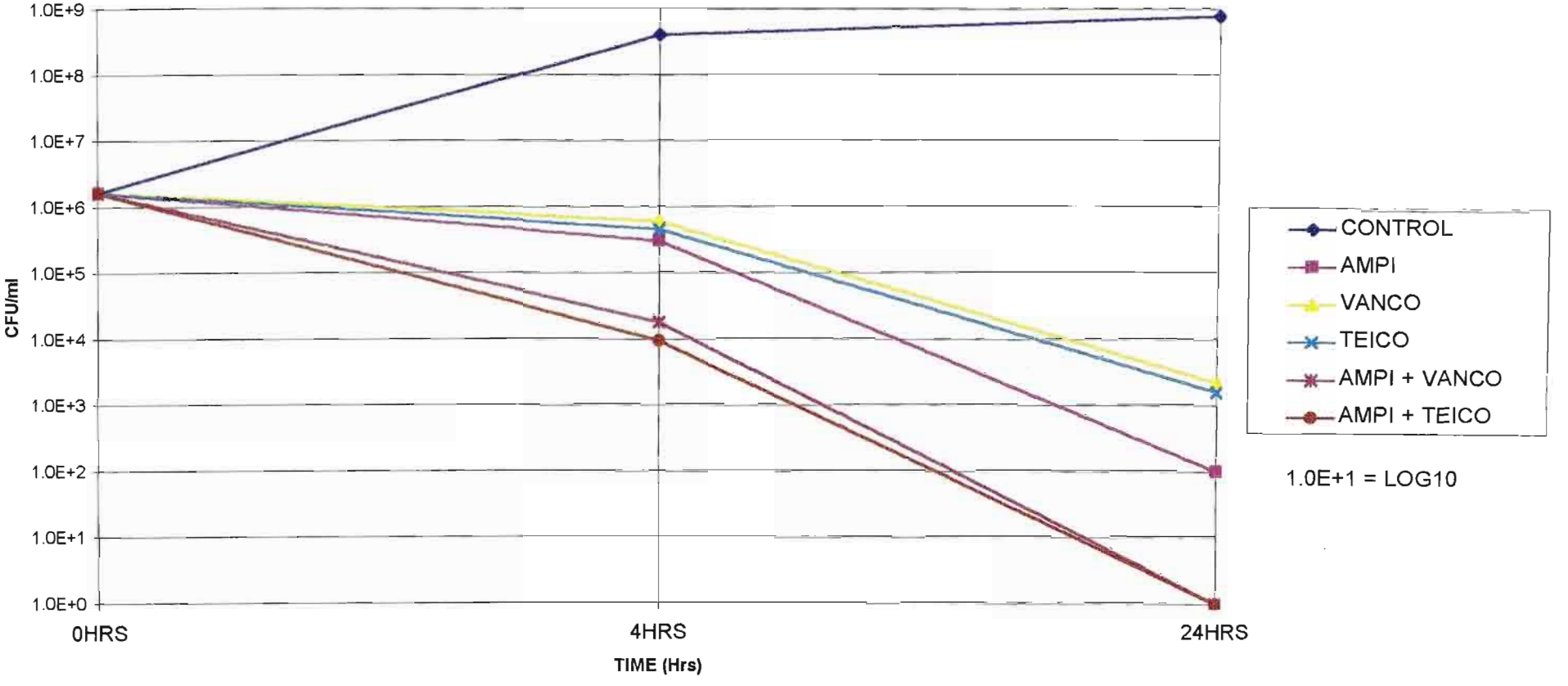


Fig.13.1 Time-kill study of *E.faecalis* showing synergism of ampicillin and vancomycin or teicoplanin. This isolate displayed high-level gentamicin resistance

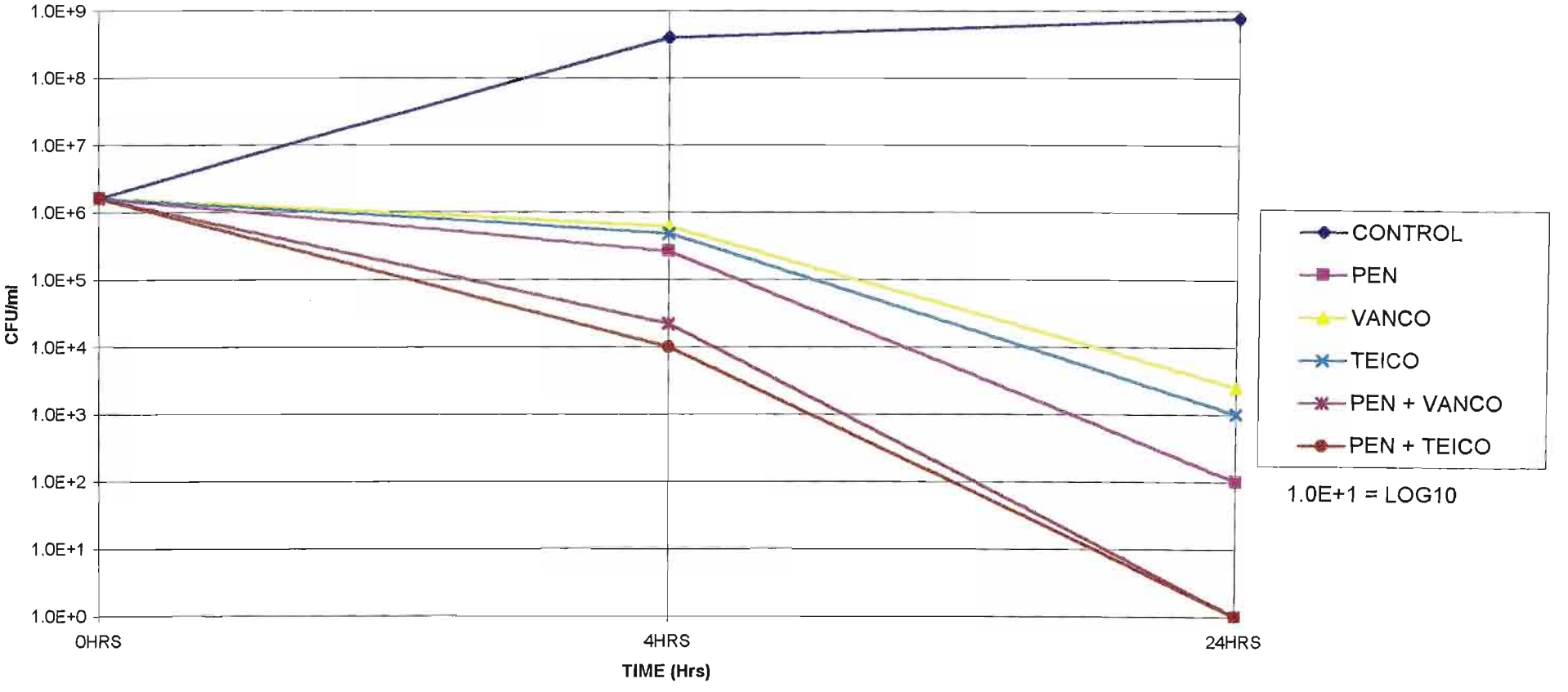


Fig.13.2 Penicillin in combination with vancomycin or teicoplanin displaying synergism in the time-kill study of *E.faecalis* with high-level gentamicin resistance

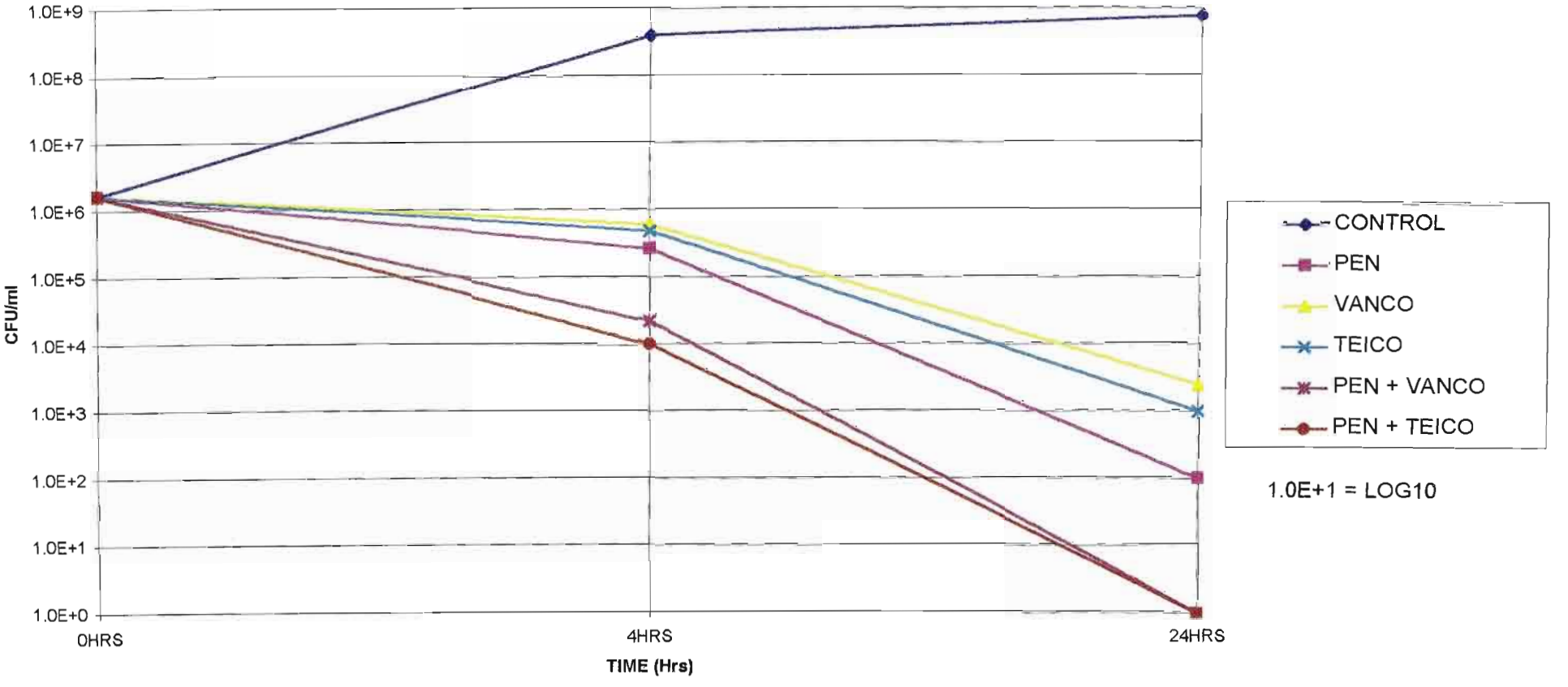


Fig.13.3 Time kill study showing synergism against *E.faecalis* in the presence of high-level gentamicin resistance when combined with penicillin and vancomycin or teicoplanin

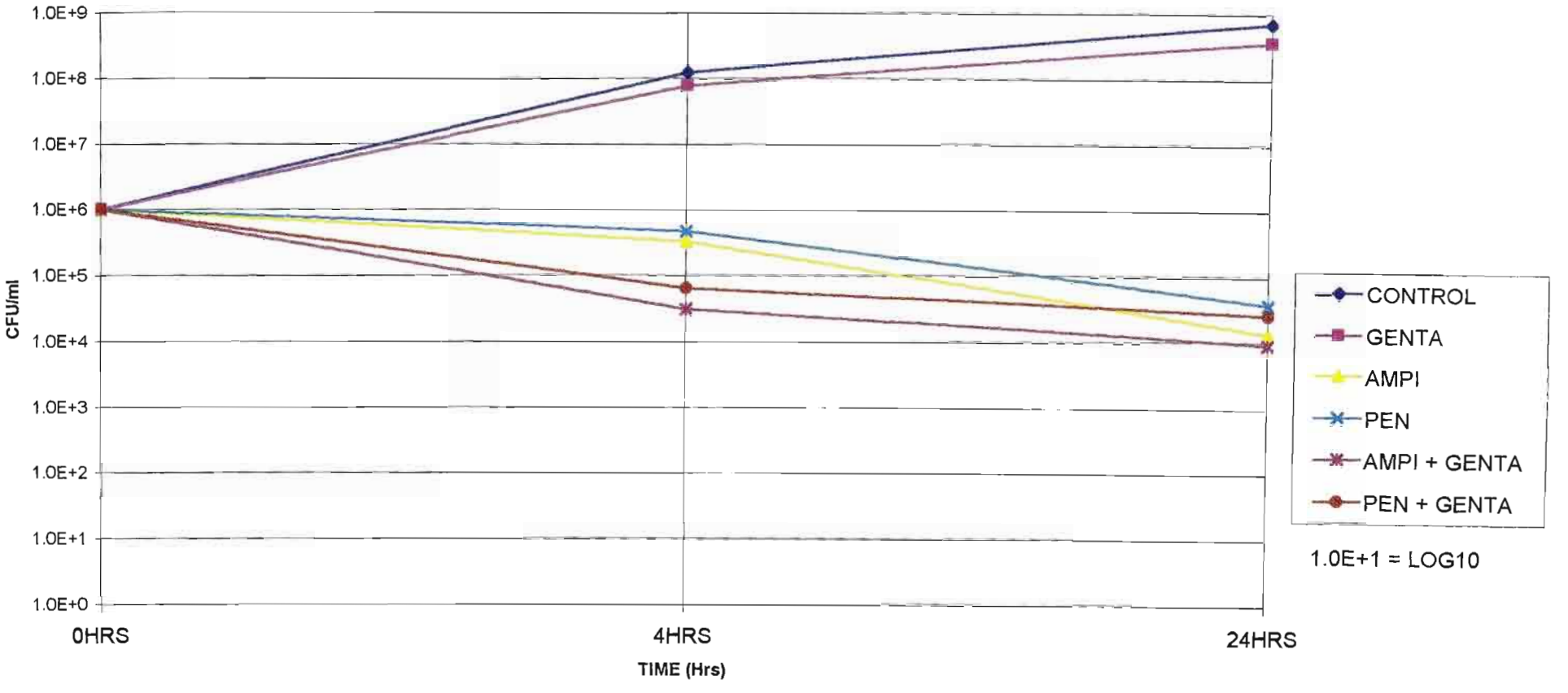


Fig.14.1 Time kill study of*E.faecium* failing to show synergism. An additive phenomenon was noted with ampicillin or penicillin in combination with gentamicin. This isolate displayed low-level resistance to gentamicin

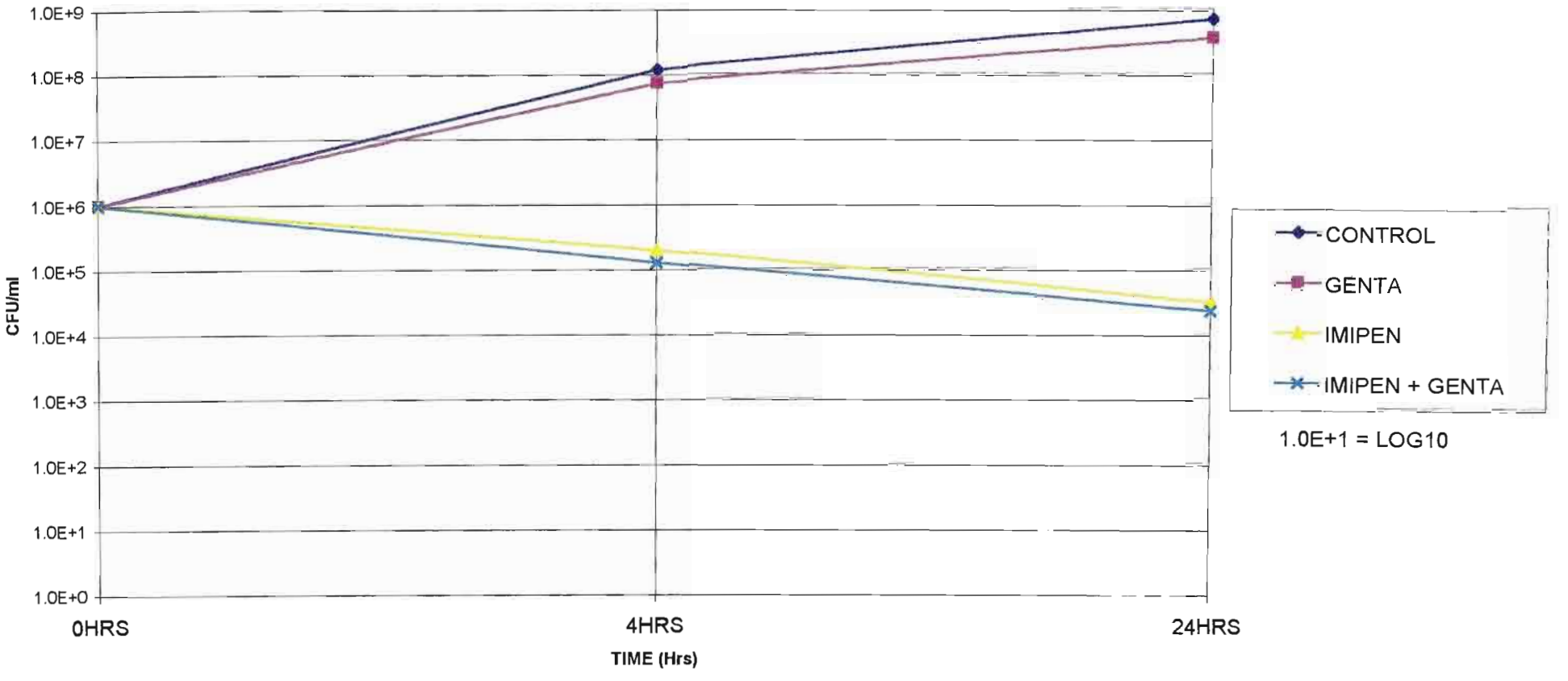


Fig.14.2 Time-kill study showing an additive effect against *E.faecium* when combined with imipenem and gentamicin. This isolate displayed low-level resistance to gentamicin

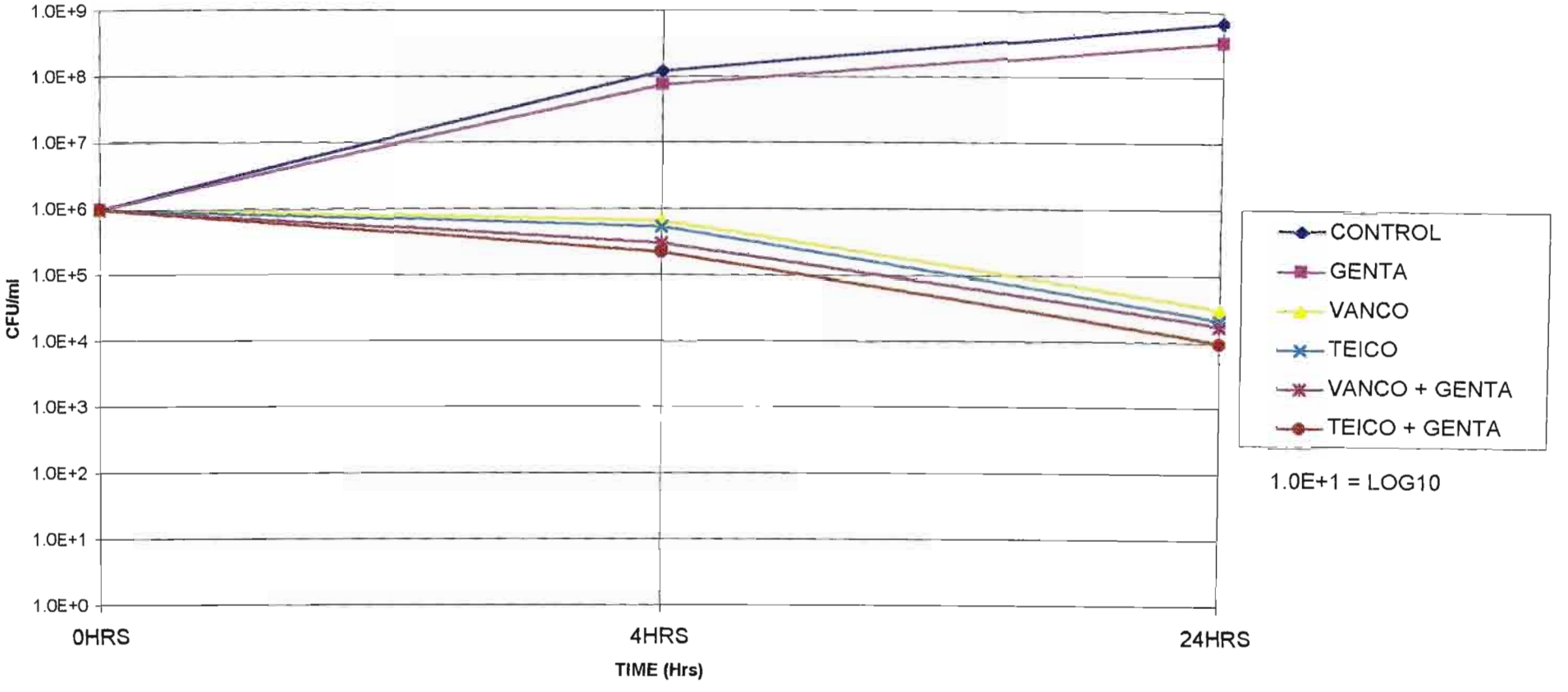


Fig.14.3 Vancomycin or teicoplanin when combined with gentamicin displaying an additive phenomenon against *E.faecium* in the time-kill curves. This isolate showed low-level resistance to gentamicin

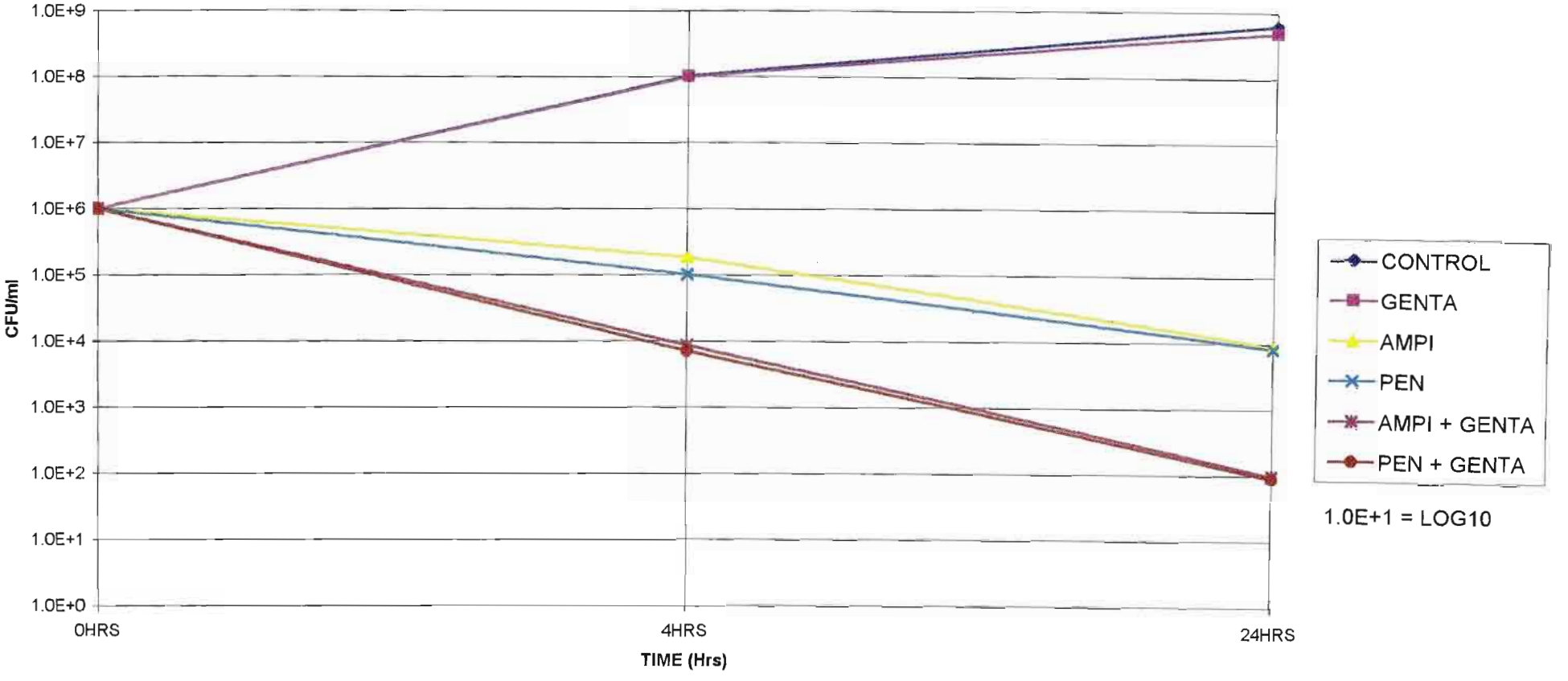


Fig.15.1 Time-kill study showing synergism against *E.faecium* when ampicillin or penicillin was combined with gentamicin. This isolate displayed low-level resistance to gentamicin

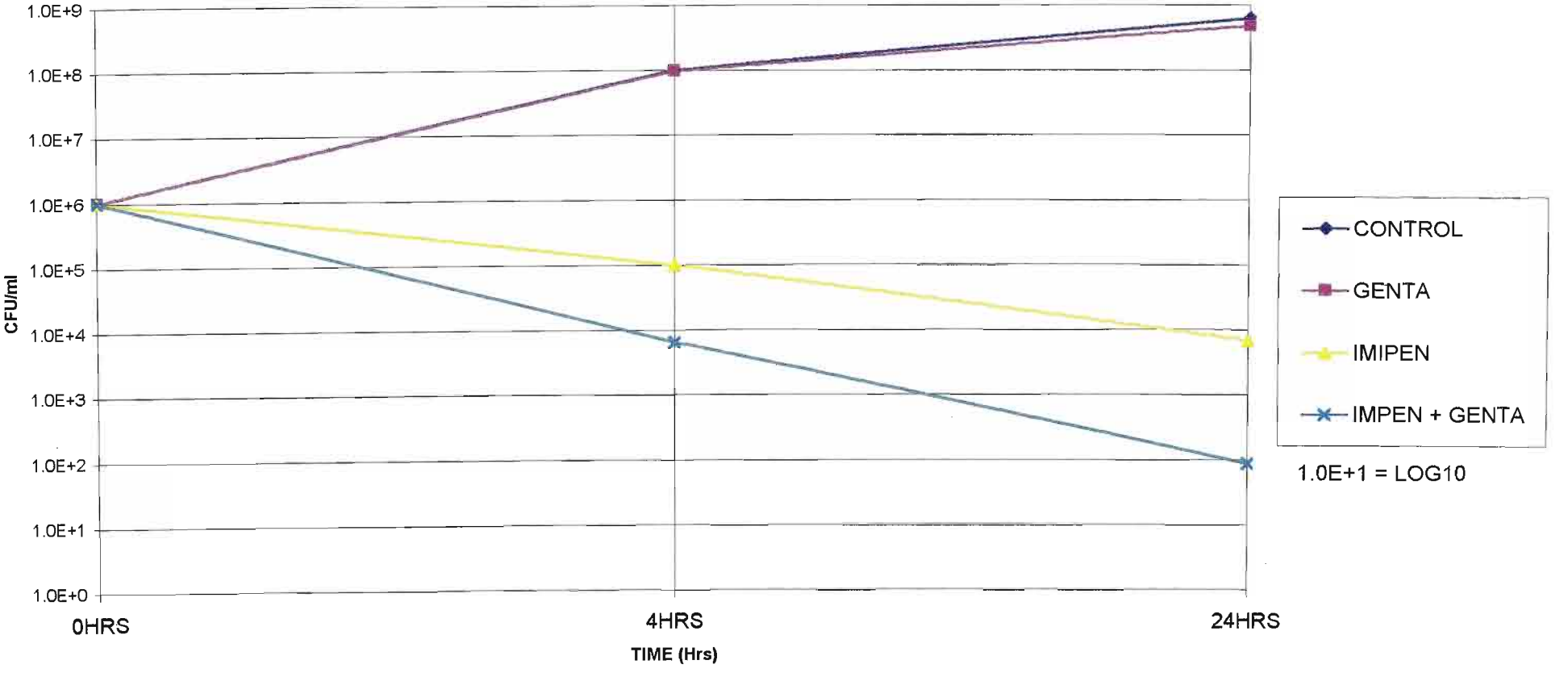


Fig.15.2 *E.faecium* isolate displaying synergism when combined with imipenem and gentamicin as shown by the time-kill curves. This isolate showed low-level resistance to gentamicin.

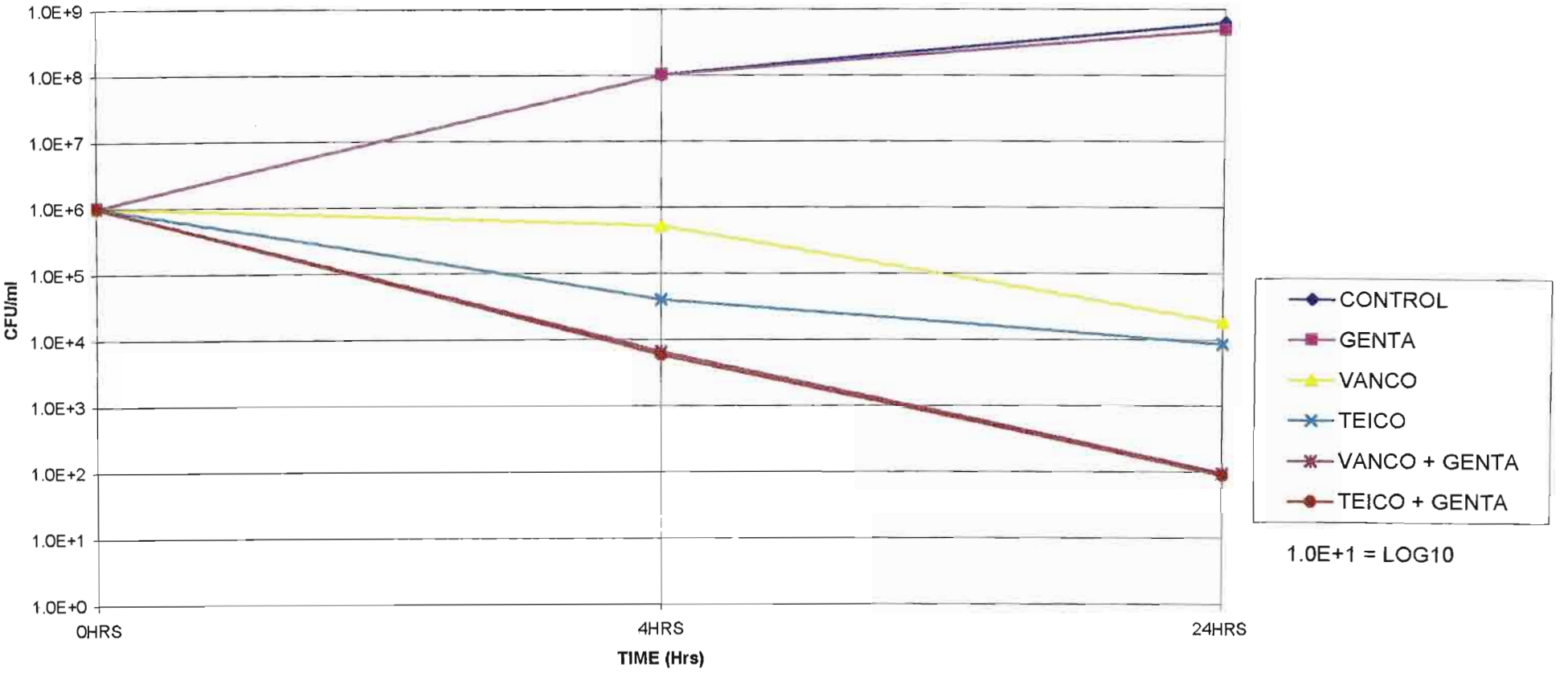


Fig.15.3 Time-kill study displaying synergism against *E.faecium* with low-level gentamicin resistance when combined with vancomycin or teicoplanin

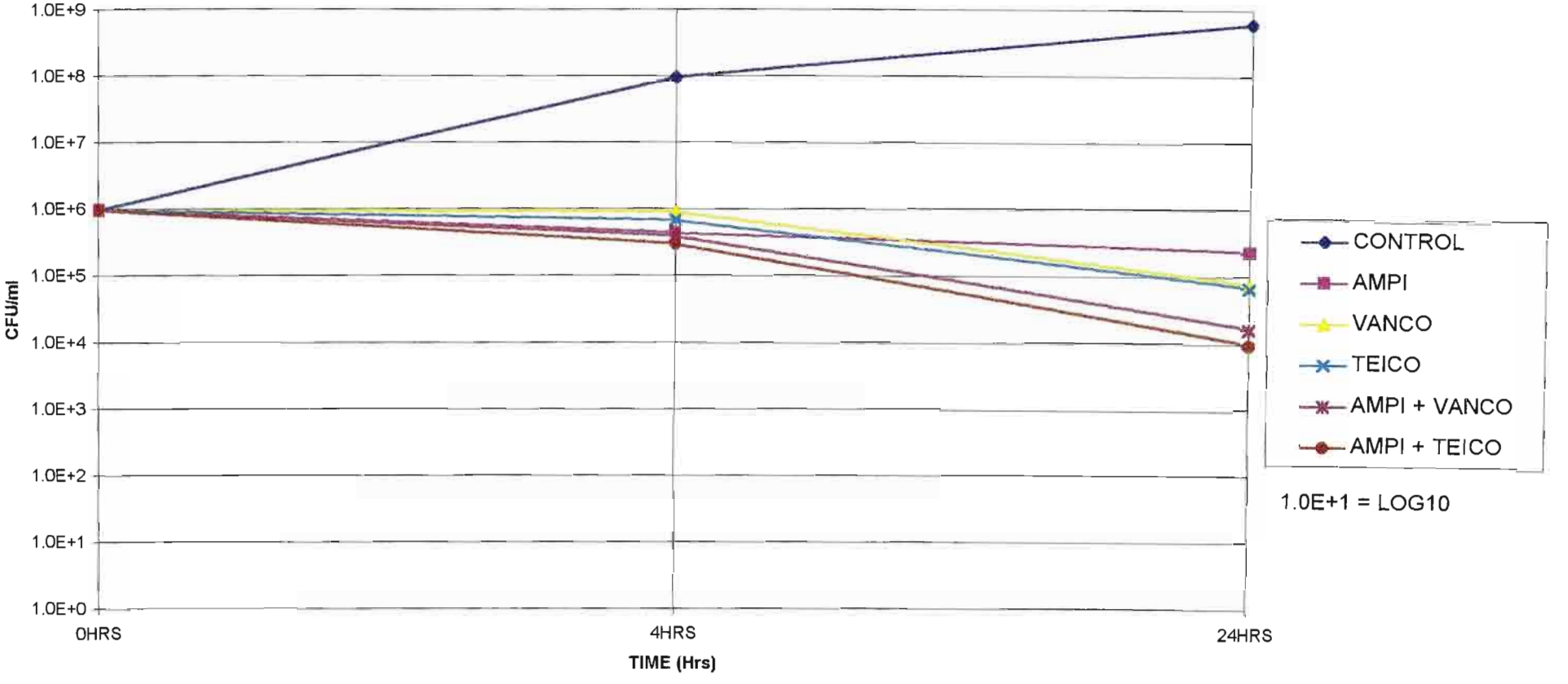


Fig.16.1 Time-kill curves showing an additive action against *E.faecium* when ampicillin was combined with vancomycin or teicoplanin in the presence of high-level resistance to gentamicin and ampicillin

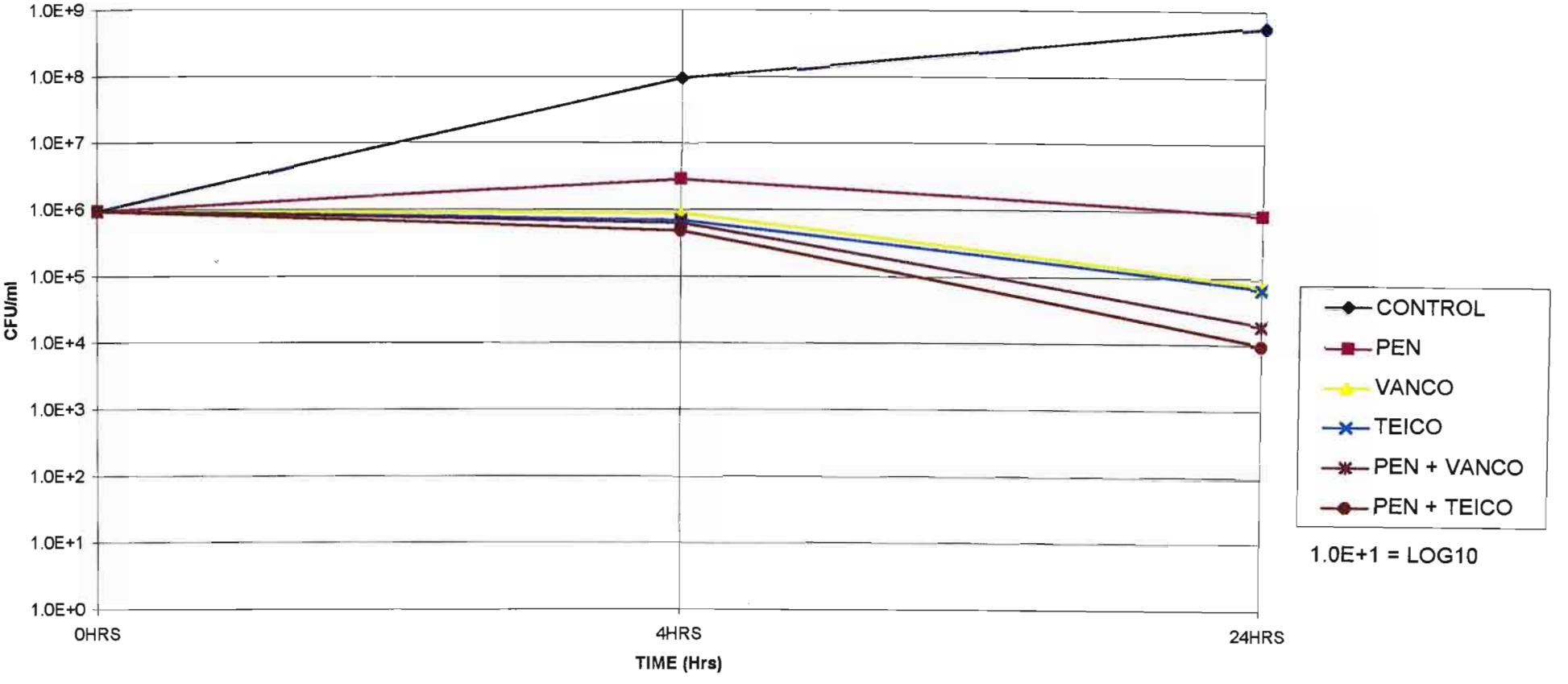


Fig.16.2 Time-kill study showing an additive phenomenon against *E.faecium* when penicillin was combined with vancomycin or teicoplanin. This isolate showed high-level resistance to gentamicin and penicillin

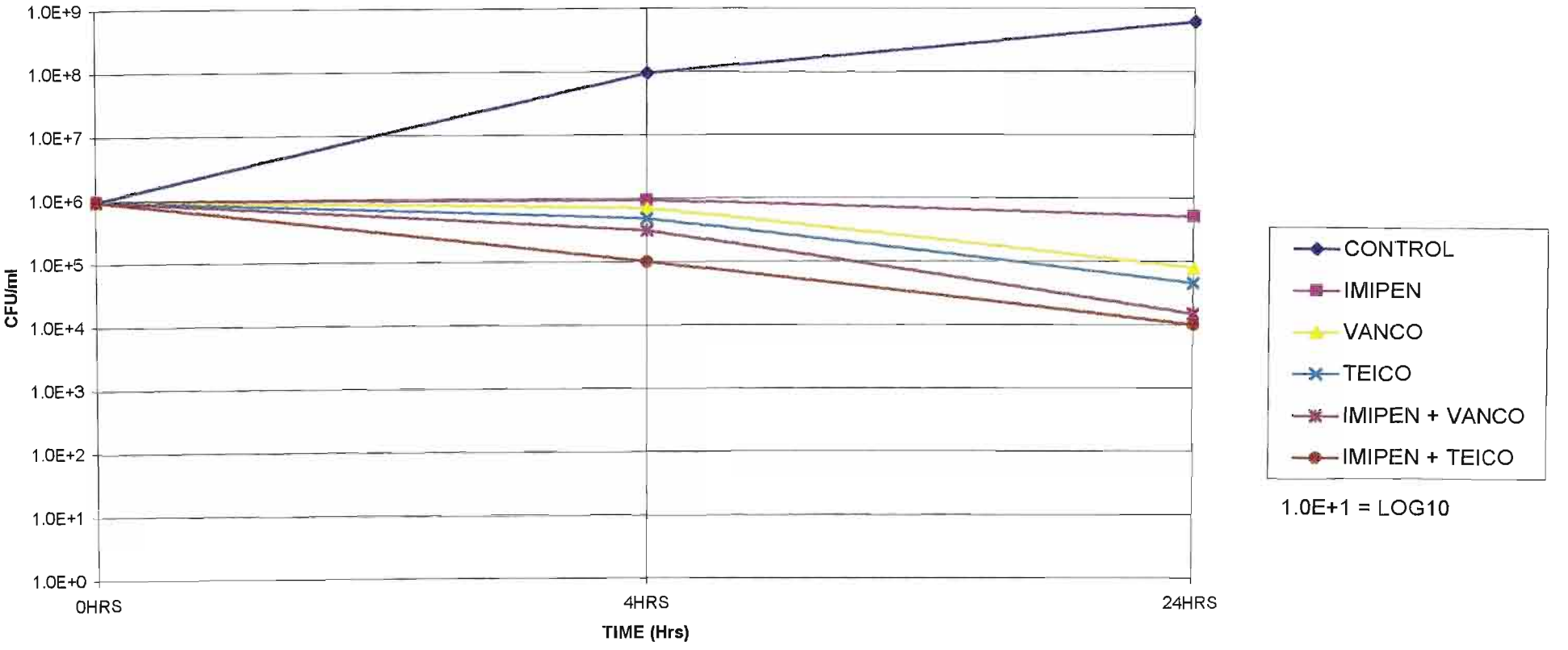


Fig.16.3 Imipenem and vancomycin or teicoplanin showing an additive action against *E.faecium* as shown by the time kill curves

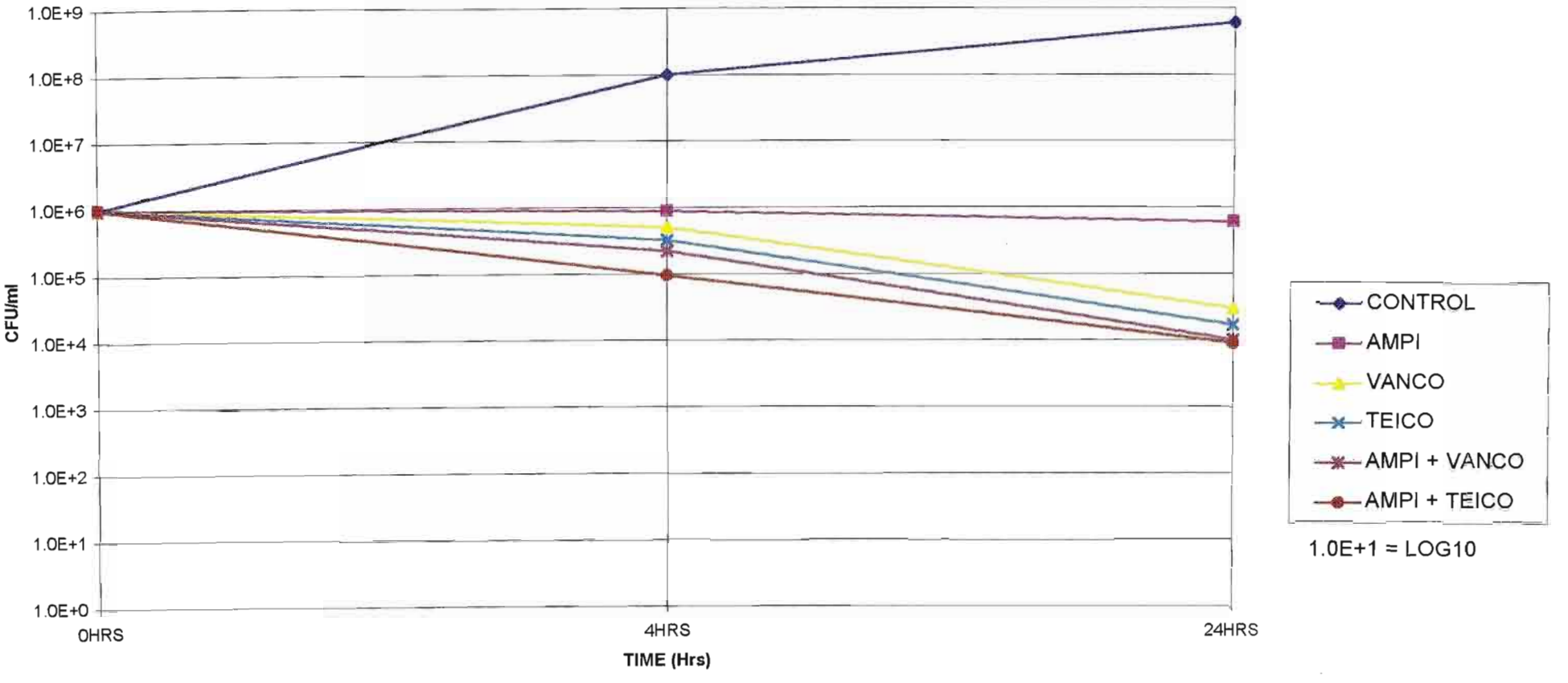


Fig.17.1 Time-kill study showing an additive effect when ampicillin was combined with the glycopeptides. This *E.faecium* isolate displayed high-level resistance to both ampicillin and gentamicin

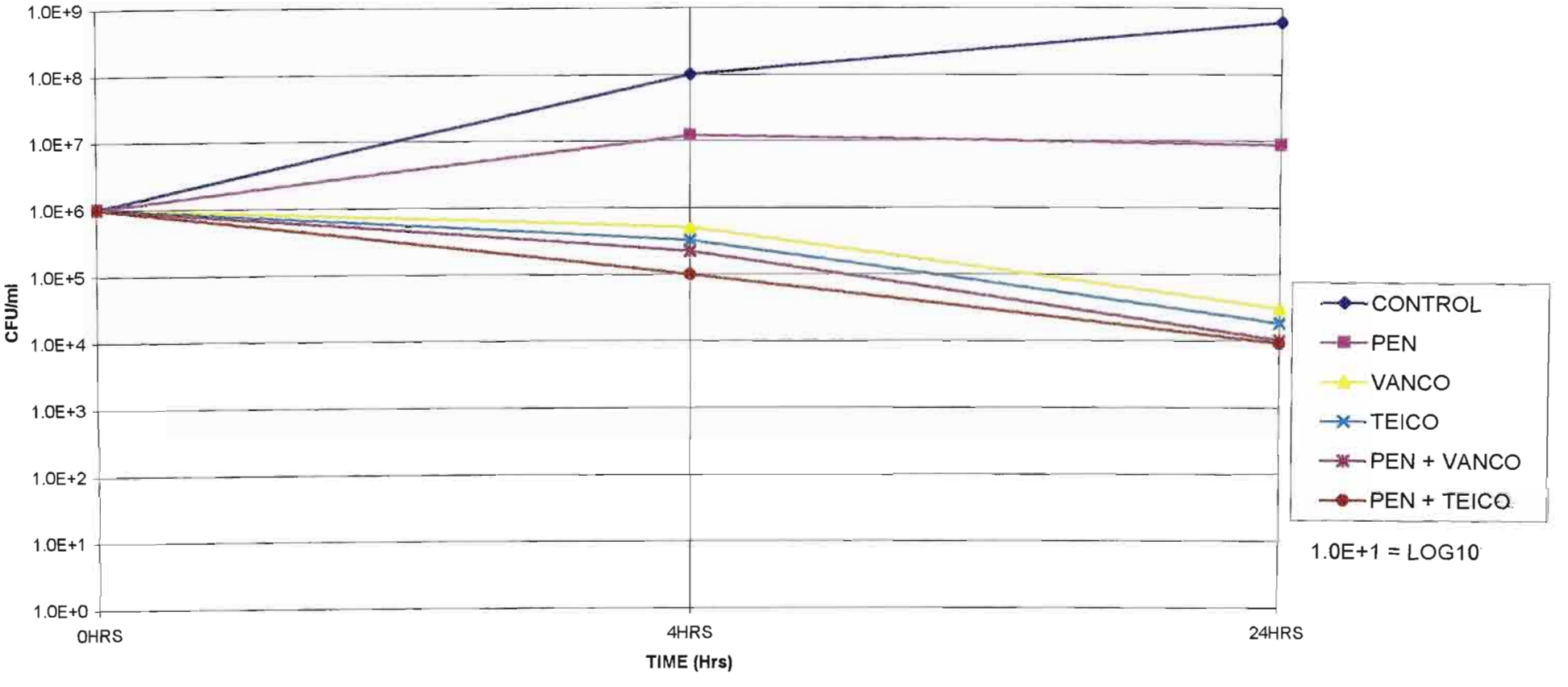


Fig.17.2 Vancomycin and teicoplanin showing an additive effect against *E.faecium* which was highly resistant to gentamicin and penicillin as shown by the time kill curves

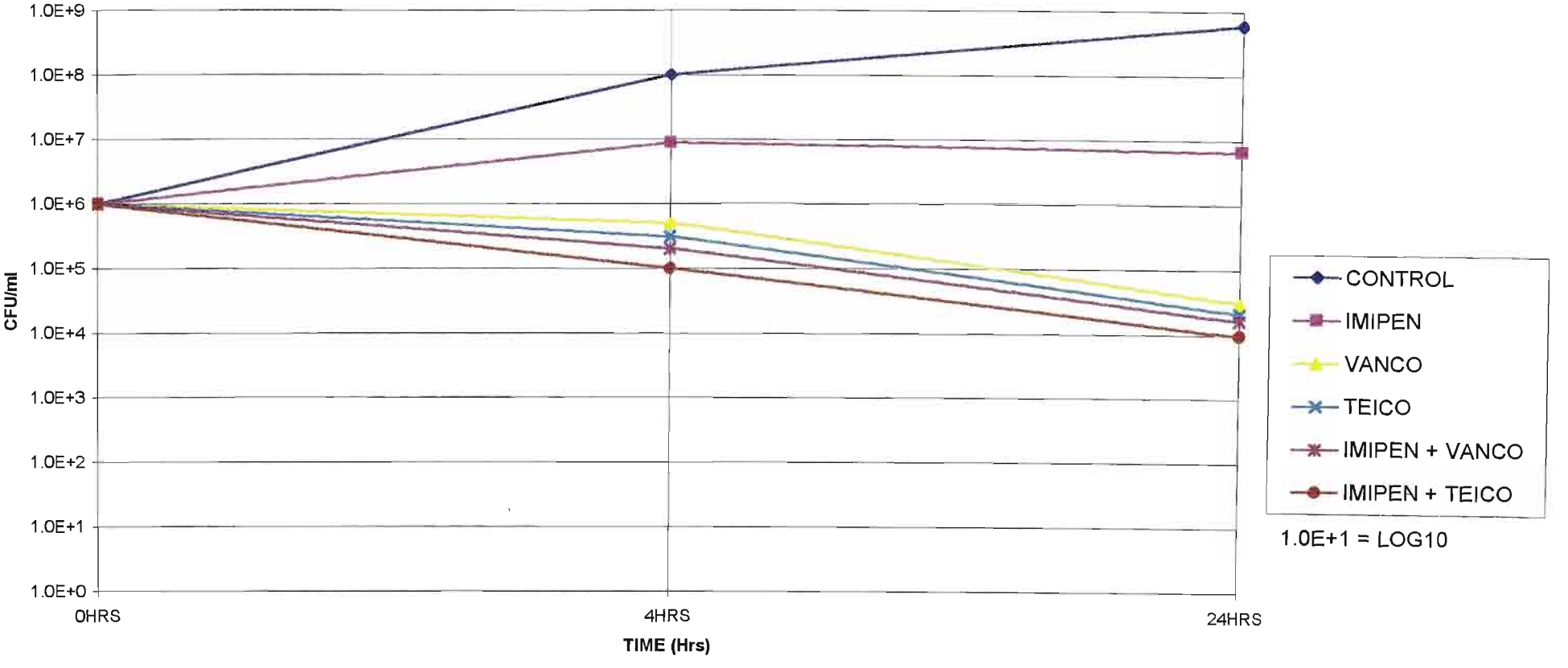


Fig.17.3 Time-kill curves showing an additive effect against *E.faecium* when imipenem was combined with vancomycin or teicoplanin. This isolate displayed high-level resistance to gentamicin and imipenem

The time-kill studies suggest that penicillin, ampicillin or imipenem that were susceptible when combined with gentamicin displayed synergy against *E.faecalis* with low-level gentamicin resistance (MIC≤500μg/ml), and showed susceptibility to these beta - lactams (MIC≤1μg/ml).

In the presence of high-level gentamicin resistance (MIC ≥2000μg/ml), beta-lactam susceptible *E.faecalis* (MIC ≤1μg/ml), when exposed to a combination of the beta-lactams and glycopeptides, displayed synergism.

Time-kill studies performed on *E.faecium* isolates with high-level resistance to ampicillin, penicillin, imipenem (MIC 32-128μg/ml) and gentamicin (MIC ≥2000μg/ml) showed an additive effect only, although an increased concentration of the beta-lactams were used.

Ampicillin, penicillin or imipenem combined with vancomycin also failed to show synergy against these *E.faecium* isolates. The result was an additive effect although the isolates were susceptible (MIC's of ≤1μg/ml) to the glycopeptides.

4.7 PULSED-FIELD GEL ELECTROPHORESIS

Plates 1 and 2 show chromosomal digestion patterns of *E.faecalis* and *E.faecium.*

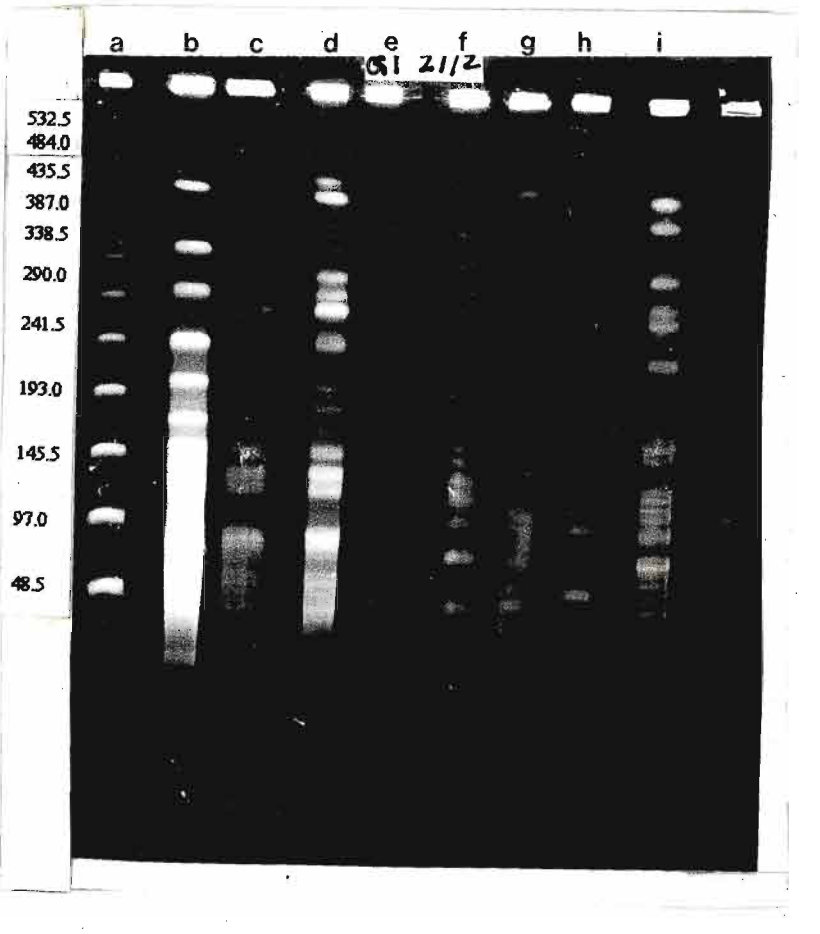


Plate 1 Pulsed-field gel electrophoresis of *Sma* I digested chromosomal DNA of *E.faecalis*

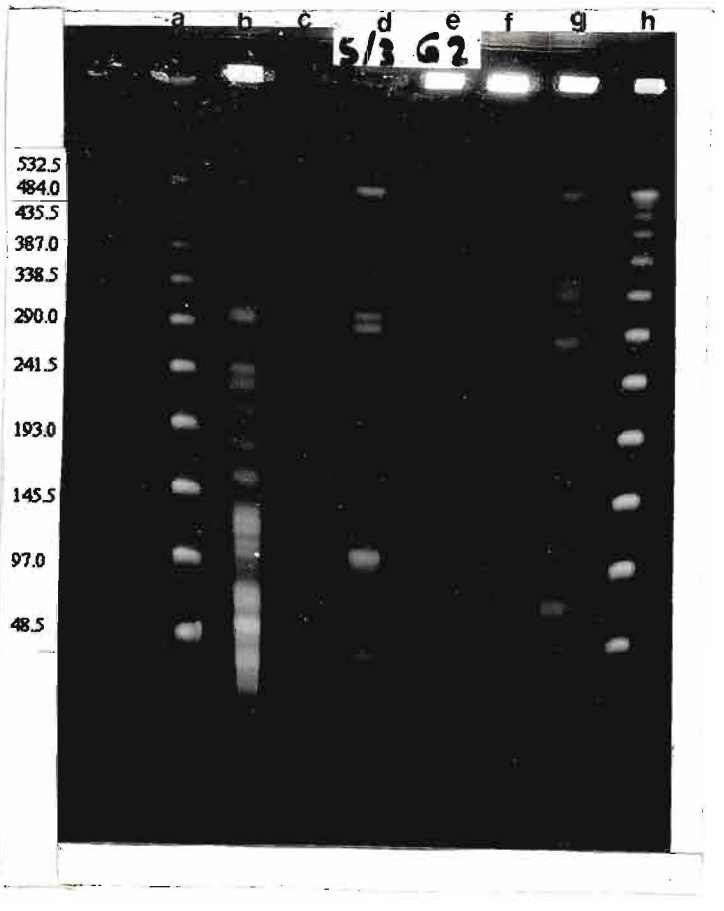


Plate 2 Pulsed-field gel ecectrophoresis of *Sma I* digested chromosomal DNA of *E.faecium*

The restriction endonuclease digestion patterns of *E.faecalis* isolates studied are shown in plate 1. The molecular sizes are given in the left-hand margin as kilobases (kb). Lane a shows the lambda ladder standard (λ concatemer). Lanes b, c and d show isolates that displayed high-level resistance to gentamicin and streptomycin. Lane e shows the negative control (TBE buffer). Lanes f and g were *E.faecalis* isolates that showed high-level resistance to gentamicin. Lanes h and i were strains that were highly resistant to streptomycin.

Plate 2 shows the pulsed-field gel electrophoretic patterns of *Sma*I digested chromosomal DNA from isolates of *E.faecium*. Lanes a and h show the lambda ladder standard (λ concatemer). Lane b displayed an isolate showing high-level resistance to gentamicin and streptomycin.
Lane c was the TBE buffer (control). Lane d, e and f showed *E.faecium* isolates that displayed high-level resistance to gentamicin.

Results of the genetic study showed that there was a wide variation in the restriction endonuclease fragment patterns among the *E.faecalis* and *E.faecium* isolates.

CHAPTER 5

DISCUSSION

5.1 IDENTIFICATION

The genus *Enterococcus* contains Gram positive cocci that are presumptively identified by their ability to hydrolyse aesculin and their tolerance to 40% bile and 6.5% NaCl. They are further identified by their ability to grow at extreme temperatures of 10 and 40°C and survive for a period of 30 minutes at 60°C. Most enterococci contain the Lancefield group D antigen. Hydrolysis of pyrrolidonyl-β-naphthylamide (PYR) is a characteristic feature that is also seen with group A streptococci but not with other streptococci.

Although the above screening tests appeared to be sufficient in the past to identify enterococci presumptively it is now recognised that other less commonly encountered Gram positive cocci can also give a positive reaction in some of these tests.
For example, some *Lactococcus, Aerococcus, Pediococcus* and *Leuconostoc* species are bile aesculin positive or can grow in 6.5% NaCl or both. Strains of *Pediococcus* and *Leuconostoc* species can react with Lancefield group D antigen and some *Lactococcus* and *Aerococcus* species are PYR positive.

In serious infections such as endocarditis, differentiation between *E.faecalis* and *E.faecium* (the 2 most commonly encountered enterococcal species) can be useful because of the naturally occurring differences in the susceptibility patterns of these species. *E.casseliflavus* and *E.faecium* showed similar susceptibility patterns. *E.faecalis* , *E.raffinosus and E.gallinarium* differed only slightly in their susceptibility patterns.

Also important for species identification of enterococci is that it may be useful for epidemiologic surveillance in hospitals.

Studies have shown that the *Enterococcus* is a nosocomially spread pathogen (78, 91, 116). Speciation and further typing within a genus assists in outbreak control.

The API 20 Strep system using the analytical profile index or the software profile recognition program to identify the enterococci was not able to identify the less common species accurately ie. *E.casseliflavus*, *E.raffinosus* and the beta-haemolytic enterococci.

It is important to note that some of the commercial or computer software identification systems for the species of enterococci were probably evaluated before some of the newer species were recognised and may therefore not be accurate.

By using the conventional identification system devised by Facklam and Collins, most if not all enterococci can be easily identified to species level. Fifteen biochemical tests which are easily prepared in the laboratory are used for this identification system. These media are cheaper than the API identification strips.

If the *Enterococcus* belongs to one of the common species such as *E.faecalis* or *E.faecium* the API 20 Strep system or the Facklam scheme would be able to accurately identify the isolate. In the case of one of the uncommon species of enterococci the best system to use is the Facklam scheme since it only requires the availability of the Facklam media. Laboratories should therefore consider using this system.

5.2 ANTIMICROBIAL SUSCEPTIBILITY TESTING

5.2.1 Susceptibility tests results

One of the most important factors in *in-vitro* testing for antimicrobial activity is the standardization of the method used. This is achieved by the use of a control (ATCC or NCTC) bacterial organism. It is the correct susceptibility test result that is useful in predicting the response of the patients treated with the appropriate antimicrobial agents.

In a diagnostic laboratory, a convenient method of performing antimicrobial susceptibility testing is required and in one form or another a disc-diffusion method is most commonly used. It is less time consuming and easily performed in the laboratory.

Common problems encountered with the disc-diffusion tests is the inactivation of labile antibiotics such as co-amoxiclav, due to inadequate storage or handling in the laboratory. This may be indicated by a gradual decrease in zone size. Too heavy or too light an inoculum may be indicated by a general decrease or increase in zone sizes. Errors in transcribing the result or measuring zone sizes may also occur. Problems with medium may be encountered eg. high pH will produce larger zones with aminoglycosides and erythromycin and smaller zones with tetracycline and fusidic acid. The reverse may occur if the pH is too low.

The two disc-diffusion methods compared were Kirby-Bauer and Stokes method. Both the methods showed similar results for most of the antibiotics tested. The 3 antibiotics that showed a discrepancy between the two methods were penicillin, chloramphenicol and cotrimoxazole. This is because of the higher breakpoint used for the Kirby-Bauer method which resulted in a much higher percent of the isolates being susceptible. For ciprofloxacin the same concentration of antibiotic is used for both the methods but the Stokes method showed a slightly higher percent of resistance than the Kirby-Bauer method.

In the breakpoint method of susceptibility testing fixed concentrations of antimicrobial agents are incorporated into the agar. One or more concentration of an antibiotic chosen is intended to conform to agreed cut-off points of bacterial susceptibility or resistance.

The breakpoint method has considerable practical advantages. Large numbers of isolates can easily be tested by the use of a multi-point inoculation technique. The clear cut end-points ie. either growth or no growth of a bacterial isolate removes interpretation difficulties.
The chief disadvantage of this method is that it does not provide information as to whether an organism is highly susceptible or highly resistant; this limitation is usually important in the assessment of degrees of resistance.

Clerical errors can be made in transcribing the results from the multi-point inoculating plates since the isolates inoculated are very close together.

Comparison of the Stokes disc diffusion with the breakpoint method results for penicillin showed that 79.4% were resistant with the disc diffusion test and only 13.1% with the breakpoint method. This discrepancy is because of the lower concentration of penicillin used for the Stokes method. The Kirby-Bauer disc diffusion and the breakpoint methods showed identical susceptibility test results for penicillin with only 13.1% being interpreted as resistant. Both the disc diffusion methods and the breakpoint method showed identical results for ampicillin, imipenem, piperacillin, vancomycin and teicoplanin.

To quantitatively measure the in-vitro activity of an antimicrobial agent against a bacterial culture, a broth or agar dilution technique may be used to determine the minimum inhibitory concentration of an antibiotic (MIC).

MICs are still generally considered to be the reference standard for comparison and evaluation of other susceptibility tests and the efficacy of all antimicrobials are described in terms of MIC.

The greatest use of the MIC is to compare new antimicrobial agents against a battery of bacterial strains.
However, MIC values are still required in routine laboratories as a guide for determining therapy whenever the susceptibility of a pathogen is unpredictable ie. it is difficult to determine whether that isolate is susceptible, intermediate or resistant by means of breakpoint methodology (disc diffusion or agar incorporation).

MIC values are also important when an infection is not responding to apparently appropriate therapy particularly when the organism is reported being susceptible to the antibiotics tested. Louie et al., reported that the MIC_{50} and MIC_{90} (μg/ml) for 34 *E.faecalis* strains tested for ampicillin was 2 and 4μg/ml, imipenem 8 and 16μg/ml, vancomycin 2 and 4μg/ml and teicoplanin 0.25 and 0.5μg/ml respectively. For the 103 *E.faecium* strains, the MIC_{50} and MIC_{90} (μg/ml) for ampicillin was 8 and 64μg/ml, imipenem was 32 and >64μg/ml, vancomycin was 2 and 8μg/ml and teicoplanin was 0.5 and 1μg/ml respectively (58).

Gordon *et al.*, showed lower MIC_{50} and MIC_{90} (μg/ml) results than those cited by Louie *et al.* They tested 633 *E.faecalis* isolates. MIC_{50} and MIC_{90} (μg/ml) for penicillin was 2 and 4μg/ml, ampicillin was 1 and 2μg/ml, imipenem was 1 and 2μg/ml, vancomycin was 2 and 4μg/ml and teicoplanin was 0.25 and 0.5μg/ml respectively (33) and lower than those found by Louie *et al.* (58).

The MIC_{50} and MIC_{90} (μg/ml) for the 58 *E.faecium* strains tested were as follows :penicillin 32 and >128μg/ml, ampicillin 8 and 64μg/ml, imipenem 16 and 32μg/ml, vancomycin 1 and 2μg/ml and teicoplanin 0.5 and 1μg/ml respectively (33).

Our studies showed that the 70 *E.faecalis* isolates tested had identical MIC_{50} and MIC_{90} (μg/ml) results, for penicillin, ampicillin and imipenem. MIC_{50} and MIC_{90} (μg/ml)for penicillin and ampicillin was 1 μg/ml and imipenem was 0.5μg/ml.
The MIC_{50} μg/ml for the *E.faecium* strains tested was 16 μg/ml for the beta-lactams and the MIC_{90} (μg/ml) was 64 μg/ml for penicillin and 32 μg/ml for ampicillin and imipenem. The *E.faecium* MIC_{50} and MIC_{90} (μg/ml) were much higher than the *E.faecalis* isolates (33,58). Our results were similar to those reported by Gordon *et al.*, (33).

5.2.2 Effect of blood in the medium for susceptibility testing

Jenkins *et al.* reported that disc diffusion tests on 5% sheep blood enriched Mueller-Hinton agar gave enlarged zone sizes that falsely indicated susceptibility (40). For gentamicin and tobramycin 70% and 72% respectively of the isolates changed from susceptible to resistant when these isolates were tested on Mueller-Hinton agar only (40). Amikacin showed a false susceptibility of 4% and kanamycin 2% on media supplemented with blood; agar dilution MIC's for gentamicin and tobramycin showed only 6-10% false susceptibility (40).

Amikacin and kanamycin MICs remained the same or decreased by one dilution only on unsupplemented media (40). In most cases the discrepancy was not sufficient to change the MIC interpretation.

Results obtained in this study for the aminoglycosides on blood enriched media for both the disc diffusion methods showed a false susceptibility of 50% for gentamicin and 32% for tobramycin and netilmicin.

With the breakpoint method the presence of blood showed a false susceptibility of 49.6%, 41% and 37.4% for gentamicin, tobramycin and netilmicin. Amikacin remained unaffected.

MIC results obtained in this study confirmed this. Gentamicin, tobramycin and netilmicin dilutions were two fold lower and amikacin was unaffected when tested on Mueller-Hinton agar +5% lysed blood.

It has been reported that the presence of 5% lysed blood and the type of culture media used give false susceptibility test results for certain second and third generation cephalosporins (98,109). Disc-diffusion comparison of the *E.faecalis* tested by Sahm *et al.* (45 strains) on media supplemented with blood showed discrepancies that did not exceed 4% for cephalothin, cefamandole, cefoperazone (98,109). In contrast, there was a much greater discrepancy 18% for cefotaxime and 14% for cefuroxime, ceftizoxine (98,109).
MICs for cephalothin, cefamandole, cefoperazone were not substantially influenced by the type of culture media or the addition of blood to the medium. MICs remained the same or was one dilution lower (98,109).

Comparitively, MICs for cefuroxime, ceftizoxime, ceftriaxone and cefotaxime varied both with a brand of media used and the addition of 5% lysed blood. MICs were 8-16 times greater with blood supplemented media (98,109).

Results obtained in this study for both the disc diffusion methods showed very similar results on blood enriched media for the cephalosporins.

Cefotaxime was the only cephalosporin that was greatly affected, showing a false susceptibility of 29%. Cefuroxime was less affected with a false susceptibility of 18%.

Cefotaxime was also the only cephalosporin that was greatly affected by the presence of blood in the medium with the breakpoint method, resulting in a false susceptibility of 23.3%. Cefuroxime displayed a false susceptibility of 12.1%, cefamandole was unaffected and the cephalosporin that was slightly affected 2.8% was cephalothin on blood enriched media.

Results of this study for MICs showed that the only cephalosporin that was markedly affected by blood supplemented media was cefotaxime. The MIC was 3 times lower on unsupplemented media. MICs for cefamandole and cephalothin with and without blood remained the same except for cefuroxime that was 2 dilutions lower in the presence of media supplemented with 5% lysed blood.

It is important to note that false susceptibility in the presence of blood for enterococci does occur with certain aminoglycosides and cephalosporins. According to Sahm *et al.* (98), false susceptibility was shown to be caused by heme. It has been postulated that the heme effect is related to the catalytic cleavage of intracellular H_2O_2 which results in lipid peroxidation which reduces zones of inhibition.

Enterococci grow well and give accurate susceptibility results on Mueller-Hinton agar without blood supplementation and because of the problem of false susceptibility with certain aminoglycosides and cephalosporins, it is recommended that enterococci routinely be tested on media without the addition of blood.

5.3 HIGH-LEVEL AMINOGLYCOSIDE RESISTANCE

Recognition of the importance of high-level aminoglycoside resistant enterococci (MIC ≥ 2000μg/ml) has clearly increased during this past decade. Medical centres in the United States and elsewhere are reporting resistant rates ranging from 4.5 to 55% (42,61,117). Keddy *et al.* from Johannesburg in South Africa showed that the high-level gentamicin resistance was 26.5% for *E.faecalis* and 20% for *E.faecium* isolates. 144 *E.faecalis* and 15 *E.faecium* strains were tested (113).

The results of this study showed that 39 (36.4%) of the enterococci tested displayed high-level resistance to gentamicin and streptomycin. All isolates that were resistant to 500μg/ml of gentamicin were also resistant to 2000μg/ml of gentamicin. A lower concentration of gentamicin ie.500μg/ml is a good screen to detect high-level gentamicin resistance.

The aminoglycoside inactivating enzyme that is responsible for streptomycin high-level resistance is the phosphorylating enzyme (APH3"). The acetylating enzyme, AAC' inactivates gentamicin and tobramycin. AAC6', another acetylation enzyme confers high-level resistance to gentamicin, amikacin, tobramycin and kanamycin.
AAC3-1, AAC3-2 inactivates gentamicin, tobramycin and kanamycin.
The adenylating enzyme, ANT2" confers high-level resistance to gentamicin, tobramycin and kanamycin. Streptomycin is also inactivated by the adenylating enzyme AAD3" (66). Since most clinical laboratories do not perform full MICs routinely and certainly not synergism studies, screening tests that predict high-level aminoglycoside resistance should be routinely performed considering the high percent of aminolglycoside resistance being observed.

A number of simple screening methods are available for detecting the presence of high-level aminoglycoside resistance. These methods include a disc diffusion method using high content aminoglycoside discs, a broth or agar screen supplemented with a particular aminoglycoside to a final concentration of 500 and/or 2000μg/ml and finally more recently the introduction of the Etest. According to Leclercq *et al.* high-level resistance to aminoglycosides in enterococci is conveniently detected by the disc diffusion technique using high content discs with a concentration of 1000μg for streptomycin and 500μg for gentamicin (51).

Lower concentrations of aminoglycosides may also be used to detect aminoglycoside resistance as based on the concentrations chosen by Rosenthal and Freundlick ie. 120μg for gentamicin and 300μg for streptomycin (95).

According to Sahm *et al.*, discs can accurately discriminate between low-level and high-level of resistance. Zone diameters of $\leq$ 6-7mm for the 300 and 120μg discs is a reliable indicator of aminoglycoside resistance. Zone diameters of $\geq$ 10 is indicative of synergy susceptibility ie. these isolates show synergy with a beta - lactam aminoglycoside combination (95). A zone diameter of $<$ 11mm is indicative of high-level gentamicin resistance and a zone diameter of $<$ 12 is indicative of high-level streptomycin resistance when using a high content disc of 500μg (51).

If there is no growth after 24 hours, it is advisable to reincubate the agar plates or broth for a further 24 hours before final results can be determined (3). About 95% of the isolates that were tested showed growth in 24 hours, only 5% showed growth after a further 24 hour incubation.

There is usually little need to test aminoglycosides other than streptomycin and gentamicin since these are the two agents for which there are most clinical data and are most commonly used for synergism.
Most gentamicin resistant organisms described thus far, are also highly resistant to kanamycin, amikacin, tobramycin and netilmicin, therefore there is no indication for routine testing of these agents (66).

High-level gentamicin resistance makes it difficult to identify a regimen that will be bactericidal. This difficulty is compounded by the increasing frequency of acquired resistance to beta-lactams and glycopeptide agents among enterococci.

The consequence of such resistance for effective treatment of serious enterococci infections makes screening designed to detect high-level aminoglycoside resistance among the most relevant of the susceptibility testing procedures performed. It is thus recommended that all enterococcal isolates be screened for high-level aminoglycoside resistance especially where synergism between a beta-lactam and an aminoglycoside will be required such as for bacterial endocarditis. Evaluation and establishment of test accuracy when performing screening for high-level aminoglycoside resistance is therefore of major importance.

5.4 BETA-LACTAMASE PRODUCTION

For susceptibility testing, inoculum should be approximately 10^7-10^8CFUs/ml for the organism to produce a large enough amount of enzyme that can inactivate the antibiotic and thus make it resistant.

Whether all ampicillin susceptible enterococci should be tested for the production of beta-lactamase is still debatable. Beta-lactamase producing strains may remain undetected since disc susceptibility may fail to detect those isolates if the inoculum size is too low ie. $< 10^6$ CFU/ml. It seems reasonable however, to test all enterococcal isolates for beta-lactamase production from very serious infections such as bacterial endocarditis. Testing with the chromogenic cephalosporin, nitrocephin is a simple method which can be easily performed in the laboratory.

All isolates tested were beta-lactamase negative, how long it would remain so locally is yet to be seen.

5.5 THE STUDY OF SYNERGISM

Time-kill studies were performed on 4 *E.faecalis* isolates and 4 *E.faecium* isolates to determine the minimal concentration required to obtain a synergistic effect with a fixed concentration of 5μg/ml for gentamicin and teicoplanin and 10μg/ml for vancomycin.

According to the minimum inhibitory concentration (MIC) of the isolates, the following concentration of antibiotics were chosen for ampicillin, penicillin and imipenem.

6-10μg/ml : MIC of less than 32μg/ml.

50μg/ml : MIC between 32 and 128μg/ml.

100μg/ml : MIC of greater than or equal to 256μg/ml

From the time-kill study graphs it was seen that in the enterococcal isolates that were susceptible to ampicillin, penicillin and imipenem (MIC $\leq$1 μg/ml), with low-level gentamicin resistance (MIC $\leq$ 500μg/ml) showed effective synergistic killing by the combination of ampicillin plus gentamicin or penicillin plus gentamicin. Thus the combination of ampicillin plus gentamicin or penicillin plus gentamicin showed a synergistic effect. Imipenem plus gentamicin has not been often used, thus clinical data is lacking, but could be successfully used judging by the results. Ampicillin and penicillin alone also produced very good activity on all the *E.faecalis* isolates tested as was demonstrated by the time-kill synergy studies.

Ampicillin, penicillin or imipenem (MIC <1.0μg/ml) in combination with the glycopeptides displayed synergism in the presence of high-level gentamicin resistance (MIC $\geq$ 2000μg/ml).

Vancomycin or teicoplanin combined with an aminoglycoside provides an effective alternative therapy for serious life threatening infections that occur in patients that cannot tolerate the penicillin-class antibiotics.

The glycopeptides are also recommended as the drugs of choice for treatment of enterococci with high-level ampicillin and penicillin resistance (MIC ≥ 128μg/ml). Vancomycin and teicoplanin alone showed very good activity against all the *E.faecalis* isolates tested.

For the one *E.faecium* strain that had an MIC of 1024μg/ml for gentamicin, 32μg/ml for ampicillin, 128.0μg/ml for penicillin and imipenem, a higher concentration of ampicillin, penicillin and imipenem (50μg/ml) was needed for the time-kill study. In this setting even the increased concentration of antibiotic still failed to show synergism between ampicillin, penicillin or imipenem plus gentamicin. An additive or indifferent interaction was observed.

Although the MIC for vancomycin and teicoplanin for this isolate was susceptible (MIC 1.0 and 0.25μg/ml respectively), synergism was not observed between the glycopeptides plus gentamicin. This combination also appeared indifferent.

Time-kill studies were performed on an *E.faecium* isolate that had an MIC of 64.0μg/ml for ampicillin and 128.0μg/ml for penicillin and imipenem indicating resistance. This strain also displayed high-level gentamicin resistance (MIC≥2000μg/ml). Due to the high MIC values, 50μg/ml of ampicillin, penicillin and imipenem was used for the time-kill studies. This isolate produced an additive effect only.

Synergy studies combining any one of these beta-lactams with gentamicin showed no synergy; only an additive effect was observed.

The combination of ampicillin, penicillin or imipenem plus vancomycin or teicoplanin also resulted in the lack of synergism although vancomycin and teicoplanin had susceptible MIC values (1.0 and 0.5μg/ml respectively).

One-hundred μg/ml of ampicillin, penicillin and imipenem were used for time-kill testing of a very resistant *E.faecium* strain with a MIC of 64μg/ml for ampicillin, 256ug/ml for penicillin and imipenem. This isolate also had a gentamicin MIC of $\geq$2000μg/ml. The isolate was susceptible to the glycopeptides with MICs of 1.0μg/ml for vancomycin and 0.5μg/ml for teicoplanin.

Ampicillin, penicillin and imipenem plus vancomycin or teicoplanin failed to show synergism against this isolate. An indifferent phenomenon was observed.

For these *E.faecium* strains displaying high-level aminoglycoside and beta-lactam resistance, a trend towards an additive interaction was observed. Thus the glycopeptides plus increased dosages of the beta-lactam antibiotics or imipenem has little to offer against these emergent nosocomial pathogens.

The increasing numbers of enterococcal strains displaying high-level resistance to antibiotics used as standard therapies for serious infections as well as the absence of synergistic activity with combinations of these antimicrobial agents is very disturbing.

New bactericidal antibiotics or combinations of antibiotics are needed to treat serious enterococcal infections that display multiple antibiotic resistance especially among the *E.faecium* strains.

5.6 PULSED-FIELD GEL ELECTROPHORESIS

The availability of reliable molecular strain typing methods such as PFGE may be a critical factor in determining the success of an epidemiologic investigation (1). PFGE may also be used to identify related isolates which are selected for their common resistance patterns.

Since isolates from this study were not from an outbreak, it was therefore not meant to analyse epidemiological observations but to determine the genetic profile between the different high-level aminoglycoside resistance patterns of a selected number of *E.faecalis* and *E.faecium* strains.

For analytic purposes, restriction fragment analysis of genomic DNA using PFGE are regarded as genetically related if they are undistinguishable (identical) from each other or are so similar that they are presumed to be derived from a common source. These isolates have restriction endonuclease digestion patterns that have the same number of bands and the corresponding bands have the same apparent size (1)

An isolate is considered to be closely related to an outbreak or resistant strain if its PFGE pattern differs only by changes consistent with a single genetic event which may be a point mutation whereby a change in the PFGE pattern shows a lack of one fragment present in an outbreak or resistant pattern and have 2 new smaller fragments approximately the size of the larger fragment. Such changes typically result in 2 or 3 band differences (1).
Point mutations may also result in the loss of a restriction site whereby the altered pattern would have a new larger fragment and would lose 2 smaller fragments (1).

Insertion of DNA into an existing restriction fragment results in isolates that are closely related with the genetic pattern showing a lack of one small fragment and a new fragment of a larger size (1).

Deletion of DNA from a fragment shows a new fragment of a smaller size and loss of a larger fragment also indicating closely related strains to an outbreak or isolates that have common resistant patterns (1).

Isolates are considered to be possibly related if its PFGE pattern differs by changes consistent with 2 independent genetic events ie. 4 to 6 band differences as compared to an outbreak isolate or an isolate with a particular resistant pattern (1).

If the restriction endonuclease pattern differ by changes consistent with 3 or more independant genetic events, generally 7 or more band differences, these isolates are considered genetically unrelated (1).

A situation that can make interpretation of chromosomal DNA patterns difficult and in some instances unknown is how to interpret small differences (79).

The genetic study showed that there was considerable restriction endonuclease fragment differences among the isolates tested with similar and different resistance patterns. Fragment sizes ranged from ≤48.5 to 532.5 kilobases.

The genomic profile of the 3 *E.faecalis* isolates displaying high-level aminoglycoside resistance and susceptible to penicillin and ampicillin, showed a large band of 484.0 kilobases. These isolates could possibly be related because there was a difference of between 4 to 6 bands.

The 2 *E.faecalis* isolates showing high-level gentamicin resistance with ampicillin and penicillin being susceptible were genetically unrelated. There was a difference of about 7 to 8 bands between these 2 strains.

The restriction endonuclease digestion pattern of the 2 streptomycin high-level resistance *E.faecalis* strains that were susceptible to the beta-lactam antibiotics, also showed an unrelated genetic profile. There was at least a difference of 7 bands.

The one *E.faecium* isolate that displayed high-level resistance to both the aminoglycosides as well as ampicillin and penicillin showed a large fragment of 532.5 kilobases and did not resemble the *E.faecalis* strains that were highly resistant to gentamicin and streptomycin.

The chromosomal digestion patterns of the 3 *E.faecium* isolates that displayed high-level streptomycin resistance and was susceptible to ampicillin and penicillin could possibly be genetically related. There was a difference of at least 5 to 6 fragments.

From the PFGE results it is concluded that none of the *E.faecalis* or *E.faecium* isolates were either similar or closely related therefore it can be presumed that the genetic elements leading to resistance occurred independently. It also suggests that PFGE analysis of enterococcal isolates during a suspected nosocomial outbreak, would be a useful tool to confirm spread of a particular strain.

CHAPTER 6

CONCLUSION

The API 20 Strep system using either the analytical profile index or the computer API profile recognition program can accurately identify the commonly isolated enterococci ie. *E.faecalis* and *E.faecium*. *E.raffinosus* was mis-identified as *E.faecalis* and *E.casseliflavus* as *E.faecium*. Thus this system cannot be relied on to accurately identify uncommon enterococci.

The scheme described by Facklam and Collins enables microbiologists to identify accurately most *Enterococcus* species with a minimum number of conventional tests for phenotypic characteristics. For appropriate management of serious enterococcal infections, the identification to species level is important since enterococcal species such as *E.faecium* and *E.casseliflavus* are resistant to a range of antimicrobial agents and express high-level resistance to penicillin and ampicillin (MIC >128.0μg/ml).

The use of an inoculum $<10^7$-10^8 CFUs/ml for susceptibility testing of enterococci results in false susceptibility to penicillin and ampicillin. It is therefore recommended that beta-lactamase testing be performed on all serious enterococcal infections since a false susceptibility report can result in the patient being treated inappropriately with ampicillin with devastating consequences for the patient.

With the high percent of false susceptibility for aminoglycosides and cephalosporins reported with blood enriched media, prompted the testing of aminoglycosides and cephalosporins.
On average 55% of the isolates showed false susceptibility to gentamicin and 35% to tobramycin and netilmicin with media supplemented with blood.

The only cephalosporins affected was cefotaxime and cefuroxime with a false susceptibility of 28% and 17% respectively. These antibiotics were the same as described by others to be affected by blood enriched media.

False susceptibility for the aminoglycosides was not as high as described by others. The reason for this might be that all the isolates that showed a few colonies around the zone of inhibition were interpreted as resistant on blood enriched susceptibility agar plates.

All laboratories should be aware that false susceptibility for enterococcal isolates does occur for gentamicin, tobramycin, netilmicin, cefotaxime and cefuroxime when media supplemented with blood are used.

Information regarding the mechanism through which the discrepancy in susceptibility testing of gentamicin, tobramycin, cefotaxime and cefuroxime occurs would facilitate the formulation of new types of media or more accurate and reliable *in-vitro* methods for testing these antibiotics with enterococci.

For now media such as Mueller-Hinton agar without the addition of blood should be used to test aminoglycosides and cephalosporins for enterococcal isolates, since these organisms grows well in this media.

Tests for the detection of high-level gentamicin resistance, which precludes bactericidal synergy with clinically achievable concentrations of the antibiotic, make time consuming and labour-intensive synergy tests such as time-kill studies and checker-board titrations unnecessary.

The high frequency of high-level resistance to gentamicin (26.2%) and streptomycin (31.8%) as shown from the study is a matter of concern. The alternative treatment regimens for infection with such strains are limited to single therapy with one of the glycopeptides.

Thus screening of enterococci for high-level aminoglycoside resistance is an essential test for management of patients with serious enterococcal infections such as bacterial endocarditis.

The high content disc, broth or agar plate (with a concentration of 500 or 2000μg/ml of gentamicin) and the Etest methods appear highly reliable for the detection of such resistance to gentamicin. Any one of these methods may be used depending on the facilities available in the laboratory.

The study of synergism which is determined by the killing rate or the establishment of a killing curve have become redundant and can be replaced for high-level organism resistance.

On the basis of studies presented, it appears that *E.faecalis* strains that demonstrate intrinsic low-level gentamicin resistance (MIC $\leq$ 500μg/ml) and are susceptible to penicillin and ampicillin (MIC $\leq$1.0μg/ml) show a synergistic effect between the beta-lactams and gentamicin. This still represent the optimal therapy for severe enterococcal infections.

In the presence of high-level resistance to gentamicin (MIC $\geq$2000μg/ml) *E.faecalis* isolates that were susceptible to ampicillin and penicillin (MICs of $\leq$1.0μg/ml), the combination of penicillin plus vancomycin or teicoplanin, ampicillin plus vancomycin or teicoplanin displayed synergism. These combinations might be successful for the treatment of high-level aminoglycoside resistant strains of enterococci.

In the *E.faecium* strains that displayed high-level gentamicin resistance with MICs of 128.0 or 256μg/ml for penicillin and 64 or 128μg/ml for ampicillin; synergism was not observed, although an increased concentration of beta-lactam antibiotic was used.

Vancomycin and teicoplanin are bactericidal antibiotics. These antimicrobial agents inhibit cell wall synthesis and are able to kill susceptible micro-organisms. In general bactericidal antimicrobial agents produce MBCs within 2 two-fold dilutions ie. 4 times the MIC. With *E.faecalis* and *E.raffinosus* the MBCs for the glycopeptides were 3-4 two-fold dilutions ie. 8-16 times greater than the MIC for *E.faecalis* and *E.raffinosus.* This indicates a bacteriostatic effect for the glycopeptides at a concentration obtained *in-vivo*.

E.faecium and *E.casseliflavus* displayed tolerance which is when the MBC:MIC ratio is $\geq$32 after 24 hours of incubation. Tolerance renders a normally bactericidal agent bacteriostatic. Although vancomycin and teicoplanin display bacteriostatic effect for *E.faecalis* and *E.raffinosus* and a tolerant effect for *E.faecium* and *E.casseliflavus* , these antimicrobial agents have a very good therapeutic effect against such organisms.

In view of reports of vancomycin resistant strains in various parts of the world, the universal susceptibility of enterococci to vancomycin no longer exists.

Routine susceptibility testing of this antibiotic is thus a necessity for clinically relevant enterococcal isolates.

All isolates tested for vancomycin are susceptible at this stage. It is vitally important that laboratories perform beta-lactamase tests to accurately identify penicillin and especially ampicillin resistance.

Use of molecular strain typing such as pulsed-field gel electrophoresis (PFGE) has now become a standard practice in hospital infection control to evaluate the epidemiology of nosocomial infections. PFGE is one of the most widely used tools for investigating bacterial outbreaks and for studying of relationships between multi-resistance or high-level resistance of bacteria. It provides information on genetic relations between isolates. Our study by means of pulsed-field gel electrophoresis (PFGE) of *E.faecalis* and *E.faecium* with high-level resistance to gentamicin and / or streptomycin showed that the isolates with the same susceptibility patterns had different restriction endonuclease patterns. That is, there was no evidence of a common origin of these isolates.

Thirteen percent of the enterococcal isolates showed high-level resistance (MIC 256μg/ml) to penicillin, ampicillin and imipenem. Nine (8.4%) were *E.faecium* and 5 (4.7%) were *E.casseliflavus* and none were *E.faecalis.* Ten (9.3%) of these isolates displayed high-level aminoglycoside resistance. Seven (6.5%) showed high-level resistance to gentamicin and streptomycin, 2 (1.9%) to streptomycin only and 1 isolate to gentamicin only. All 4 (3.7%) of the *E.casseliflavus* strains showed high-level resistance to both the aminoglycosides. Of the 6 (5.6%) *E.faecium* strains, 4 displayed high-level resistance to gentamicin and streptomycin, 1 isolate was only resistant to gentamicin and the other to streptomycin only.

There are no alternatives to treat enterococcal infections if the organism displays high-level aminoglycoside resistance, is resistant to the penicillins as well as the glycopeptides. There is a strong need for research, to leap ahead in order to understand the mechanism of how enterococci seem to acquire multiple resistance to antibiotics at such an alarming rate. In the meantime, the only alternative for high-level resistance to penicillin, ampicillin and vancomycin resistance especially for the *E.faecium* strain seems to be teicoplanin or daptomycin.

From these observations the following recommendations can be made for susceptibility testing of enterococci. For the aminoglycosides and cephalosporins susceptibility testing should be performed on media without the addition of blood. Screening for high-level resistance to gentamicin can be determined by the disc agar or Etest method that seem to be highly reliable. Beta-lactamase tests should be performed routinely for all enterococcal isolates.

The unbounded promise of the glycopeptides for therapy of multi-resistant enterococci is being threatened as seen in other countries. Considering what the *Enterococcus* has done in the 1990's, we should be concerned about what it will accomplish in the 2000's.

CHAPTER 7

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APPENDICES

SECTION A : IDENTIFICATION OF ENTEROCOCCI

API Identification

(i) A dense suspension with a turbidity of greater than a 4 McFarland standard was made from a pure culture of the test organism in 2mls of sterile distilled H_2O.

(ii) Approximately 150 μl of this suspension was used to rehydrate and inoculate the enzymatic substrates (pyruvate) Voges-Proskauer (VP) to L-leucine-2-naphthyl-amide (LAP).

(iii) The tube portion only was filled (ie.½ way) for the arginine (ADH) test.

The metabolic end products produced during the period of incubation were revealed through indicator change or by the addition of reagents.

The fermentation tests comprises the second half of the strip.

(iv) Tests ribose (RIB) to glycogen (GLYG) were inoculated with an enriched medium to which approximately 0.5mls of the test suspension was added. This reconstitutes the sugar substrates.

(v) The cupules of the tests arginine (ADH) to glycogen (GLYG) were overlayed with mineral oil to create an anaerobic atmosphere.

Fermentation of carbohydrates was detected by a shift in the pH indicator.

(vi) Inoculated API strips were incubated at 35 - 37°C for 4 hours to obtain a first reading and if necessary, 24 hours to obtain a second reading.

(vii) After the incubation period, the following reagents were added :

Voges-Proskauer test (VP) : 1 drop VP1 and VP2.

Hippurate Test (HIP) : 2 drops Ninhydrin solution (Nin)

Pyrrolidonyl-2-naphthylamide (PYRA), 6-Bromo-2-naphthyl-α-D-galactopyranoside (α-Gal) , Naphthol AS-BI β-D-gluconate (β-Gur), 2-naphthyl-β-D-galactopyranoside (β-Gal), 2-naphthylphosphate (Pal), L-leucine-2-naphthylamide (Lap) Tests : 1 drop ZYM A and ZYM B.

After 10 minutes, reactions were read by referring to the colour chart or interpretation table. Results were recorded on a report sheet.

(viii) Identification of the organism was obtained by using the API 20 Strip Analytical Profile Index.

(ix) On the report sheet, tests are separated into groups of 3 and a number 1,2,4 is indicated for each group.

(x) By adding the numbers corresponding to only the positive reactions within each group, 7 digits are obtained which constitute the profile number.

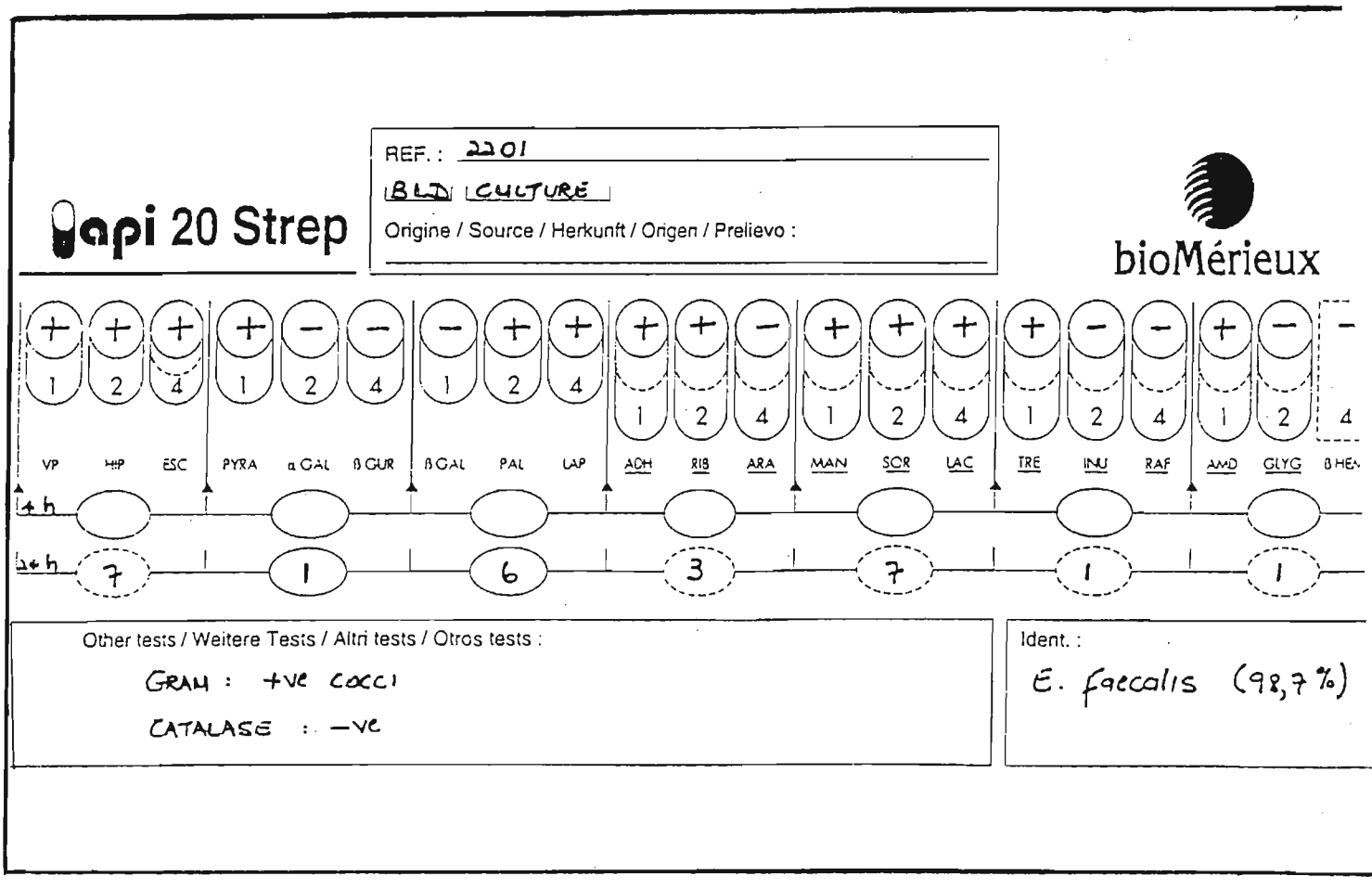

Fig. 18 Identification of enterococci using the API 20 strip analytical profile index

Identification by conventional biochemical tests

Salt tolerance test

Broth test

Procedure

(i) Inoculate 2 to 3 colonies of test culture into 6.5% NaCl broth.

(ii) Incubate culture at 35-37°C aerobically overnight.

Result : Examine culture for evidence of growth (indicated by turbidity) after 24- 48 hours.

Positive reaction : Growth in the medium within 24-48 hours indicated the strain was salt tolerant.

Negative reaction : No growth in 6.5% NaCl broth..

Controls : Positive control : *E. faecalis* ATCC 29212

Negative control : *S. bovis* (ATCC 33317)

Agar plate test

Procedure

(i) 2 to 3 colonies of test culture was inoculated onto 6,5% NaCl agar plate.

(ii) Culture plate was incubated overnight at 35-37°C in an atmosphere of 5-10% CO_2 for 24 hours.

(iii) Agar plate was examined for the presence of bacterial growth.

Positive reaction : Visible growth seen on the agar surface.

Negative reaction : No growth visible after 24-48 hours incubation.

Controls : Positive control : *E. faecalis* ATCC 29212
Negative control : *S. bovis* (ATCC 33317)

Bile aesculin test

Agar plate method

Procedure

(i) Inoculated 40% bile aesculin medium with 2 to 3 colonies of the test organism.

(ii) Agar plate was incubated at 35-37°C overnight aerobically.

(iii) Plates were examined for evidence of growth and blackening of the medium (aesculin hydrolysis).

Positive reaction : Growth on the agar surface with diffuse blackening of the medium after 24-48 hours of incubation.

Negative reaction : No growth or growth without blackening of the medium.

Controls : Positive control : *E. faecalis* ATCC 29212
Negative control : *S.mutans* ATCC 35668

Arginine deamination

Procedure

(i) A single colony of the test isolate was inoculated into 5mls of brain-heart infusion broth.

(ii) Incubated inoculated broth at 35-37°C overnight.

(iii) Arginine was inoculated with 1 to 2 drops of the inoculated brain-heart infusion broth. A layer of sterile mineral oil was added to the broth.

(iv) Tests were incubated at 35-37°C for 48 hours.

(v) Broths were observed for a change in colour.

Positive reaction : Violet to deep purple colour.
Negative reaction : No change in colour of the broth (yellow).

Controls : Positive control : *E.faecalis* ATCC 29212
Negative control : *S.mutans* ATCC 35668

Pyruvate utilization

Procedure

(i) Pyruvate medium was inoculated with 1 to 2 drops of overnight incubated brain-heart infusion broth with the test organism.
(ii) Incubated inoculated pyruvate medium at 35-37°C for 24-48 hours.
(iii) Tests were observed for a change in colour.

Positive reaction : Yellow (A yellow colour with only a hint of green in interpreted as positive).
Negative reaction : Green or greenish yellow.

Controls : Positive control : *E. faecalis* ATCC 29212
Negative control : *S.mutans* ATCC 35668

Tellurite tolerance

Procedure

(i) 2 to 3 colonies of the test organism were inoculated directly onto the medium.

(ii) Inoculated plates were incubated at 35-37°C for 24-48 hours.

(iii) Plates were examined for the presence of black colonies.

Positive reaction : Black colonies on the medium usually after 24-48 hours.

Negative reaction : No growth or growth without blackening of colonies.

Controls : Positive control : *E. faecalis* ATCC 29212

Negative control : *E. faecium* ATCC 35667

Hippurate hydrolysis

Procedure

(i) Inoculated 2 to 3 colonies of the test isolates into 0.5mls of sodium hippurate broth.

(ii) Tests were incubated at 35-37°C for 2 hours.

(iii) 2 drops of ninhydrin was added. Mix well.

(iv) Tests were reincubated for 10 minutes at room temperature.

(v) Examined for the development of the purple colour.

Positive reaction: Purple colour within 10 minutes after addition of ninhydrin.

Negative reaction : Colourless to pale yellow colour broth.

Controls : Positive control : *S.agalactiae* (ATCC 13813)

Negative control : *S.pyogenes* (ATCC 19615)

Starch hydrolysis

Procedure

(i) Inoculated starch agar with 2 to 3 colonies of the test organism.
(ii) Culture plates were incubated at 35-37°C aerobically for 48 hours. When the organisms were grown well, the surface of the agar plate was flooded with 0.5mls of Gram's iodine.

Positive reaction : Complete clearing around the organisms indicates hydrolysis of starch. A partially cleared area surrounding the growth indicates partial hydrolysis and was interpreted as a weak positive reaction.
Negative reaction : If the plate stained uniformly dark purple because of the reaction of the iodine and starch, a negative reaction was recorded.

Controls : Positive control : *S.bovis* (ATCC 33317)
Negative control : *E. faecalis* ATCC 29212.

Voges-Proskauer (VP) test (Coblentz)

Procedure

(i) Inoculated VP broth with an inoculum standardized to a turbidity of a McFarland number 4 standard from an overnight culture plate to be tested.
(ii) Incubated medium at 30°C for 6 to 7 hours.
(iii) 0.6mls of α-naphthol, followed by 0.2ml of 40% KOH containing 0.3% creatine was added.
(iv) Tubes were shaken vigorously for 30 seconds to 1 minute.
(v) To determine a colour change, broths were read within 15 minutes.

Positive reaction : Pink or red colour at the surface of the broth.

(Strong positive resulted in a diffusion of a deep red colour throughout the broth. Faint pink around the surface edge was a weak positive reaction).

Most positive reactions wee apparent within 15 minutes.

Final results were read after 1 hour.

Negative reaction : No colour development (colourless)

Controls : Positive control : *E. faecalis* ATCC 29212

Negative control : *E. coli* ATCC 25922

Carbohydrate fermentation tests (1%)

The following carbohydrates were tested : mannitol, sorbose, sorbitol, inulin, arabinose, melibiose, sucrose, trehalose, lactose, glycerol, salicin, maltose, raffinose, glucose.

Inoculation of carbohydrates

(i) A single colony of the test isolate was inoculated into 5mls of brain-heart infusion broth.

(ii) Incubated inoculated broths at 35-37°C overnight.

(iii) All the carbohydrate media were inoculated with 1 to 2 drops of the inoculated brain-heart infusion broth culture.

(iv) Tests were incubated at 35-37°C and read after 24-48 hours.

(v) Broths were observed for a colour change.

Positive reaction : Yellow.

Negative reaction : Purple (no change in colour).

Controls : Positive control : *E.faecalis* ATCC 29212

Negative control : *E.faecium* ATCC 35667

Test or growth at 10°C and 45°C

Procedure

(i) A single colony of the test organism was inoculated into brain-heart infusion broth.

(ii) Inoculated broth was incubated at 35-37°C overnight.

(iii) A drop of the overnight broth culture was placed into 5mls sterile BHI broth and incubated at the respective temperatures ie. 10 and 45°C for 24 hours.

(For the 10°C incubator, a refrigerator was adjusted to a temperature of 10°C).

(iv) Broths were examined for the presence of bacterial growth by plating out a loopful of the test organism onto a blood agar plate.

Positive reaction : Presence of growth of the organism on the agar plate at both temperatures (10 and 45°C).

Negative reaction : No growth of the test organism at both the above mentioned temperatures.

Controls : Positive control : *E. faecalis* ATCC 29212

Negative control : *S.mutans* ATCC 35668

Survival at 60°C for 30 minutes

Procedure

(i) Organism to be tested was picked up into duplicate 0.5 ml volumes of brain-heart infusion broth.

(ii) 1 tube containing the test organism was incubated in a waterbath at 60°C for 30 minutes and the control tube was left unincubated.

(iii) After 30 minutes, both the tubes ie. the incubated and the unincubated were plated out onto blood agar plates and incubated at 35-37°C for 24 hours.

(iv) After overnight incubation, both the plates were examined for the presence of growth.

Positive reaction : Bacterial growth on the test and the control agar plate.

Negative reaction : No growth on the agar plate incubated at 60°C for 30 minutes. Growth on the unincubated agar plate (control).

Controls: Positive control : *E. faecalis* ATCC 29212

Negative control : *S.mutans* ATCC 35668

Type of haemolysis

Procedure

(i) A single colony of the test organism was plated out onto a blood agar plate and the agar was stabbed with the inoculating loop to test for haemolysis.

(ii) Inoculated plates were incubated at 35-37°C for 24 hours in an atmosphere of 5-10% CO_2.

(iii) After overnight incubation the blood agar plates were examined for the type of haemolysis present, ie. the destruction of erythrocytes around the stab in the agar.

Results

If destruction of some but not all of the erythrocytes is apparent, the haemolytic reaction was recorded as alpha.

If destruction of all of the erythrocytes occurs, the haemolytic reaction was recorded as beta.

If no destruction of cells is apparent, this indicated a non-haemolytic reaction.

Motility

Procedure

(i) The medium was inoculated from an overnight incubated broth with an inoculating straight wire.

(ii) The straight wire was inserted into the centre of the medium in the tube (± 2.5 cm).

(iii) The inoculated tube was placed in a 30°C incubator (since some strains become non-motile at 37°C but are motile from temperatures between 25 and 30°C).

Results

Motile strains grow outward to the edge of the tube and downward towards the bottom of the tube.

Motility medium was incubated for 24-48 hours until good growth was observed.

Non motile strains grew on the line of inoculum only.

Controls : Positive control : *E. gallinarium* (Lab control strain)

Negative control : *E. faecalis* ATCC 29212

Detection of L-pyrrolydonylpeptidase (PYR)

Procedure

(i) PYR disc was moistened with 10μl distilled water.

(ii) With a sterile loop, about 10 colonies of the test organism from an overnight culture plate was smeared gently in the centre of the moistened disc.

(iii) After inoculation, test was incubated at room temperature for 2 minutes.

(iv) A drop of colour developer was dispensed onto the test area of the disc and observed for the development of a pink colour.

(v) Results were read after 1 minute.

Positive reaction : Inoculated portion of disc turned bright pink, orange-pink or cherry red within 10 seconds to 1 minute after applying the colour developer.

Negative reaction : No colour change. Reactions after 1 minute is considered negative.

Controls: Positive control : *E. faecalis* ATCC 29212

Negative control : *S.mutans* ATCC 35668

Determination of Lancefield group antigen

Procedure

(i) 4-5 colonies of test organism were picked up into a tube containing 0.4mls of extract enzyme.

(ii) Suspension was incubated at 35-37°C for 30 minutes.

(iii) The group D latex suspension was resuspended by shaking vigorously for a few seconds.

(iv) Using a sterile Pasteur-pipette, 1 drop of extract was placed on a circle on the Reaction Card.

(v) 1 drop (20μl) of group D latex suspension was added next to the extract on the Reaction Card.

(vi) The contents in the circle was mixed with a mixing stick and spread gently to cover the complete area of the circle.

(vii) The card was rocked gently for about 1 minute.

(viii) Test was observed for the presence of agglutination.

Positive reaction : Agglutination showing clearly visible clumping of the latex particle

Negative reaction : Milky appearance of the latex particles.

Controls: Positive control : *E faecalis* ATCC 29212

Negative control : *S.mutans* ATCC 35668

SECTION B : TEST FOR BETA-LACTAMASE PRODUCTION

Chromogenic Cephalosporin Method

Procedure

(i) 5μl of nitrocefin was dotted onto a Whatman 3MM filter paper.

(ii) 2-3 colonies of the test organism was smeared onto the filter paper using a mixing stick

(iii) Filter paper was observed for a colour change.

Positive reaction : Dark pink to red.

Positive reactions usually appear within 15 seconds to 5 minutes.

Negative reaction : No colour change (remains yellow)

Controls : Positive control : *S.aureus* NCTC 11561 (Beta-lactamase producing).

Negative control : *S.aureus* NCTC 6571 (Non beta-lactamase producing).

SECTION C : ANTIMICROBIAL SUSCEPTIBILITY TESTING

8.3.1 Disc diffusion susceptibility testing

8.3.1.1 Modified "Stokes" method

Media

Mueller-Hinton agar plus 5% lysed horse blood. In addition Mueller-Hinton agar only was used for testing aminoglycosides and the cephalosporins.

Procedure

Preparation of inoculum

A suspension of the inoculum was made by touching 4 to 5 colonies and emulsifying it in 10mls of sterile distilled H_2O.

The inoculum gave semi-confluent growth of the colonies on the plate after overnight incubation.

Inoculations

Using the rotary plate method, control strain (*S.aureus* NCTC 6571) was inoculated to the centre of the plate by means of using a sterile cotton wool swab leaving an uninoculated band of 1.5cm around the edge of the plate. The test organism was seeded evenly to the 1.5cm band using a sterile swab immersed in the suspension containing the test isolate.

There was a central band of 3 to 4 mm left uninoculated between the test and control organisms.

Antibiotic discs

Discs were applied with forceps on the uninoculated band between the test and control organisms. Discs were gently pressed onto the agar surface to ensure even contact with the medium.

Incubation

Test and control organisms were incubated at 35-37°C for 16-18 hours in an atmosphere of 5-10% CO_2.

Reading of zones of inhibition

If the test zones are obviously larger than or equal to the control, it was not necessary to perform any measurements. If there was any doubt, zones were measured with calipers.
Zones were measured from the edge of disc to the edge of the zone.

Interpretation

Susceptible : Zone size equal to, wider than, or not more than 3mm smaller than the control.

Intermediate : Zone size greater than 3mm, but smaller than the control by more than 3mm.

Resistant : Zone size 3mm or less.

Kirby-Bauer method

Media

Mueller-Hinton agar supplemented with 5% lysed horse blood.

Mueller-Hinton agar only was used for testing the aminoglycosides and the cephalosporins as an additional medium to compare the results of these antibiotics on agar plates with and without the addition of blood.

Procedure

Preparation of inoculum

At least 4 to 5 morphologically similar colonies of the test organisms were touched with a wire loop and transferred to a tube containing 5ml of sterile tryptic soy broth .

Inoculated tubes were incubated for 2 to 5 hours at 35°C to produce a bacterial suspension of moderate turbidity. Inoculum was standardized to match the turbidity of a 0.5 McFarland standard (approximately 10^8 CFU/ml) by diluting the inoculum with sterile broth.

Inoculation

Plates were inoculated within 15 minutes of preparation of the standardized suspension so that the density of the inoculum remained the same. For the inoculation of the medium, a sterile cotton-wool swab was dipped into the suspension and the surplus removed by rotation of the swab against the side of the tube above the fluid level. The medium is inoculated by even streaking of the swab over the entire sterile surface in 3 directions. The plate was rotated at 60° each time to ensure even distribution of inoculum. This resulted in uniformly circular inhibition zones and a confluent lawn of growth.

Antibiotic discs

After the inoculum was dried, drug impregnated discs were applied with forceps.

Discs were gently pressed onto the agar surface to ensure complete contact.

Incubation

Plates are incubated 16-18 hours at 35-37°C in an atmosphere of 5-10% CO_2.

Reading of zones of inhibition

Diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc, using calipers. The endpoint was taken as the area showing no obvious, visible growth.

Interpretation

Each zone size of inhibition was interpreted by referring to a reference table. (zone diameter interpretation standard- NCCLS Document M_{100}-S_5).

Organisms were reported as either susceptible, moderately susceptible or resistant.

Controls

S. aureus ATCC 25923 was tested with each batch of test organisms against all the antibiotics tested and the results recorded.

The control results are compared to the NCCLS control limit table for monitoring antimicrobial disc susceptibility tests zone diameter(mm) limits

(NCCLS Document M_{100}-S_5).

Disc diffusion susceptibility test results

Table XXV **Analysis of Kirby-Bauer susceptibility test results according to NCCLS zone diameter interpretation standards (107 isolates tested)**

ANTIBIOTIC	CONC.	S	I	MS	R
Penicillin	10units	nil	nil	93(87%)	14(13.1%)
Ampicillin	10μg	nil	nil	93(87%)	14(13.1%)
Tetracycline	30μg	43(40%)	1(0.9%)	nil	63(59%)
Chloramphenicol	30μg	75(70%)	11(10%)	nil	21(19.6%)
Cotrimoxazole	10μg	9(8.4%)	nil	nil	98(91.6%)
Clindamycin	24μg	nil	8(7.5%)	nil	99(92.5%)
Rifampicin	5μg	20(18.7%)	12(11.2%)	nil	75(70%)
Imipenem	10μg	92(86%)	1(0.9%)	nil	14(13.1%)
Piperacillin	75μg	85(79.4%)	8(7.5%)	nil	14(13.1%)
Ciprofloxacin	5μg	nil	nil	55(51.4%)	52(48.6%)
Vancomycin	30μg	nil	nil	107(100%)	nil
Teicoplanin	30μg	107(100%)	nil	nil	nil

S : Susceptible

I : Intermediate

MS : Moderately susceptible

R : Resistant

Table XXVI **Analysis of Stokes susceptibility test results according to the criteria set out by the Journal of Antimicrobial Chemotherapy (JAC) [107 isolates tested]**

ANTIBIOTIC	CONC.	S	I	R
Penicillin	1μg	nil	22(20.6%)	85(79.4%)
Ampicillin	10μg	nil	93(87%)	14(13.1%)
Tetracycline	10μg	11(10.3%)	34(31.8%)	62(57.9%)
Erythromycin	5μg	16(14.9%)	33(30.8%)	58(54.2%)
Chloramphenicol	10μg	19(17.8%)	48(44.8%)	40(37.4%)
Cotrimoxazole	25μg	23(21.5%)	55(51.4%)	29(27.1%)
Clindamycin	2μg	nil	2(1.9%)	105(98.1%)
Rifampicin	5μg	nil	65(60.7%)	42(39.3%)
Imipenem	10μg	nil	93(86.9%)	14(13.%)
Piperacillin	75μg	nil	93(86.9%)	14(13.1%)
Ciprofloxacin	5μg	nil	73(68.2%)	34(31.8%)
Fusidic acid	2μg	7(6.5%)	83(77.6%)	17(15.8%)
Vancomycin	30μg	107(100%)	nil	nil

SECTION D : MINIMUM INHIBITORY CONCENTRATION (MIC)

Agar dilution procedure

The antimicrobial agent was incorporated into the agar medium, with each plate containing a different concentration of the antibiotic.

The inocula was applied to the agar surface using an inoculum replicating apparatus.

Media

Mueller-Hinton agar supplemented with 5% lysed horse blood.

An additional medium ie. Mueller-Hinton agar without blood was used for testing of the aminoglycosides and cephalosporins.

Preparation of agar plates

(i) A two-fold doubling dilution of antimicrobial solutions (final concentration of 512-0.03μg/ml) was added to molten test agar that was allowed to equilibrate in a water bath at 50°C.

(ii) The agar and antimicrobial solution was thoroughly mixed and the mixture was poured into petri dishes on a level surface.

(iii) Plates were poured as quickly as possible after mixing to prevent cooling and partial solidification in the mixing bottle (agar depth was between 3 and 4 mm).

(iv) The agar was allowed to solidify at room temperature.

Appropriate reference control strains were tested with each batch of tests performed.

Quality control

Reference strain *S. aureus* ATCC 29213 was used as a control organism. The control organism was inoculated onto the agar medium incorporating the antimicrobial agents, containing a different concentration of the antibiotic. The reference strain results were compared to the NCCLS Document M_{100}-S_5 table of acceptable quality control ranges of minimum inhibitory concentration (MIC).

Control plates

Drug free control plates with and without supplements were prepared.

Preparation of inoculum

(i) Inoculum was prepared by touching the top of the at least four or five colonies morphologically similar and inoculating them into a tube containing 5.0ml of Mueller-Hinton broth.

Incubated the bacterial suspension for 2-5 hours at 35-37°C until it was visibly turbid.

Density of the broth culture was adjusted to a turbidity equivalent to a 0.5 McFarland standard by adding sterile broth.

(ii) Cultures adjusted to this standard contained approximately 10^8CFUs/ml. Adequate light source was essential for the adjustment of the turbidity of the inoculum. The adjusted suspension was diluted 1:10 in sterile broth to obtain the desired inoculum concentration of 10^7 CFU/ml. The inoculum replicator deposited approximately 1 to 2μl on the agar surface. The final inoculum on the agar area was approximately 10^4CFUs in an agar area whose diameter is 5 to 8mm.

Inoculating agar plates

(i) The surface of the agar medium was dried before inoculating it. Plates were placed with the lids ajar in a 35-37°C incubator for about 30 minutes. 400μl of each suspension was placed into the corresponding well in the replicator seed block. Well 37 was used as a marker.
A known control strain was added with each batch of tests.
(ii) 1 to 2μl of each inoculum was applied to the agar surface with an inocula-replicating device.
(iii) Inoculated a control plate (no antibiotic present) first and then, starting with the lowest concentration, plates containing the different antibiotic concentrations. A second control plate was inoculated last to ensure that there was no contamination or antimicrobial carryover during the inoculation process.

Incubation

(i) Inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots were absorbed onto the agar surface, ie. until the spots were dry.
The plates were inverted and incubated for 16-20 hours at a temperature of 35-37°C in an atmosphere of 5-10% CO_2.

Determining endpoints

(i) Plates were laid on a dark non-reflecting surface and the results read. A plate reader with a light was used.
MIC was recorded as the lowest concentration of antimicrobial agents that completely inhibited growth.
(ii) Subcultured to check purity of culture.

Broth microdilution procedure

Media

(i) 50μl of Mueller-Hinton broth supplemented with 25mg of Ca^{2+} and 12.5mg Mg^{2+}/l was added from wells 2-11.

(ii) 50μl of the antimicrobial agent was added to wells 1 and 2.

Starting concentration of antibiotic was 512μg/ml.

(iii) 50μl of suspension (antibiotic plus broth) from well 2 was transferred to well 3.

(iv) Continued double diluting until well 11.

Discarded 50μl of suspension from well 11.

Inoculum

(i) Inoculum was standardized to match a 0.5 McFarland standard (approximately 10^8CFUs/ml). Inoculum was further diluted 1:10 to yield a final inoculum of 10^7CFUs/ml. 50μl of this suspension was inoculated into all wells (ie. 1-12).

The final concentration of bacteria was approximately $5x10^5$or $5x10^4$CFUs/well.

After addition of inoculum the final starting concentration of antimicrobial agent was 256μg/ml.

Incubation

(i) Microtitre trays were covered with a plastic cover to prevent drying and incubated at 35-37°C for 16-20 hours in ambient air.

Interpretation of results

(i) MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye.

Quality control

(i) Amount of growth in the wells containing the antibiotic was compared to the amount of growth in the growth control well (ie. well 12).

(ii) Quality control strain ATCC 29212 was set up with each batch of tests. (MIC result is only valid if the Q.C. reference strain falls within the NCCLS acceptable Q.C. range).

A row of sterility broth control was set up with each batch of tests.

This uninoculated broth must be free of any growth.

Table XXVII **Minimum inhibitory concentration (MIC) results of the beta-lactam and glycopeptides**

MIC (μg/ml)	PENICILLIN	AMPICILLIN	IMIPENEM	VANCOMYCIN	TEICOPLANIN
0.25	5(4.7%)	8(7.5%)	6(5.6%)	nil	20(18.7%)
0.5	17(15.9%)	75(70.1%)	29(27%)	19(17.7)	65(60.7%)
1.0	62(59.9%)	10(9.3%)	53(49.5%)	62(57.9%)	22(20.6%)
2.0	2(1.9%)	nil	1(0.9%)	22(20.6%)	nil
4.0	7(6.5%)	nil	4(3.7%)	4(3.7%)	nil
8.0	nil	nil	nil	nil	nil
16.0	2(1.9%)	4(3.7%)	2(1.9%)	nil	nil
32.0	nil	5(4.7%)	nil	nil	nil
64.0	6(5.6%)	5(4.7%)	6(5.6%)	nil	nil
128.0	4(3.7%)	nil	4(3.7%)	nil	nil
256.0	2(1.9%)	nil	2(1.9%)	nil	nil
512.0	nil	nil	nil	nil	nil

Table XXVIII **Minimum inhibitory concentration (MIC) of aminoglycosides and cephalosporins on Mueller-Hinton agar only**

MIC (μg/ml)	GENTA	TOBRA	NETIL	AK	CTX	CEFAM	CEFUR	CEPHA
0.25	nil	nil	nil	nil	nil	nil	nil	nil
0.5	nil	nil	nil	nil	nil	nil	nil	nil
1.0	nil	nil	nil	nil	nil	nil	nil	4 (3.7%)
2.0	nil	nil	nil	nil	nil	nil	nil	15 (14%)
4.0	4 (3.7%)	nil	nil	nil	nil	3 (2.8%)	1 (0.9%)	20 (18.7%)
8.0	26 (24.6%)	20 (19.7%)	23 (21.5%)	nil	2 (1.9%)	2 (1.9%)	1 (0.9%)	16 (14.9%)
16.0	27 (25.2%)	30 (28%)	27 (25.2%)	nil	18 (16.8%)	17 (15.9%)	16 (14.9%)	nil
32.0	18 (16.8%)	15 (14%)	16 (14.6%)	20 (18.7%)	30 (28%)	27 (25.2%)	32 (29.9%)	nil
64.0	6 (5.6%)	3 (2.8%)	3 (2.8%)	42 (39.2%)	10 (9.3%)	7 (6.5%)	6 (5.6%)	nil
128.0	nil	7 (6.5%)	4 (3.7%)	25 (23.4%)	11 (10.3%)	11 (10.3%)	10 (9.3%)	9 (8.4%)
256.0	nil	10 (9.3%)	10 (9.3%)	10 (9.3%)	11 (10.3%)	14 (13.1%)	16 (14.9%)	17 (15.9%)
>256.0	26 (24.3%)	22 (20.6%)	24 (22.4%)	10 (9.3%)	25 (23.4%)	26 (24.3%)	25 (23.4%)	26 (24.3%)

Table XXIX **Minimum inhibitory concentration (MIC) of aminoglycosides and cephalosporins with Mueller-Hinton agar (MHA) supplemented with 5% lysed horse blood and Mueller-Hinton agar only**

MIC (μg/ml)	GENTA	TOBRA	NETIL	AK	CTX	CEFAM	CEFUR	CEPHA
0.25	nil	nil	nil	nil	nil	nil	nil	nil
0.5	nil	nil	nil	nil	nil	nil	nil	nil
1.0	nil	6 (5.6%)	nil	nil	nil	nil	nil	nil
2.0	10 (9.3%)	8 (7.5%)	6 (5.6%)	nil	10 (9.3%)	nil	4 (3.7%)	18 (16.8%)
4.0	34 (31.8%)	30 (28%)	26 (24.3%)	nil	16 (14.9%)	2 (1.9%)	8 (7.5%)	22 (20.6%)
8.0	12 (11.2%)	14 (13.1%)	8 (7.5%)	nil	4 (3.7%)	3 (2.8%)	6 (5.6%)	15 (14%)
16.0	11 (10.3%)	12 (11.2%)	14 (13.1%)	nil	nil	nil	nil	2 (1.9%)
32.0	9 (8.4%)	10 (9.3%)	15 (14.0%)	22 (20.6%)	15 (14%)	18 (16.8%)	10 (9.3%)	4 (3.7%)
64.0	5 (4.7%)	6 (5.6%)	9 (8.4%)	44 (41.1%)	10 (9.3%)	14 (13.1%)	10 (9.3%)	4 (3.7%)
128.0	2 (1.9%)	3 (2.8%)	5 (4.7%)	20 (18.7%)	20 (18.7%)	28 (26.2%)	30 (28%)	10 (9.3%)
256.0	2 (1.9%)	1 (0.9%)	3 (2.8%)	10 (9.3%)	12 (11.2%)	17 (15.9%)	15 (14%)	10 (9.3%)
>256.0	22 (20.6%)	17 (15.9%)	21 (19.6%)	11 (10.3%)	20 (18.7%)	25 (23.4%)	24 (22.4%)	22 (20.6%)

Comparison of MIC with and without supplemented blood for the aminoglycoside showed that there was a false susceptibility of 50% for gentamicin, 40% for tobramycin and 38% for netilmicin in blood enriched media. Amikacin was the only aminoglycoside that remained unaffected by the presence of 5% lysed blood in the media. Cefotaxime displayed a false susceptibility of 26%. Cefuroxime 15% , cephalothin 3% and cefamandole was unaffected.

Table XXX **MICs for vancomycin performed using the broth microdilution method to determine MBCs**

VANCOMYCIN		
CONC.(μg/ml)	MIC	MBC
0.12		
0.25		
0.5	20 (18.7%)	
1.0	65 (60.1%)	
2.0	22 (20.6%)	
4.0		
8.0		48 (44.8%)
16.0		40 (37.4%)
32.0		4 (3.7%)
64.0		2 (1.9%)
128.0		4 (3.7%)
>128.0		9 (8.4%)

Table XXXI **MICs for teicoplanin performed using the broth microdilution method to determine MBCs**

TEICOPLANIN		
CONC.(μg/ml)	MIC	MBC
0.12	17 (15.9%)	
0.25	42 (39.2%)	
0.5	48 (44.8%)	
1.0		
2.0		
4.0		
8.0		49 (45.8%)
16.0		39 (36.4%)
32.0		6 (5.6%)
64.0		6 (5.6%)
128.0		1 (0.9%)
>128		6 (5.6%)

Comparing the MICs of the agar dilution and the microtitre method, MICs were the same or one dilution lower with the microtitre method. MBCs were 3-4 two-fold dilutions ie. 8-16 times greater than the MIC. These isolates had an MBC:MIC ratio <32μg/ml indicating that vancomycin and teicoplanin are bacteriostatic for *E.faecalis* and *E.raffinosus*. Isolates that had an MBC:MIC ratio ≥32μg/ml were *E.faecium* and *E.casseliflavus* strains indicating that these strains were tolerant.

SECTION E : MINIMUM BACTERICIDAL CONCENTRATION (MBC)

Procedure

(i) After the broth MIC was performed, MBC was determined.

(ii) Contents of each well showing no visible growth was mixed to achieve a uniform suspension.

(iii) 20μl(0.02ml) from each well showing no visible growth was removed and plated out onto duplicate blood agar plates.

(iv) Sample was placed over the entire plate surface with a bent (hockey-stick) glass rod.

Incubation

(i) Plates were inverted and incubated 35-37°C for 24 hours.

Interpretation of results

(i) Each subculture plate was examined and the number of colonies recorded.

The mean of both the plates were taken as the final count.

(ii) The MBC is defined as the lowest concentration of the antimicrobial agent showing ≥99.9% killing.

(iii) From the initial MIC inoculum the number of colonies allowable for a 99.9% MBC endpoint was calculated.

(iv) MBC was calculated as follows :

Initial inoculum x aliquot plated x allowable viable percent

$5x10^5$(CFUs/ml) x 0.02(ml) x 0.001%

= 10CFUs

A plate showing >10 CFUs did not meet the 99.9% endpoint.

≤10 colonies indicated a ≥99.9% killing.

SECTION F : BREAKPOINT SUSCEPTIBILITY TESTING

The following concentrations for antibiotics to be tested were chosen according to NCCLS Document M_7-A_2.

Penicillin	: 16μg/ml and 256μg/ml	Gentamicin	: 8μg/ml
Ampicillin	: 8μg/ml and 256μg/ml	Amikacin	: 32μg/ml
Imipenem	: 4μg/ml	Streptomycin	: 32μg/ml
Piperacillin	: 4μg/ml	Cephalothin	: 16μg/ml
Ciprofloxacin	: 1 and 8μg/ml	Cefuroxime	: 16μg/ml
Vancomycin	: 4μg/ml	Cefamandole	: 16μg/ml
Teicoplanin	: 4μg/ml	Cefotaxime	: 32μg/ml

Media

Mueller-Hinton agar supplemented with 5% lysed horse blood.

In addition, Mueller-Hinton only was used to test the aminogylcosides and the cephalosporins.

Procedure

(i) Agar plates were prepared with the appropriate antibiotic at the chosen concentrations (either 1 or 2 plates).

(ii) Reference strains *E.coli* ATCC 25922 and *S.aureus* ATCC 29213 were included with each batch of tests and compared to a NCCLS control range.

(iii) Inoculated pure cultures of test and control organisms into Mueller-Hinton broth and incubated at 35-37°C for 2-5 hours until faintly turbid.

Cultures were adjusted to match a 0.5 McFarland standard that would contain (approximately 10^8CFU/ml) using sterile broth.

Inoculum was further diluted 1:10 to achieve a final inoculum of approximately 10^7CFU/ml.

The multipoint inoculum replicator apparatus deposits 1 to 2μl on the agar surface, thus the final inoculum on the agar was approximately 10^4CFUs per spot.

(iv) After drying the surface of agar plates to be inoculated , 1 to 2μl of each culture was inoculated on the appropriate plates by means of the multipoint inoculator, delivering 37 spots per plate including 2 control strains and a marker.

(v) An antibiotic free control plate was included with each set of inocula.

(vi) Inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots were dry.

(vii) Incubated plates at 35-37°C in an atmosphere of 5-10% CO_2 for 18-24 hours.

(viii) Results were recorded as growth or no growth for each inoculum spot.

Interpretation

No growth at either concentration is interpreted as susceptible.

Growth at both concentrations indicates resistance.

Growth only at the lower concentration is interpreted as intermediate.

SECTION G: DETECTION OF HIGH-LEVEL AMINOGLYCOSIDE RESISTANCE (HLAR)

Screening method

Agar screen technique

Concentration of aminoglycosides used :

Gentamicin : 500 and 2000μg/ml

Streptomycin : 2000μg/ml

Media

Brain-heart infusion agar incorporated with the appropriate aminoglycoside at the concentration stated above.

Preparation of inoculum

(i) Inoculum was prepared by touching 4 to 5 colonies of similar morphology from an overnight incubated culture plate and suspended in 0.85% normal saline.

(ii) The inoculum was standardized to match the turbidity of a 0.5 McFarland standard (approximately 10^8CFU/ml).

Inoculation of agar plates

(i) Plate supplemented with the aminoglycoside was divided into 4 quadrants.

(ii) Inoculated each quadrant with 10μl of the standardized suspension of 10^8CFU/ml to achieve a final inoculum of 10^6CFUs.

(iii) Inoculated plates were allowed to stand undisturbed at room temperature until the inoculum was absorbed into the agar before plates were inverted.

Incubation

Medium incubated at 35-37°C in an ambient atmosphere for 18-24 hours; incubation was prolonged for a further 24 hours on the plates showing no growth.

Results

Plates examined for the presence of bacterial growth (any growth was considered significant). (If bacterial growth was evident, the relevant amount ie. light to heavy was noted).

Quality control

Positive growth control : Media lacking an aminoglycoside supplement (ie. brain-heart infusion agar only) was inoculated for each test organism.

Gentamicin and streptomycin agar plate controls (2000μg/ml)

Positive control : *E.faecalis* Q.C. 2423 (MIC : 2048μg/ml)

Negative control : Lab control strain of *E.faecalis* (MIC :1024μg/ml)

Gentamicin agar plate control (500μg/ml)

Positive control : Lab control strain *E. faecalis* (MIC :512μg/ml)

Negative control : Lab control strain *E.faecalis* (MIC :128μg/ml)

Interpretation of results

Results were interpreted as follows :

No growth in the presence of 500 and 2000μg/ml of gentamicin indicates the absence of high-level resistance to gentamicin.

No growth in the presence of 2000μg/ml of streptomycin indicates the absence of high-level resistance to streptomycin.

Growth in the presence of 500 and 2000μg/ml of gentamicin indicates the presence of high-level gentamicin resistance.

Growth in the presence of 500 (not 2000μg/ml) of gentamicin indicates the presence of high-level gentamicin resistance.

Growth in the presence of streptomycin indicates high-level streptomycin resistance.

SECTION H: THE STUDY OF SYNERGISM USING TIME-KILL CURVES

Concentration of antibiotics used:

Killing curves were performed using the following antibiotics at the stated concentrations.

Gentamicin : 5μg/ml (Fixed)

Vancomycin : 10μg/ml (Fixed)

Teicoplanin : 5μg/ml (Fixed)

Penicillin : 6μg/ml (MIC < 32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC ≥ 256μg/ml)

Ampicillin : 10μg/ml (MIC < 32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC ≥ 256μg/ml)

Imipenem : 10μg/ml (MIC < 32μg/ml)
50μg/ml (MIC 32-128μg/ml)
100μg/ml (MIC ≥ 256μg/ml)

Drug concentrations selected for testing was based on clinically achievable levels in blood. Each of the antibiotics were tested alone and in different combinations.

Example : Ampicillin tested alone
Ampicillin plus gentamicin (Gentamicin MIC < 500μg/ml)
Vancomycin and penicillin tested individually
Penicillin plus vancomycin

Procedure

(i) Test organisms were grown in brain-heart infusion (BHI) broth .

(ii) Inoculated broth was incubated at 35-37°C overnight.

(iii) Adjusted turbidity of overnight broth by adding fresh sterile broth to match a 0.5 McFarland standard (approximately 10^8 CFU/ml).

(iv) From the adjusted inoculum (10^8 CFU/ml), 0.1ml was removed and added to 0.9ml of fresh brain-heart infusion broth.

(This gave a final inoculum of 10^7CFU/ml).

N.B. (i) Prior to inoculation, each tube of fresh BHI broth was supplemented with the appropriate aminoglycoside, beta-lactam or glycopeptide antibiotic either alone or in combination.

Calculation of concentration required : 5μg/ml of gentamicin

A stock solution of gentamicin containing 500μg/ml was made.

0.1ml of gentamicin stock of 500μg/ml plus 9.9mls broth containing 10^7CFU/ml of organisms was added.

This gave a final concentration of 5μg/ml of gentamicin.

Positive growth control

(i) A BHI broth tube with only test organisms 10^7CFU/ml (no antibiotic) was set up as a growth control.

(ii) Inoculated broths were incubated at 35-37°C aerobically.

(iii) At 0, 4, 24 hour intervals after inoculation 0.1ml portion was removed from the BHI broth tube and diluted (ie. a serial dilution bacterial colony count perfomed).0.9ml distilled water was added to tubes 1 to 8. 0.1ml of inoculum 10^7CFU/ml was added to tube 1.

Transferred 0.1ml of diluent from tube 1 to tube 2, 0.1ml from tube 2 to 3, continued double diluting until the last tube.

The last 0.1ml was discarded.

(iv) 0.1ml of diluent from each tube was plated out onto duplicate blood agar plates.

(v) In the same fashion, 0.1ml of inoculum was removed, after 4 and 24 hours of incubation, diluted and 0.1ml of diluent was plated out onto 2 purity plates.

Colony counts were performed.

(vi) By using the viable colony count determined at each time interval , a 24 hour time kill curve was established for each isolate tested.

Interpretation

Susceptibility to an aminoglycoside, beta-lactam or glycopeptide synergy is defined as a >100 fold increase in killing by the drug combination over the killing accompanied by the most active of the two drugs when tested separately.

Resistance to synergy is a <100 fold increase in killing.

Time-kill study graph data for *E. faecalis*

Table XXXII(A) Refer to Fig. 10.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.5X10^6$	$1.8X10^8$	$5.8X10^8$
Genta	32.0	LLR	64.0	5	$1.5X10^6$	$9.0X10^7$	$4.6X10^8$
Ampi	0.5	S	1.0	10	$1.5X10^6$	$3.8X10^5$	$1.8X10^2$
Pen	1.0	S	64.0	6	$1.5X10^6$	$2.0X10^5$	$1.0X10^2$
Ampi + Genta					$1.5X10^6$	$7.0X10^1$	$1.0X10^0$
Pen + Genta					$1.5X10^6$	$5.0X10^1$	$1.0X10^0$

LLR : Low-level resistance

HLR : High-level resistance

MIC : Minimum inhibitory concentration

MBC : Minimum bactericidal concentration

S : Susceptible

R : Resistant

MIC INT : Minimum inhibitory concentration interpretation

CFU/ml : Colony forming units per millilitre

ANTIB. CONC : Antibiotic concentration (μg/ml)

Table XXXII(B) Refer to Fig. 10.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.5X10^6$	$1.8X10^8$	5.8X18
Genta	32.0	LLR	32.0	5	$1.5X10^6$	$9.0X10^7$	$4.6X10^8$
Imipen	1.0	S	>64	10	$1.5X10^6$	$1.5X10^5$	$1.0X10^2$
Imipen + Genta					$1.5X10^6$	$3.0X10^1$	$1.0X10^0$

Table XXXII(C) Refer to Fig. 10.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.5X10^6$	$1.8X10^8$	$5.8X10^8$
Genta	32.0	LLR	64.0	5	$1.5X10^6$	$9.0X10^7$	$4.6X10^8$
Vanco	1.0	S	16.0	10	$1.5X10^6$	$9.0X10^5$	$1.0X10^4$
Teico	0.25	S	4.0	5	$1.5X10^6$	$7.0X10^5$	$8.4X10^3$
Vanco + Genta					$1.5X10^6$	$2.0X10^2$	$1.0X10^0$
Teico + Genta					$1.5X10^6$	$1.0X10^2$	$1.0X10^0$

Table XXXIII(A) Refer to Fig. 11.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.8X10^6$	$1.4X10^8$	$6.4X10^8$
Genta	32.0	LLR	64.0	5	$1.8X10^6$	$9.8X10^7$	$4.0X10^8$
Ampi	0.5	S	2.0	10	$1.8X10^6$	$8.0X10^4$	$1.5X10^2$
Pen	1.0	S	64.0	6	$1.8X10^6$	$4.0X10^4$	$1.0X10^2$
Ampi + Genta					$1.8X10^6$	$4.0X10^2$	$1.0X10^0$
Pen + Genta					$1.8X10^6$	$3.0X10^2$	$1.0X10^0$

Table XXXIII(B) Refer to Fig. 11.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$18X10^6$	$1.4X10^8$	$6.4X10^8$
Genta	32.0	LLR	64.0	5	$1.8X10^6$	$98X10^8$	$4.0X10^8$
Imipen	0.25	S	>64	10	$1.8X10^6$	$2.1X10^4$	$2.0X10^2$
Imipen + Genta					$1.8X10^6$	$2.2X10^2$	$1.0X10^0$

Table XXXIII(C) Refer to Fig. 11.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.8X10^6$	$1.4X10^8$	$6.4X10^8$
Genta	32.0	LLR	64.0	5	$1.8X10^6$	$9.8X10^7$	$4.0X10^8$
Vanco	1.0	S	16.0	10	$1.8X10^6$	$2.8X10^5$	$2.0X10^4$
Teico	0.5	S	4.0	5	$1.8X10^6$	$2.0X10^5$	$1.2X10^4$
Vanco + Genta					$1.8X10^6$	$2.2X10^3$	$1.0X10^0$
Teico + Genta					$1.8X10^6$	$1.4X10^3$	$1.0X10^0$

Table XXXIV(A) Refer to Fig. 12.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$2.5X10^6$	$1.6X10^8$	$8.0X10^8$
Genta	≥2000	HLR					
Ampi	0.5	S	1 .0	10	$2.5X10^6$	$6.0X10^5$	$1.0X10^3$
Vanco	1.0	S	16.0	10	$2.5X10^6$	$1.6X10^5$	$5.5X10^3$
Teico	0.25	S	4.0	5	$2.5X10^6$	$8.8X10^4$	$4.0X10^3$
Ampi + Vanco					$2.5X10^6$	$4.3X10^4$	$1.0X10^0$
Teico + Vanco					$2.5X10^6$	$2.5X10^4$	$1.0X10^0$

Table XXXIV(B) Refer to Fig. 12.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$2.5X10^6$	$1.6X10^8$	$8.0X10^8$
Genta	≥2000	HLR					
Pen	0.5	S	64.0	6	$2.5X10^6$	$4.8X10^5$	$1.0X10^3$
Vanco	1.0	S	16.0	10	$2.5X10^6$	$1.6X10^5$	$5.0X10^3$
Teico	0.25	S	4.0	5	$2.5X10^6$	$8.4X10^4$	$4.1X10^3$
Pen + Vanco					$2.5X10^6$	$6.0X10^4$	$1.0X10^0$
Pen + Teico					$2.5X10^6$	$3.0X10^4$	$1.0X10^0$

Table XXXVIC Refer to Fig. 12.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$2.5X10^6$	$1.6X10^8$	$8.0X10^8$
Genta	≥2000	HLR					
Imipen	0.5	S	>64	10	$2.5X10^6$	$3.8X10^5$	$7.0X10^2$
Vanco	1.0	S	16.0	10	$2.5X10^6$	$1.6X10^5$	$5.5X10^3$
Teico	0.25	S	4.0	5	$2.5X10^6$	$8.8X10^4$	$4.0X10^3$
Imipen + Vanco					$2.5X10^6$	$5.0X10^4$	$1.0X10^0$
Imipen + Teico					$2.5X10^6$	$3.0X10^4$	$1.0X10^0$

Table XXXV(A) Refer to Fig. 13.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.6X10^6$	$4.0X10^8$	$7.7X10^8$
Genta	≥2000	HLR					
Ampi	0.5	S	1.0	10	$1.6X10^6$	$3.0X10^5$	$1.0X10^2$
Vanco	1.0	S	32.0	10	$1.6X10^6$	$6.0X10^5$	$2.2X10^3$
Teico	0.5	S	4.0	5	$1.6X10^6$	$4.5X10^5$	$1.6X10^3$
Ampi + Vanco					$1.6X10^6$	$1.8X10^4$	$1.0X10^0$
Ampi + Teico					$1.6X10^6$	$9.4X10^3$	$1.0X10^0$

Table XXXV(B) Refer to Fig. 13.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.6X10^6$	$4.0X10^8$	$7.7X10^8$
Genta	≥2000	HLR					
Pen	0.5	S	32.0	6	$1.6X10^6$	$2.6X10^5$	$1.0X10^2$
Vanco	1.0	S	32.0	10	$1.6X10^6$	$6.0X10^5$	$2.5X10^3$
Teico	0.5	S	4.0	5	$1.6X10^6$	$4.8X10^5$	$1.0X10^3$
Ampi + Genta					$1.6X10^6$	$2.2X10^4$	$1.0X10^0$
Pen + Genta					$1.6X10^6$	$1.0X10^4$	$1.0X10^0$

Table XXXV(C) Refer to Fig 13.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$1.6X10^6$	$4.0X10^8$	$7.7X10^8$
Genta	≥2000						
Imipen	0.25	S	>64	10	$1.6X10^6$	$3.0X10^5$	$1.5X10^2$
Vanco	1.0	S	32.0	10	$1.6X10^6$	$6.5X10^5$	$2.4X10^3$
Teico	0.5	S	4.0	5	$1.6X10^6$	$4.5X10^5$	$2.0X10^3$
Imipen +Vanco					$1.6X10^6$	$2.9X10^4$	$1.0X10^0$
Imipen + Teico					$1.6X10^6$	$2.0X10^4$	$1.0X10^0$

Time kill study graph data for *E.faecium*

Table XXXVI(A) Refer to Fig. 14.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.2X10^8$	$6.8X10^8$
Genta	1024	LLR	≥2000	5	$9.9X10^5$	$7.5X10^7$	$3.5X10^8$
Ampi	32.0	R	>256	50	$9.9X10^5$	$3.2X10^5$	$1.3X10^4$
Pen	128	R	>256		$9.9X10^5$	$4.6X10^5$	$3.6X10^4$
Ampi + Genta					$9.9X10^5$	$3.0X10^4$	$9.0X10^3$
Pen + Genta					$9.9X10^5$	$6.2X10^4$	$2.5X10^4$

Table XXXVI(B) Refer to Fig. 14.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.2X10^8$	$6.8X10^8$
Genta	1024	LLR	≥2000	5	$9.9X10^5$	$7.5X10^7$	$3.5X10^8$
Imipen	128	R	>256	50	$9.9X10^5$	$2.0X10^5$	$3.0X10^4$
Imipen + Genta					$9.9X10^5$	$1.3X10^5$	$2.2X10^4$

Table XXXVIC Refer to Fig. 14.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.2X10^8$	$6.8X10^8$
Genta	1024	LLR	≥2000	5	$9.9X10^5$	$7.5X10^7$	$3.5X10^8$
Vanco	1.0	S	32.0	10	$9.9X10^5$	$6.5X10^5$	$3.2X10^4$
Teico	0.25	S	16.0		$9.9X10^5$	$5.3X10^5$	$2.2X10^4$
Vanco + Genta					$9.9X10^5$	$3.0X10^5$	$1.8X10^4$
Teico + Genta					$9.9X10^5$	$2.2X10^5$	$1.0X10^4$

Table XXXVII(A) Refer to Fig. 15.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.0X10^8$	$6.2X10^8$
Genta	8.0	LLR	64.0	5	$9.9X10^5$	$9.8X10^7$	$4.8X10^8$
Ampi	0.5	S	1.0	10	$9.9X10^5$	$1.8X10^5$	$9.0X10^3$
Pen	0.5	S	16.0	5	$9.9X10^5$	$9.9X10^7$	$8.2X10^3$
Ampi + Genta					$9.9X10^5$	$8.5X10^3$	$9.8X10^1$
Pen + Genta					$9.9X10^5$	$7.0X10^3$	$9.0X10^1$

Table XXXVII(B) Refer to Fig. 15.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.0X10^8$	$6.2X10^8$
Genta	8.0	LLR	64.0	5	$9.9X10^5$	$9.9X10^7$	$4.8X10^8$
Imipen	0.25	S	32	10	$9.9X10^5$	$1.0X10^5$	$6.8X10^3$
Imipen + Genta					$9.9X10^5$	$6.6X10^3$	$8.7X10^1$

Table XXXVII(C) Refer to Fig. 15.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.9X10^5$	$1.0X10^8$	$6.2X10^8$
Genta	8.0	LLR	64.0	5	$9.9X10^5$	$9.8X10^7$	$4.8X10^8$
Vanco	1.0	S	32.0	10	$9.9X10^5$	$5.1X10^5$	$1.8X10^4$
Teico	0.25	S	16.0	5	$9.9X10^5$	$4.0X10^5$	$8.5X10^3$
Vanco + Genta					$9.9X10^5$	$6.5X10^3$	$9.5X10^1$
Teico + Genta					$9.9X10^5$	$5.8X10^3$	$8.8X10^1$

Table XXXVIII(A) Refer to Fig. 16.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.5X10^5$	$9.5X10^7$	$6.0X10^8$
Genta	≥2000	HLR					
Ampi	64.0	R	>256	50	$9.5X10^5$	$4.4X10^5$	$2.4X10^5$
Vanco	1.0	S	64.0	10	$9.5X10^5$	$9.0X10^5$	$7.8X10^4$
Teico	0.5	S	32.0	5	$9.5X10^5$	$6.8X10^5$	$7.0X10^4$
Ampi + Vanco					$9.5X10^5$	$3.9X10^5$	$1.7X10^4$
Ampi + Teico					$9.5X10^5$	$3.0X10^5$	$1.0X10^4$

Table XXXVIII(B) Refer to Fig. 16.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.5X10^5$	$9.5X10^7$	$6.0X10^8$
Genta	≥2000	HLR					
Pen	128	R	>256	50	$9.5X10^5$	$2.9X10^6$	$8.8X10^5$
Vanco	1.0	S	64.0	10	$9.5X10^5$	$9.0X10^5$	$7.8X10^4$
Teico	0.5	S	32.0	5	$9.5X10^5$	$7.0X10^5$	$7.0X10^4$
Pen + Vanco					$9.5X10^5$	$6.4X10^5$	$2.0X10^4$
Pen + Teico					$9.5X10^5$	$4.9X10^5$	$1.0X10^4$

Table XXXVIIIC Refer to Fig. 16.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.5X10^5$	$9.5X10^7$	$6.0X10^8$
Genta	≥2000	HLR					
Imipen	128	R	>256	50	$9.5X10^5$	$9.2X10^5$	$5.0X10^5$
Vanco	1.0	S	4.0	10	$9.5X10^5$	$7.0X10^5$	$7.8X10^4$
Teico	0.5	S			$9.5X10^5$	$4.8X10^5$	$4.5X10^4$
Imipen + Vanco					$9.5X10^5$	$2.1X10^4$	$1.5X10^4$
Imipen + Teico					$9.5X10^5$	$1.0X10^5$	$1.0X10^4$

Table XXXIX(A)Refer to Fig. 17.1

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.8X10^5$	$1.0X10^8$	$5.9X10^8$
Genta	≥2000	HLR					
Ampi	64.0	R	>256	100	$9.8X10^5$	$8.6X10^5$	$6.0X10^5$
Vanco	1.0	S	32.0	10	$9.8X10^5$	$5.0X10^5$	$3.0X10^4$
Teico	0.5	S	16.0	5	$9.8X10^5$	$3.2X10^5$	$1.7X10^4$
Ampi + Vanco					$9.8X10^5$	$2.2X10^5$	$1.0X10^4$
Ampi + Teico					$9.8X10^5$	$9.5X10^4$	$9.0X10^3$

Table XXXIX(B)Refer to Fig. 17.2

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.8X10^5$	$1.0X10^8$	$5.9X10^8$
Genta	≥2000	HLR					
Pen	256.0	R	>256	100	$9.8X10^5$	$1.2X10^7$	$8.5X10^6$
Vanco	1.0	S	32.0	10	$9.8X10^5$	$5.0X10^5$	$3.0X10^4$
Teico	0.5	S	16.0	5	$9.8X10^5$	$3.2X10^5$	$1.8X10^4$
Pen + Vanco					$9.8X10^5$	$2.2X10^5$	$1.0X10^4$
Pen + Teico					$9.8X10^5$	$9.8X10^4$	$9.0X10^3$

Table XXXIX(C) Refer to Fig. 17.3

				TIME-KILL STUDY			
ANTIBIOTIC	MIC	MIC INT.	MBC	ANTIB. CONC.	0 HRS (CFU/ml)	4 HRS. (CFU/ml)	24 HRS (CFU/ml)
Control					$9.8X10^5$	$1.0X10^8$	$5.9X10^8$
Genta	≥2000	HLR					
Imipen	256.0	R	>256	100	$9.8X10^5$	$8.8X10^6$	$6.5X10^6$
Vanco	1.0	S	32.0	10	$9.8X10^5$	$5.0X10^5$	$3.0X10^4$
Teico	0.5	S	16.0	5	$9.8X10^5$	$3.1X10^5$	$2.1X10^4$
Imipen + Vanco					$9.8X10^5$	$2.0X10^5$	$1.6X10^4$
Imipen + Teico					$9.8X10^5$	$1.0X10^5$	$9.4X10^3$

SECTION I. TYPING OF ENTEROCOCCI USING PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

The procedure used for the pulsed-field gel electrophoresis was according to Murray et al. (79).

(i) Enterococci were grown overnight in 5mls of brain-heart infusion broth at 37°C overnight.

(ii) Cells were harvested and suspended in an equal vol of PVI buffer (1M NaCl, 10mM Tris-HCl) [pH 7.6].

(iii) A portion (2.5mls) of this suspension was mixed with 2,5mls of 1.6% low melting - temperature agarose in water at 40-50°C.

The agarose was then pipetted into a plug mold to allow to solidify.

(iv) For lysis, five plugs were placed in 10mls of fresh solution (which was made up just before use). The lysis solution contained 6mM Tris-HCl (pH 7.6), 1M NaCl, 100mM EDTA (pH 7.5), 0.5% Brij, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20μg RNase (DNase free) per ml and 1mg of lysozyme per ml.

(v) Plugs were incubated overnight at 35-37°C with gentle shaking.

This solution was replaced with 10ml ESP (Proteinase K 50μg/ml, 1% Sarkosyl, 0.5M EDTA (pH 9.0). and then incubated overnight at 50°C with gentle shaking.

(vi) The plugs were washed 3 times for 30minutes each time with 15ml TE solution (10mM Tris-HCl [pH 7.5], 1mM EDTA).

The plugs were stored at 4°C until their use.

(vii) Digestion with restriction enzyme *Sma* I was performed by placing a small slice (about 1-2mm thick) of an agarose plug in a microfuge tube with 200μl of distilled water, followed by 25μl of reaction buffer and 2μl of *Sma* I (Boeringer-Mannheim).

This was incubated at 25°C for 12 hours.

The slices were washed with 1ml of TE for 1 hour at 37°C.

They were then melted at 55 to 65°C and loaded into wells of 1.2% agarose gels (Sea Plaque GTG agarose) in 0.5x TBE buffer (0.089M Tris-HCl, 0.089 boric acid, 0.0025M EDTA).
Lambda concatemers were used as a size standard.
Electrophoresis was performed with the Contoured-Clamped Homogeneous Electric Field apparatus (CHEF-DRII; Bio-Rad).
Pulse time was increased from 5 to 35 seconds over 24 hours at 200V for 32 hours.
Gels were stained for 30 minutes with ethidium bromide followed by 12 hours of destaining with distilled water.
They were photographed with UV radiation.