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The Epidemiology of Resistant Nosocomial Gram-Negative Bacteria in an Intensive Care Unit

by

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ABSTRACT

Widespread use of antimicrobials in hospitals has resulted in increasing resistance in many bacterial species. This study focused on the epidemiology of resistant nosocomial gram-negative organisms among 89 patients admitted to the ICU of the Calgary General Hospital between December, 1994 and June, 1995. The prevalence of resistant gram-negative organisms in this population was about ten percent. Colonized patients were more likely to have received first or second generation cephalosporins or metronidazole. Significant risk factors for colonization were use of chest tubes and prior surgery. Genotypic typing showed differences between isolates of the same species (endogenous acquisition) except for *Acinetobacter* species which were all similar (exogenous acquisition). Effective ways to control resistant organisms in the present situation should focus on appropriate antibiotic stewardship, including cyclic antimicrobial use, synergistic combination therapy, and optimized pharmacokinetic dosing. In addition, focused microbiological surveillance and infection control would be of enormous benefit.

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DEDICATION

To

Mikaela

who is truly a

GIFT from God

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CHAPTER ONE

1.1 Introduction

Since the turn of the century medical advances (such as the development of vaccines) and effective public health programs (such as improved sanitation, hygiene and nutrition, and standard of living) have played a major role in the decrease of many infectious diseases. The discovery of antibiotics has been called the single most important therapeutic discovery in the history of medicine and it is generally conceded that the development and use of antimicrobial agents was one of the most significant measures employed in the control of bacterial diseases in the 20th century. The ability of antimicrobials to effect rapid cures for previously fatal infections led to their being referred to as "miracle drugs" (Levy, 1992). Antimicrobial agents have had a major impact on public health and mortality due to infectious diseases has declined dramatically in populations where antimicrobials are used (Cohen, 1992; Gaynes, 1995).

Today there are countless numbers of antibiotics: more than 50 penicillins, 70 cephalosporins, 12 tetracyclines, 8 aminoglycosides, 1 monobactam, 3 carbapenems, 9 macrolides, 2 streptogramins, and 3 dihydrofolate reductase inhibitors (Neu, 1992). In developed countries the concept of untreatable infectious diseases is almost unthinkable and many bacteria remain fully susceptible to commonly used antimicrobial agents. Antibiotics are still the major therapeutic source for curing and preventing infectious diseases but this situation is not secure and is continually changing. In many parts of the world cost effective inexpensive antibiotics are no longer successful because of the development of bacterial resistance to them (Levy, 1992). The seemingly endless

miracles attributed to these drugs have led to misuse and overuse and widespread application of antibiotics has resulted in bacteria finding ways to become resistant and in spite of all the antibiotics available today a person could still die in a modern hospital as a result of a resistant bacterial infection.

There have been indications in recent years that the frequency and spectrum of antimicrobial-resistant infections have increased both in the hospital and the community largely because the bacterial agents that cause them have developed resistance to antibiotics (Pechére, 1994). The emergence of resistant bacteria has resulted in widespread demand for antibiotics with the result that parenteral and oral antibiotics have become the second most commonly used class of drugs in the United States surpassed only by cardiovascular drugs (Pallares, Dick, Wenzel et al., 1993). Despite the successes of antimicrobials in the last fifty years, the development of any new antimicrobial has almost always been followed by the development of resistance. The recent emergence of multiple drug resistance in certain bacterial species poses a serious public health threat and in many instances has made currently available antimicrobials ineffective. This raises the specter of untreatable infections as they were encountered in the pre-antimicrobial era. It is in fact believed by many that the post-antimicrobial era is on our doorstep.

Factors associated with the development of antimicrobial resistance include antibiotic overuse and abuse, bacterial selection and transmission of resistance phenotypes and resistance genes, environmental conditions promoting persistence or dissemination of resistance determinants, and the presence of bacterial reservoirs.

In many countries the major impact of antimicrobial resistance is seen in the

hospital environment where infections caused by multi-resistant nosocomial organisms are encountered (Murray, 1992). About 5 to 10% of all hospitalized patients develop a nosocomial infection and Intensive Care Units (ICUs) probably represent the single largest identifiable source for these infections in the hospital (Emmerson, 1990; Bergogne-Berezin, Decre, and Joly-Guillou, 1993). Patients in ICUs may be temporarily immunocompromised and are thus at high risk from acquiring infection caused by multi-resistant organisms. Most ICU patients are subjected to several invasive procedures and devices which tend to breach natural lines of defense such as skin, bronchial and urethral barriers thus facilitating ease of entry of pathogenic organisms. These are usually gram-negative bacilli many of which are highly resistant to antibiotics (Emmerson, 1990).

The primary objective of this study therefore, was to describe the epidemiology of resistant nosocomial gram-negative organisms among patients admitted to the ICU using conventional phenotypic methods as well as newer molecular epidemiological tools. The following specific objectives were determined:

1. The prevalence and incidence of colonization and/or infection with resistant gram-negative organisms in ICU patients.

2. The characterization of resistant organisms by phenotypic and genotypic typing methods.

3. The association between the development of resistance and underlying disease, exposure to antimicrobials, invasive devices, and invasive procedures.

CHAPTER TWO

HISTORICAL OVERVIEW OF ANTIMICROBIAL DEVELOPMENT AND RESISTANCE

2.1 The Pre-Antibiotic Era

In ancient times it was believed that everything had a spirit and that people got sick because they had somehow offended the spirit(s). Healing was sought through a healer who would make sacrifices or offerings to the gods to appease their wrath, thereby returning the patient to a harmonious relationship. This non-medicinal approach was based on the power of suggestion that could mobilize processes in the human body to help relieve symptoms (Levy, 1992). Nowadays this is sometimes called "the placebo effect."

As time passed new discoveries led to changes in therapeutic approach. There is evidence in ancient Egyptian papyri and Babylonian cuneiform tablets (about 2000 BC or earlier) that many natural products were mixed into a potion to treat illnesses. These potions were used to drive out evil spirits that had invaded the body. Details of pathology and disease with complete descriptions of treatments have been discovered in Egyptian papyri as early as the 17th century BC. These medications included whole parts of plants and animals. A special chant usually accompanied the application of the medication with a verbal appeal being an important component of the therapy (Levy, 1992).

Early Greek physicians defined illness as the imbalance of four body humors - blood, phlegm, yellow bile, and black bile. They also recognized four cardinal

features of infection: dolor (pain), calor (heat), rubor (redness), and tumor (swelling). In the 4th century BC Hippocrates and later Aristotle articulated these concepts in their teachings. In order for health to be restored something had to be replaced or removed. This led to experimentation with countless numbers of natural substances such as plant leaves, roots etc. which are now known to contain active chemical ingredients including antibiotics. These substances were responsible for the relief of symptoms and cure of disease. In the 2nd century AD a Greek physician named Galen (130 AD - 200 AD) used other substances that were known as "galenicals." His teachings greatly influenced medicine and this continued for almost fourteen centuries. Basically, he chose medicinals that were the opposites of the disease symptoms for treatment and gave it to the patient based on its ability to restore what he considered absent or off balance. Following Galen, Paracelsus introduced methods for extracting the active components of medicinals. He simplified and gave uniformity to Galen's prescriptions insisting that the diseased organ and not the symptom should be treated (Levy, 1992). In contrast to Galen who believed in "opposites," Paracelsus believed in "similars" so the flower or leaf of a plant that most closely resembled the diseased organ was often selected to heal the organ. This was in fact how digitalis, one of the most important drugs used to treat heart disease today, was discovered. The extract of the heart-shaped foxglove leaf was used to treat symptoms then that is now known to have been related to the heart. The practice of medicine in the Middle Ages and during the Renaissance continued to be a mixture of superstition from ancient times and experimentation by physicians of the day. Belief that diseases were caused by evil spirits still remained entrenched in peoples' minds well into the 18th century.

In the 19th century a significant development in the understanding of disease

appeared which led to yet another change in the direction of the discovery of drugs. Although the concept of a diseased state of man rather than a causative agent was still the current belief, Louis Pasteur, a French chemist in Paris, produced convincing evidence that microorganisms were the causative agents of human disease. This observation was a major step into the era of modern medicine (Levy, 1992). The "germ theory" of disease set the scene for tremendous advancement in medical science and enabled scientists to begin searching for drugs as defined therapeutic entities to fight bacteria.

2.2 The Antibiotic Era

The acceptance of the "germ theory" of disease was the first step in a change in the goals of treatment. and the search for drugs that could kill "germs" resulted in the discovery of antibiotics. Pyocyanase was the first naturally occurring antibiotic product discovered in 1888 by de Freudenreich in Germany. He found that the blue pigment released by *Bacillus pyocaneus* in broth culture (now called *Pseudomonas aeruginosa*) halted the growth of other bacteria in the tube. Extensive trials followed and it was found that pyocyanase was capable of killing a wide variety of pathogenic organisms. Unfortunately, pyocyanase was found to be unstable and highly toxic for patients (Levy, 1992).

In 1910, Ehrlich developed a chemical dye linked to arsenic which he called Salvarsan. It was shown to help patients afflicted with syphilis, but the toxicity of the arsenic derivative caused severe and painful debilitating side effects. The first decades of the 20th century however, passed without much advancement in the search for antibacterial substances and the failure of earlier compounds probably had a discouraging effect on the search for more effective antibacterials.

In 1928 Alexander Fleming observed that colonies of *Staphylococcus aureus* were lysed when they grew in the proximity of *Penicillium notatum* mold. This led to the discovery of the first true antibiotic, penicillin, but its use as a therapeutic agent for treatment of human infection did not occur until 1942. Fleming attributed this delay to a lack of biochemical and microbiological expertise at the time (Levy, 1992).

In 1932 Gerhard Domagk, a researcher from Germany, found that Prontosil, a newly patented dye cured diseases caused by streptococci when injected into diseased mice. It was however ineffective against bacteria growing in a test tube. It was later discovered that the sulphonamide part of the molecule was released during metabolism of the dye in the body and this is what killed bacteria. The successes of sulphonamides prompted new interest in discovering other antibacterial agents and soil became the focus of these new searches. In 1939 René Dubos isolated for the first time *Bacillus brevis*, an antibiotic-producing soil organism. This discovery led to the first clinically useful antibiotic which was called Gramicidin. Its major drawback was that it was severely toxic when given intravenously. When Fleming discovered penicillin in 1928 he continued to work on it but was unable to purify enough to test it in animals or humans. In 1942 Howard Florey and Ernest Chain learned how to extract the substance, keep it stable, and then mass produce it for use in animals. Their results were spectacular and penicillin eventually became available to the general public by 1944. This discovery was hailed as a miracle and people began to assume that it could cure any disease - it became whatever people wanted it to be.

The first useful antibiotic from soil was discovered by Selman Waksman in 1943. It was produced by *Streptomyces griseus* and subsequently called streptomycin. This

antibiotic was active against a wide variety of bacteria and was the first drug to offer hope to victims of tuberculosis. As with earlier antibiotics however, it was nephrotoxic and its use also led to temporary and even persistent deafness (Levy, 1992). A further problem was that bacteria frequently became resistant to streptomycin during therapy at an alarming rate.

In 1947 Paul Burkholder discovered a drug that was active against both gram-positive and gram-negative bacteria. This drug was produced by *Streptomyces venezuela*, a soil bacteria, and named chloramphenicol. It was the first of the class of so-called broad spectrum antibiotics and had spectacular results against typhus, Rocky Mountain Spotted Fever, and typhoid fever. Its success however, was marred by its toxic effect on bone marrow cells. Soon after the discovery of chloramphenicol, Benjamin Duggar in 1948 found that *Streptomyces aureofaciens* produced a substance with antibiotic properties which was later called chlortetracycline. This antibiotic had low toxicity and broad spectrum activity against many bacteria. Many more antibiotics were discovered, tested, and introduced for human use following the discovery of these first generation antibiotics. In the 1970s and 1980s, synthetic antimicrobials such as trimethoprim, nalidixic acid and the fluoroquinolones were introduced for use. The chronology of events in the antibiotic era is shown in table 2.1.

2.3 The Evolution of Antimicrobial Resistance

The potential for bacterial resistance to antibiotics has probably existed since the beginning of time and bacterial populations isolated before the antibiotic era most likely contained antibiotic-resistant organisms. Penicillinase for example, was first described in 1940 when no penicillin was yet available for treating patients

Table 2.1 Major Antimicrobial Discoveries of the Antibiotic Era

Antimicrobial Name	Derived From	Year Discovered	Year Introduced	Country
Pyocyanase	*P.aeruginosa*	1888	1889	Germany
Salvarsan	Chemical dye	1910	1910	Germany
Penicillin	*Penicillium notatum*	1928	1942	England
Prontosil	Chemical dye	1932	1935	Germany
Gramicidin	*Bacillus brevis*	1939	1939	USA
Streptomycin	*Streptomyces griseus*	1944	1944	USA
Bacitracin	*Bacillus licheniformis*	1944	1945	USA
Chlortetracycline	*Streptomyces aureofaciens*	1944	1945	USA
Cephalosporins (1st gen.[1])	*Cephalosporium acremonium*	1945	1964	Italy
Chloramphenicol	*Streptomyces venezuelae*	1947	1948	USA
Neomycin	Streptomyces species	1949	1949	USA
Oxytetracycline	*Streptomyces rimosus*	1950	1950	USA
Erythromycin	*Streptomyces erythreus*	1952	1952	USA
Tetracycline	Synthetic	1953	1953	USA
Vancomycin	*Streptomyces orientalis*	1956	1958	USA
Kanamycin	*Streptomyces kanamyceticus*	1957	1957	USA/Japan

[1] gen=generation

Table 2.1 (continued)

Antimicrobial Name	Derived From	Year Discovered	Year Introduced	Country
Methicillin	Synthetic	1960	1960	USA/UK
Spectinomycin	*Streptomyces spectabilis*	1960	1961	USA
Ampicillin	Synthetic	1961	1961	USA
Nalidixic acid	Synthetic	1962	1964	USA
Gentamicin	Micromonospora species	1963	1964	USA
Doxycycline	Synthetic	1966	1966	USA
Clindamycin	Synthetic	1967	1967	USA
Tobramycin	*Streptomyces tenebrarius*	1971	1971	USA
Cephalosporins (2nd gen.)	Streptomyces species	1972	1972	USA
Cephalosporins (3rd gen.)	Synthetic	1980	1980	USA
Fluoroquinolones	Synthetic	1980-83	1980-83	USA/Japan

(Pechére, 1994). Since the introduction of antibiotics in medical practice and agriculture, antimicrobial resistance has undergone an explosive development. All antibiotics that have been developed thus far possess the potential to produce adverse side effects in the host, and the emergence of organisms resistant to its antibiotic effects has plagued every new antibiotic developed (Murray and Moellering, 1978).

Sulphonamides were the first commercially marketed antibiotics and were introduced in 1935. In the early 1940s they were extensively used in Japan against bacillary dysentery, but by 1950 over 80% of Japanese isolates of Shigella were resistant to sulphonamides (Murray and Moellering, 1978). Other antibiotics such as chloramphenicol, streptomycin, and tetracyclines were developed and used extensively in Japan but before long Shigella strains resistant to these drugs also began to appear (Murray and Moellering, 1978). By the late 1950s, there were increasing reports from Japan of multiply-resistant shigellae. When penicillin was introduced for clinical use in 1942, almost all strains of *Staphylococcus aureus* were susceptible. When it first became available orally it was a major success but as Fleming warned at the time, it was also an open invitation for misuse. Until the mid-1950s penicillin was available without a doctor's prescription and could be obtained "over the counter." This free access undoubtedly led to the emergence of resistance. By 1944 *Staphylococcus aureus* was capable of destroying penicillin by means of a penicillinase, now called a beta-lactamase. By 1951 about 73% of *S.aureus* isolates in the USA were resistant, mostly because of penicillinase production. For a period of time community acquired isolates of *S.aureus* were more susceptible to penicillin than nosocomially acquired isolates, but by the late 1960s over 80% of both community- and nosocomially-acquired strains were resistant to penicillin and resistance remained

at least this high for the next 20 years (Murray, 1989). Today in excess of 95% of *S.aureus* isolates worldwide are resistant to penicillin, ampicillin, and the anti-Pseudomonas penicillins (Neu, 1992).

In 1959 the transfer of quadruple resistance (tetracycline, streptomycin, chloramphenicol, and sulphonamides) between Shigella and *E.coli* was reported (Falkow, 1975). It was subsequently established that transfer required cell to cell contact, was not mediated by free DNA, and was independent of chromosomal transmissibility. The concept of transferable, extrachromosomal elements that contained resistance genes was thus born, and the term "R-factor" was adopted to describe this type of plasmid (Helinski, 1976). Soon afterwards reports of transferable plasmids having multiple drug resistance came from England, Germany, the USA, and elsewhere (Levy, 1992). The origin of R-factors is not known but the deluge of antibiotics that began after 1940 probably did much to select and disseminate R-factors and hasten their evolution.

By 1966, 65 to 75% of Shigella strains in Japan were resistant to tetracycline, chloramphenicol, streptomycin, and sulphonamides. Multiply-resistant Shigella strains also appeared in Israel in 1956. In 1965 transmissible resistance to ampicillin was reported in a strain of *Salmonella typhimurium*. This resistance was shown to be mediated by a beta-lactamase. Also in 1965, a combination of ampicillin, neomycin, kanamycin, tetracycline, streptomycin, chloramphenicol, and sulphonamide resistance was reported in some organisms causing an outbreak of salmonellosis in France (Murray and Moellering, 1978). Similar cases of resistance were reported from the USA and Puerto Rico at this time. Salmonellae have shown a dramatic ability to develop resistance and in the USA attention has focused on resistance in non-typhoidal salmonellae which probably have their

major reservoir in animals. Resistance in these organisms likely reflects the resistance that exists in animal husbandry areas where heavy use of antibiotics and enormous potential for fecal-oral spread exists (Murray, 1989). *S.typhi* has also developed resistance to major antibiotics used to treat typhoid fever such as chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole.

Nosocomially-acquired gram-negative bacilli have also demonstrated ability to develop antimicrobial resistance. Plasmid and/or transposon-mediated resistance in *E.coli* for example, has caused major problems first with the emergence of resistance to sulphonamides, streptomycin, tetracycline, and chloramphenicol, and then later to ampicillin, first generation cephalosporins, carboxy- and ureidopenicillins, aminoglycosides, and trimethoprim. The most recent resistance that has developed among nosocomial gram-negative bacilli is resistance to third generation cephalosporins. When these antibiotics were first introduced (about 1978 in Europe and 1981 in the USA) the vast majority of the Enterobacteriaceae and a number of other gram-negative bacteria were susceptible. However it was not long before organisms such as *Enterobacter, Serratia, Citrobacter* and *Pseudomonas aeruginosa* showed resistance, which was mostly related to the presence of a chromosomal cephalosporinase.

Plasmid-mediated resistance to the third generation cephalosporins was first reported in 1983 in Germany and since 1987, there has been a tremendous proliferation in the number of strains and species with this type of resistance, including *Klebsiella pneumoniae, E.coli*, and Salmonella species. Initial reports were from France and Germany but now include Japan, the USA, and South America (Murray, 1989).

CHAPTER THREE

THE IMPACT OF ANTIMICROBIAL RESISTANCE

3.1 The Magnitude of the Problem

Antibiotic resistance has undergone an explosive development following the introduction of antibiotics in medical practice and agriculture. Today most bacteria that were previously universally susceptible to antibiotics are resistant to at least some antibiotics, and in some cases to many different ones. This disturbing situation has to be faced now only 100 years since the recognition that bacteria cause disease, and only 50 years since the discovery of antibiotics. One of the greatest concerns today is that new organisms are being encountered that are so resistant that no antimicrobial of established efficacy is available to combat them (Murray, 1992).

The impact of antimicrobial resistance is an increase in morbidity, mortality, and costs associated with disease (Cohen, 1992). Morbidity and mortality increase because effective therapy for specific infections is delayed, especially where resistance emerges to the drug of choice for a specific situation, or to the appropriate empiric therapy for a given infection (Cohen, 1992). Increases in morbidity and mortality usually occur early in the emergence of antimicrobial resistance and have been lessened by the use of newer antimicrobial agents to which the organisms are susceptible. Excess morbidity and mortality continue to occur however, when organisms develop resistance to most or all effective antimicrobial agents.

Another important consequence of antimicrobial resistance is that resistance can lead to an increase in the incidence of the disease. This is especially true for diseases in which antimicrobial treatment of ill persons or carriers is an important strategy in the prevention of additional cases of disease. A more subtle impact on the incidence of disease occurs when a person receives an antimicrobial agent to which a potentially infecting or colonizing organism is already resistant. By killing competing organisms the antimicrobial agent provides a selective advantage that enables the resistant organism to cause disease, persist in the host for longer periods, or be spread more widely (Cohen, 1992).

A most critical consequence of antimicrobial resistance is the compromised therapy of human disease. However, another mounting and disturbing consequence of resistance revolves around the price tag for treatment. Millions of dollars are invested in the discovery, development, and animal and human testing of new antibiotics. In 1980 the total world market for all pharmaceutical products was estimated to have been $75 billion dollars and is expected to grow to $270 billion dollars by the year 2000 (Holmberg, Solomon, and Blake, 1987; Kunin, Johansen, Worning et al., 1990). In the USA alone, the total societal costs of antibiotic resistance have been estimated to range from $150 million (without deaths) to $3 billion dollars (with deaths) each year (Levy, 1992).

The situation in developing countries however is quite different. Additional costs for the newer and more expensive antibiotics can not be met when faced with antimicrobial resistance. The number of people in the USA suffering from the consequences of antibiotic resistance are relatively small when compared with the much larger numbers of people in developing countries. In the last 20 years many thousands of lives have been lost in developing countries as a result of

antimicrobial resistance. Countries in all continents are becoming increasingly aware that antibiotic resistance is not a problem unique to their country or even continent, but that it is truly a global dilemma (Levy, 1992).

3.2 Antibiotic Resistance in the Hospital Setting

Multiple antimicrobial resistance in nosocomial pathogens has become a major problem in nosocomial infections in many developed countries such as the USA as well as in Europe (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991). The major impact of antimicrobial resistance is felt in the hospital setting with infections caused by these multiresistant hospital-acquired organisms and since the 1960s, reports of antibiotic-resistant organisms have appeared with increasing frequency(Murray, 1992; Goldmann, Weinstein, Wenzel et al., 1996; Weinstein, 1992). Prior to 1965 no hospital outbreaks involving multiply resistant gram-negative bacilli were investigated by the Centers for Disease Control (CDC). During 1965 to 1975 however, 11 of 15 nosocomial epidemics of Enterobacteriaceae involved multiply resistant strains (Weinstein, 1992). Over the last two decades there has been a noticeable change in the pattern of hospital-acquired infection, partly due to the selection pressure exerted by new broad-spectrum antibiotics. In the 1980s, large families of β-lactamases that mediate resistance to newer cephalosporin antibiotics emerged in the Enterobacteriaceae. It is well known that approximately 5 to 10% of all hospitalized patients develop a nosocomial infection which can increase morbidity and mortality and add significantly to the economic burden of managing underlying diseases by prolonged hospital stay (Haley, Culver, White et al., 1985). Other factors such as the unavoidable presence of hospital microbial flora in environmental reservoirs and increasingly invasive procedures may have also contributed to changes in

patterns of bacterial species involved in nosocomial infections (Bergogne-Berezin, Decre, and Joly-Guillou, 1993). Bacterial resistance has become a fact of hospital life and is so common that it often goes unnoticed until it is either extreme or epidemic (Pittet, Herwaldt and Massanari, 1992).

3.3 Antibiotic Resistant Nosocomial Organisms in ICUs

The organisms responsible for nosocomial infections are continually evolving and although the predominant sites of nosocomial infections have not changed, the etiological agents have altered as new antimicrobials have been introduced and host factors modified. Furthermore, new pathogens have emerged and old ones revived with new virulence and resistance mechanisms (Bergogne-Berezin, Decre, and Joly-Guillou, 1993). Widespread use of broad-spectrum antibiotics in hospitals provides strong selective pressure for the emergence and persistence of bacterial antimicrobial resistance, a phenomenon seen in Europe as well as the USA (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991; Johnson and Woodford, 1993). Examples of resistance traits that have emerged in recent years include β-lactam and aminoglycoside resistance in enterococci, resistance to third-generation cephalosporins and aminoglycosides in the enterobacteriaceae, and glycopeptide resistance in enterococci and coagulase-negative staphylococci (CNS). The continuing tendency of nosocomial pathogens to develop or acquire new antibiotic-resistant traits poses significant problems regarding the treatment and control of hospital infections (Johnson and Woodford, 1993).

Gram-negative bacteria such as *Enterobacter, Serratia, Citrobacter, Pseudomonas, Stenotrophomonas (Xanthomonas),* and *Acinetobacter* species are important causes of nosocomial infections such as bacteremias, pneumonias, urinary tract infections,

and surgical wound infections. They are among the most common infecting bacteria in patients hospitalized in the intensive care setting and account for about 40% of all infections in ICUs in the USA (Flynn, Weinstein, Nathan et al., 1987; Snydman, 1991). Studies performed in Europe and the USA on the incidence of multi-resistance in gram-negative bacterial isolates from ICUs indicate that resistance rates vary from about 10 to 30 % (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991).

The National Nosocomial Infections Surveillance (NNIS) system (USA) recently conducted a survey to determine trends in the microbial etiology of nosocomial infections in the 1980s. Since 1980 there has been an overall decrease in infections caused by *E.coli* (23% in 1980 to 16% in 1986-1989) and *Klebsiella* (7% to 5%). However, antimicrobial-resistant strains of *Pseudomonas* and *Enterobacter* species were seen more frequently (Schaberg, Culver, and Gaynes, 1991). In contrast to the 1970s, major shifts in the etiology of nosocomial infections have occurred in the 1980s away from more easily treated pathogens toward more resistant pathogens with fewer options for therapy. Furthermore, changes within a given genus have also occurred, particularly in antimicrobial resistance phenotypes. For example, 91% of *P.aeruginosa* isolates in 1982 were susceptible to cefotaxime in contrast to only 65% in 1989, and 93% of *Enterobacter cloacae* in 1982 versus 76% in 1989 (Schaberg, Culver, and Gaynes, 1991). Similarly, 97% of *P.aeruginosa* were sensitive to gentamicin in 1982 versus 88% in 1989. Infection problems in the 1990s and some of the diseases caused by these gram-negative organisms are shown in table 3.1. *Enterobacter cloacae* has become a problem particularly in areas where there is extensive use of some of the newer cephalosporins (Flynn, Weinstein, and Kabins, 1988). In parts of the world *Klebsiella pneumoniae* species possess extended spectrum β-lactamases (ESBLs)

which are capable of hydrolyzing cefotaxime, ceftriaxone, and aztreonam. Clinical isolates from France showed that 30% of *Klebsiella pneumoniae* isolates in surgical ICUs and 18.3% in medical ICUs had ESBLs (Neu, 1993). In 1994 the percentage of nosocomial *E.coli* in ICUs reported to be resistant to ampicillin was 40.7% compared to 35% in isolates from non-ICUs (unpublished NNIS data). NNIS data also indicate that the percentage of Enterobacteriaceae that are resistant to ceftazidime has increased in isolates from ICUs (Gaynes, 1995). An analysis of NNIS data for imipenem resistance among *Pseudomonas aeruginosa* isolates showed that resistance was more common in ICUs, teaching hospitals, and in isolates from the respiratory tract (Gaynes, 1995).

The most recent resistance that has developed among nosocomial gram-negative organisms is resistance to third generation cephalosporins. When these antimicrobials were first introduced (1978 in Europe and 1981 in the USA), the vast majority of Enterobacteriaceae and a number of other gram-negative organisms were susceptible. However, it soon became apparent that certain organisms such as *Enterobacter, Serratia, Citrobacter,* and *Pseudomonas aeruginosa* could rather easily develop resistance (Murray, 1989). Cephalosporin resistance can also occur with *Proteus* and *Serratia* species. *Serratia marcescens* has become increasingly common as a cause of nosocomial wound, urinary tract, pulmonary, and even bacteremic infections (Neu, 1993). *Enterobacter* species have replaced *Klebsiella pneumoniae* as the third leading cause of gram-negative nosocomial infections in the USA behind *E.coli* and *Pseudomonas aeruginosa* (de Champs, Henquell, Guelon et al., 1993; Neu, 1992). *Pseudomonas aeruginosa* resistance is usually a combination of poor entry of antimicrobials as well as the presence of enzymes that inactivate or modify the antimicrobial. Resistance of *Pseudomonas aeruginosa* to imipenem and fluoroquinolones has also increased, and in 1984

Table 3.1 Infection Problems in the 1990s

Gram-Negative Organism	Diseases(examples)	Resistant to
Klebsiella	Pneumonia	Cephalosporins
Enterobacter	Bacteremia	Penicillins
Other Enterobacteriaceae	UTI[1], surgical wounds	Aminoglycosides
Pseudomonas aeruginosa	Bacteremia, pneumonia, UTI	All antimicrobials
Stenotrophomonas (Xanthomonas)maltophilia	Pneumonia	All antimicrobials

[1]UTI= Urinary tract infection

(Modified after Neu, 1993)

virtually all *Pseudomonas aeruginosa* isolates in the USA, Europe, and Japan were inhibited by $<1\mu g/ml$ of ciprofloxacin. Today 25% of *Pseudomonas aeruginosa* isolates are resistant to all the fluoroquinolones in some institutions (Neu, 1992).

Stenotrophomonas (Xanthomonas) maltophilia is resistant to virtually all antimicrobials, including the newer fluoroquinolones and aminoglycosides. *Acinetobacter baumannii* has become an important cause of nosocomial infection in the hospital setting and is resistant to β-lactams, aminoglycosides and tetracyclines. Most *Acinetobacter* species have been susceptible to imipenem but will probably become resistant to all carbapenems as have many other organisms (Neu, 1992).

3.4 Risk Factors for Colonization and Infection in Patients in ICUs

One of the primary components of modern medicine is the care of critically ill patients in special high technology units. However, life support systems which involve invasive diagnostic and therapeutic procedures disrupt normal host defense mechanisms with the result that more than one third of patients admitted to ICUs experience unexpected complications of medical care (Pittet, Herwaldt and Massanari, 1992). The incidence of nosocomial infections in ICUs is usually much higher than in other hospital units and although ICUs make up only 5% of hospital beds and care for less than 10% of hospitalized patients, infections acquired in these units account for more than 20% of nosocomial infections (Daschner, 1985; Pittet, Herwaldt and Massanari, 1992).

Natural host defense mechanisms may be impaired by underlying diseases or as a result of medical and surgical interventions. All patients admitted to an ICU

will have at least one, and often multiple vascular cannulas that break the normal skin barriers and establish direct access between the environment and the circulatory system. Administration of H_2 blockers or antacids reduce acidity and allow overgrowth of enteric flora leading to colonization of the stomach with nosocomial pathogens. This in turn increases the frequency of retrograde colonization of the oropharynx and trachea with gram-negative bacteria (Pittet, Herwaldt and Massanari, 1992). Insertion of endotracheal tubes, nasogastric tubes, and urinary catheters circumvent normal physiological mechanisms for evacuating and cleansing hollow organs thus facilitating ease of entry of potentially pathogenic nosocomial organisms. Normal food intake is often suspended in ICU patients because of the severity of their condition which may result in nutritional deprivation. The prevalence of malnutrition in ICUs is an almost universal problem and has been estimated to be as high as 10 to 50% in some US hospitals (Pittet, Herwaldt and Massanari, 1992). Furthermore, ICU patients tend to be the youngest and oldest in hospital, are given more antibiotic therapy during their hospitalization than any other group of patients, and often require prolonged hospital stays (Daschner, 1985; Donowitz, Wenzel, and Hoyt, 1982; Emmerson, 1990; Pittet, Herwaldt and Massanari, 1992). All these multiple factors contribute significantly to the increased risk these patients have of developing a resistant nosocomial infection. Whereas a general medical or surgical patient has a 6% risk of becoming infected with a nosocomial resistant pathogen during their hospital stay, the ICU patient has an 18% risk (Donowitz, Wenzel, and Hoyt, 1982).

CHAPTER FOUR

THE EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE

4.1 Introduction

The human host and its indigenous microflora is a complex ecosystem held in equilibrium by a number of factors. The surfaces of the human body harbor a complex of indigenous aerobic and anaerobic bacteria which enjoy a commensal existence with the host. The upper respiratory tract, including the oral cavity, the nasal passages, the nasopharynx and the oropharynx present various aspects of microbial colonization (Tancrede, 1992). The gastrointestinal tract is the main reservoir of bacteria and the presence of anaerobes in particular are responsible for limiting the concentration of potentially pathogenic organisms. The equilibrium of gastrointestinal microflora can be upset by antimicrobial agents ultimately leading to colonization and/or infections as a result of proliferation of antimicrobial-resistant pathogens (Bingen, Denamur, and Elion, 1994; Emmerson, 1990). This chapter describes the ecosystem of the bowel, bacterial colonization of the oropharynx, lower respiratory tract, and gastrointestinal tract, and the effects of antimicrobial agents on the intestinal flora. It also looks at the mechanisms of antimicrobial resistance and the role of molecular typing methods in characterizing resistant microorganisms.

4.2 Ecosystem of the Human Bowel

Marked variations in the concentrations and bacteriological patterns of the gastrointestinal flora are observed at different levels of the tract. The normal flora

of the bowel contains more than 400 obligate anaerobic species in a total concentration of 10^{11} to 10^{12} colony forming units (CFUs) per gram of feces (Vollaard and Clasener, 1994; Tancrede, 1992). The dominant populations of human intestinal flora are members of the genera *Eubacterium, Clostridium, Bacteroides,* and *Peptostreptococcus.* The concentration of aerobic flora is one thousand times lower, and is represented by a small number of species of gram-negative bacilli (*Escherichia coli* being the most predominant), and gram-positive cocci, notably enterococci (Pechére, 1994).

With respect to population control of microorganisms, the number of ingested bacteria is dramatically reduced by gastric acidity. A low concentration of organisms is maintained in the small bowel where the major mechanism of population control is intestinal motility. The highest concentration of bacteria are found in the colon (Tancrede, 1992). It is well known that the indigenous microflora of the gastrointestinal tract are one of the major defense mechanisms that protect the human body against colonization by invading bacteria (Tancrede, 1992). This phenomenon has been described as colonization resistance which may be defined as the limiting action of indigenous anaerobic flora on colonization of the bowel by exogenous as well as potentially pathogenic (mostly aerobic) flora in the digestive tract (Vollaard and Clasener, 1994; Apperloo-Renkema and van der Waaij, 1991). Colonization resistance may also be mediated by anatomical and physical factors such as intact mucosa, salivation, swallowing, secretion of immunoglobulin A, production of gastric acid, desquamation of cells of the mucous membranes, and normal gastrointestinal motility (Vollaard and Clasener, 1994). Even though many bacterial members of the gastrointestinal ecosystem are potentially pathogenic, the human host and its microflora coexist concordantly. This equilibrium is sometimes unbalanced by

modifications of the host and/or its microflora (Tancrede, 1992).

The normal microflora prevent colonization by non-commensal organisms by several different mechanisms:

i) **Competition for nutrients:** Endogenous bacteria in the gastrointestinal tract utilize available nutrients with the result that exogenous species can not establish themselves.

ii)**Competition for attachment sites:** Mucosal surfaces of the gastro-intestinal tract are sites of attachment for infection-producing organisms. By occupation of attachment sites, commensal organisms are able to prevent colonization by pathogenic organisms. Administration of antimicrobials suppress indigenous flora allowing potential pathogens to adhere to the mucosa of the gastrointestinal tract.

iii) **Production of volatile fatty acids:** Anaerobic bacteria produce volatile short-chain fatty acids such as acetic, butyric, and propionic acids that are toxic for invading enterobacteria and inhibit their growth. These fatty acids are changed during therapy with antimicrobials that affect anaerobes.

iv) **Bacteriocins:** These are high-molecular-weight protein antimicrobial substances produced by commensal enterobacteria, streptococci, and anaerobic bacteria. They limit the overgrowth of commensal bacteria in the gastrointestinal tract (Nord, Kager, and Heimdahl, 1984).

4.3 Bacterial Colonization, Translocation, and Infection

Bacterial colonization may be defined as the isolation of the same species of bacteria from two or more consecutive cultures from the same site without clinical signs of infection (van Uffelen, Rommes, and van Saene, 1987; Stoutenbeek, van Saene, and Zandstra, 1987). Bacterial translocation is defined as the passage of viable indigenous bacteria from the gastrointestinal tract to extraintestinal sites (Carrico, Meakins, Marshall et al., 1986; Berg, 1995; Livermore, 1987a; Tancrede, 1992). The factors that promote bacterial translocation are virulence and pathogenicity of the bacterial strain, intestinal bacterial overgrowth, permeability of the intestinal mucosa, and a lowered immune status of the host (Livermore, 1987a; Tancrede, 1992).

The most common and often the most significant cause of disturbances in the normal oropharyngeal and gastrointestinal flora is the administration of antimicrobials (Hentges, Stein, Casey et al., 1985; Barza, Giuliano, Jacobus et al., 1987). This aspect will be covered in section 4.4.

An important factor in the development of ICU-acquired infections is impairment of local host defenses by surgery and the presence of invasive monitoring devices, use of broad spectrum antibiotics with disruption of endogenous flora, and prolonged parenteral nutrition (Marshall, Christou, and Meakins, 1993). Moreover, they arise in the setting of transient but profound abnormalities of intrinsic host defenses, and their prevalence is highest in patients with the most marked degrees of organ dysfunction. Most ICU infections are caused by the patient's own flora after pathologic colonization of endogenous reservoirs (Nystrom, Frederici, and von Euler, 1988; Marshall, Christou, and Meakins,

1993; Lambert-Zechovsky, Bingen, Denamur et al., 1992). Factors that have been significantly associated with colonization in previous studies include respiratory tract disease, coma, hypotension, tracheal intubation, acidosis, azotemia, and either leucocytosis or leucopenia (Peacock, Sorrell, Sottile et al., 1988). Colonization of the respiratory tract with gram-negative bacilli apparently plays a major role in the pathogenesis of nosocomial respiratory infections (Johansen, Pierce, Sanford et al., 1972; Bryant, Trinkle, Mobin-Uddin et al., 1972; Goldmann, LeClair, and Macone, 1978). A patient's own flora, bacteria in the hospital environment, contaminated commercial products and other infected patients are the major reservoirs of nosocomial pathogens (Ismaeel, 1993). Use of invasive devices greatly amplifies transmission, colonization and susceptibility to infection. Because gram-negative bacilli are part of the normal bowel flora, the catheterized urinary tract, surgical wounds or the oropharynx readily become cross-colonized (Noone, Pitt, Bedder et al., 1983; Maki, 1978).

4.3.1 Oropharyngeal Colonization

Earlier reports from studies performed in the 1970s focused on the role of oropharyngeal colonization in the pathogenesis of nosocomial infection (Johanson, Pierce, and Sanford, 1969). The oropharyngeal cavity in a healthy individual does not normally harbor gram-negative bacteria. There are several defense factors associated with the mucosa that resist colonization by gram-negative bacteria. Serious underlying disease and advanced age are two major determinants that interfere with this mucosa-associated defense system which facilitates oropharyngeal colonization by gram-negative bacteria in ICU patients (Maki, 1978; van Uffelen, Rommes, and van Saene, 1987). Practically all other conditions impairing clearance of gram-negative bacteria are iatrogenic: operative

trauma, operations lasting longer than two hours, intubation which interferes with chewing and swallowing (van Uffelen, Rommes, and van Saene, 1987). Many of these agents are especially prevalent in patients who are ventilated for more than one week. In these severely ill, longterm ventilated patients, high colonization rates of the oropharyngeal cavity by gram-negative bacteria are recorded (van Uffelen, Rommes, and van Saene, 1987). It has also been observed that even hospitalized patients with no underlying disease and no exposure to antibiotics become colonized with gram-negative bacteria in the oropharynx. The fact that oropharyngeal flora are almost always of the same genera as those in the fecal flora point to the latter as the origin of newly appearing oropharyngeal strains (Rosenthal and Tager, 1975; LeFrock, Ellis, and Weinstein, 1979).

4.3.2 Lower Respiratory Tract Colonization

Because it bypasses the upper airway host defense system, an endotracheal tube allows direct entry of bacteria into the lower respiratory tract. Also, the presence of an endotracheal tube can interfere with multiple defense functions of the lung through its impairment of the cough reflex, its interference with the function of the mucociliary escalator and its stimulation of excessive tracheobronchial mucus secretion that leads to the pooling and stagnation of secretions. This induction of mucus can promote colonization by *Pseudomonas aeruginosa* because this organism has the capacity to bind directly to mucus (Morrison, Jr. and Wenzel, 1984; Levine and Niederman, 1991).

The respiratory tract of hospitalized patients can become colonized with gram-negative bacteria through endogenous contamination from the gastrointestinal tract or exogenous contamination from respiratory therapy and mechanical

ventilation. Such colonization appears to immediately precede a lower respiratory tract infection in up to 23% of ICU patients (Johanson, Pierce, and Sanford, 1969; Villarino, Stevens, Schable et al., 1992). Host susceptibility appears to be a more important factor than degree of contact in the acquisition of gram-negative respiratory tract flora (Rahal, Meade, Bump et al., 1970; Johanson, Pierce, and Sanford, 1969). Although it is apparent that the reservoir and mode of spread of gram-negative nosocomial infection varies from one outbreak to another, two basic epidemiological patterns are discernable. In many reported "epidemic" situations the reservoir of the pathogen is in a relatively restricted area of the environment such as inhalation equipment or contaminated solutions. Usually the outbreak can be readily controlled by identification and appropriate management of the reservoir (Grundmann, Kropec, Hartung et al., 1993). However, with "endemic" nosocomial infections, the reservoir is more diffuse and in most cases includes the endogenous flora of the patient population (Chetchotisakd, Phelps, and Hartstein, 1994). A greater variety of strains are usually observed and conventional control measures are less effective. The potential for emergence of highly drug resistant gram-negative bacteria by selective pressure of antimicrobial therapy is enhanced if the reservoir includes the patient's endogenous flora (Selden, Lee, Wang et al., 1971). Colonization of the respiratory tract is common in intubated patients requiring intensive care and in most instances precedes development of infection. Certain patho-physiologic conditions have been shown to be associated with bacterial colonization of the respiratory tract including acidosis, coma, and prior respiratory tract disease (Schwartz, Dowling, Benkovic et al., 1978). In intubated patients who no longer have the protective benefit of glottic closure and cough, the cuff of the endotracheal tube represents the final mechanical barrier guarding the trachea (Schwartz, Dowling, Benkovic et al., 1978).

4.3.3 Gastrointestinal Colonization

More recently it has been observed that the stomach and small bowel also become colonized with the organisms responsible for a significant number of nosocomial infections in critically ill patients and it has been suggested that the pathological colonization of the gastrointestinal tract may be a critical prelude to the development of multiple organ failure (MOF) syndrome (Marshall, Christou, Horn et al., 1988; Marshall, Christou, and Meakins, 1993). Overgrowth of bacteria in the stomach provides a reservoir for colonization of the esophagus, mouth, and nasopharynx, probably facilitated by de-acidification of the stomach, the inability of some patients to swallow and by the presence of a nasogastric tube. Prevention or elimination of this dangerous reservoir will depend on the maintenance of gastric acidity and restoration of gut motility (Atherton and White, 1978). The gastric contents of fasting healthy people are largely free from bacteria, and the major factor responsible for this is a gastric pH of less than four, which is a potent antagonist of bacterial growth. Studies have shown that hypochlorhydria or therapeutic alkalinization in ICU patients tends to promote bacterial overgrowth in gastric contents within four days, whereas fungal colonization of nasogastric aspirates may occur within eight days of starting antacid therapy (Garvey, McCambley, and Tuxen, 1989). When the pH of the stomach is greater than four then rapid colonization with high counts of gram-negative bacilli occurs (Hillman, Riordan, O'Farrell et al., 1982). In a study conducted in Boston, Massachusetts, USA, the number of gram-negative bacilli in gastric aspirates correlated positively with the pH of the gastric aspirates, and that treatment of seriously ill patients with antacids or cimetidine encouraged upper airway colonization and predisposition of patients to pneumonia caused by

gram-negative bacilli (Du Moulon, Paterson, Hedley-White et al., 1982). The most commonly accepted explanation of upper airway colonization is fecal-oral contamination by the patient's endogenous gut organisms, transmitted by the patient or nursing staff. However, the stomach may be just as important a reservoir (Du Moulon, Paterson, Hedley-White et al., 1982).

Bacterial adherence is a cell to cell interaction that may be a mechanism through which seriously ill patients have an enhanced susceptibility to colonization. At multiple epithelial sites throughout the body, epithelial cells have receptors for bacteria that allow organisms to adhere to the mucosa and establish a foothold from which colonization can develop. Bacteria bind to these receptors via surface appendages called adhesins. Studies have shown that patients with an increased in vitro adherence are the same persons who have an increased incidence of in vivo gram-negative bacilli colonization (Levine and Niederman, 1991).

4.4 The Effect of Antimicrobial Agents on Intestinal Microflora

The parenteral administration of broad-spectrum antibiotics may have important effects on the composition of normal fecal flora (Nord, Heimdahl, Kager et al., 1984; Giuliano, Barza, Jacobus et al., 1987). A number of adverse reactions to antibiotics have been related to changes in the fecal flora including diarrhea, overgrowth of *Clostridium difficile* and fungi, the selection of antibiotic-resistant strains, and a diminution in colonization resistance (Giuliano, Barza, Jacobus et al., 1987). Several factors influence the extent to which a given antibiotic will decimate the normal bowel flora. Predominant among these is the incomplete absorption of orally administered drugs (Sutter and Finegold, 1974). Poorly absorbed agents may reach the colon in an active form where they destroy

susceptible organisms and alter the ecological balance. Parenterally administered antibiotics that are secreted by the salivary glands, in the bile, or from the intestinal mucosa also tend to destroy the normal microbial population (Nord, Kager, and Heimdahl, 1984). Colonization and infection by nosocomial gram-negative bacilli are then likely to follow (Myerowitz, Medeiros, and O'Brien, 1971).

The influence of antibiotics on the concentrations of potentially pathogenic organisms in the bowel is determined by their influence on the flora that provides colonization resistance as well as by their direct influence on potentially pathogenic organisms (van der Waaij, de Vries-Hospers, and Welling, 1986; Vollaard and Clasener, 1994). There are four scenarios that may arise: firstly, no inhibition of potentially pathogenic organisms nor the flora providing colonization resistance occurs; secondly, potentially pathogenic organisms are inhibited whereas flora providing colonization resistance are not. This means that potentially pathogenic organisms are eliminated whilst resistant flora will not grow out since colonization resistance is not disturbed. This is called "selective decontamination" and can be achieved by the administration of 20mg of pefloxacin daily (Clasener,Vollaard and van Saene, 1987; van der Waaij and Berghuis-de Vries, 1974; Vollaard, Clasener, and Janssen, 1990). Thirdly, the flora that provide colonization resistance are inhibited whereas potentially pathogenic organisms are not which allows their overgrowth. Examples of antibiotics that can do this are amoxicillin, cefotaxime, and clindamycin (Vollaard, Clasener, and Janssen, 1990). Fourthly, both potentially pathogenic organisms as well as colonization resistance flora are inhibited. This may be called "unselective decontamination" in which susceptible potentially pathogenic organisms are inhibited but the concentration of resistant organisms increases

(Vollaard and Clasener, 1994). Table 4.1 illustrates the impact of various antimicrobial agents on the human gastrointestinal flora.

The potential of an antibiotic to change the colonization is related to its dose and pharmacokinetic properties. Oral antimicrobial agents that are poorly absorbed from the gastrointestinal tract, or absorbed but also excreted in active form in the bile or saliva, generally have a significant effect on microbial colonization. Parenterally administered antimicrobials excreted in high concentrations in the gastrointestinal tract also cause significant changes in the microflora (Nord, Kager, and Heimdahl, 1984). Oral antimicrobials that are well absorbed in the upper part of the small intestine have little impact on the microflora in the large intestine whereas poorly absorbed agents cause considerable changes. Ampicillin is one of the most widely used antimicrobials because of its broad spectrum activity, but its major disadvantages are that it is incompletely absorbed and there is a high incidence of diarrhea during therapy (Nord, Kager, and Heimdahl, 1984). Pipericillin, a fourth generation penicillin, is excreted in high concentrations in the bile and has therefore a pronounced impact on large intestinal microflora (Kager, Malmborg, Nord, et al, 1983). Third generation cephalosporins have good activity against gram-negative aerobes but they also have high biliary excretion resulting in significant changes in the normal intestinal microflora. The consequences are superinfection, diarrhea and colitis (Arvidsson, Alvan, Angelin, et al, 1983; Alestig, Carlberg, Nord, et al, 1983). Cefoxitin has wide activity against aerobic gram-positive and gram-negative bacteria but the colon microflora are affected because it is present in the large bowel in concentrations far above the minimum inhibitory concentrations of many aerobic and anaerobic bacteria (Nord, Heimdahl, Kager, et al, 1984). Colonization with cefoxitin-resistant enterococci, clostridia, *Pseudomonas* and *Enterobacter* strains can

Table 4.1 Impact of Different Antimicrobial Agents on Gastrointestinal Flora

Antibiotic	Salivary concentration	Intestinal concentration	Overgrowth of resistant strains: Oropharyngeal	Gastrointestinal
Ampicillin	Low	Moderate	Low	High
Penicillin	Low	Low	Low	Low
Pipericillin	Low	High	Low	High
Cefuroxime	Low	High	Low	High
Cefazolin	Low	High	Low	High
Cefotaxime	Low	High	Low	High
Cefoxitin	Low	Moderate	Low	Moderate
Ceftriaxone	Low	High	Low	High
Cefoperazone	Low	High	Low	High
Aztreonam	Low	High	Low	High
Imipenem	Low	Low	Low	Low
Erythromycin	Moderate	High	High	High
Doxycycline	High	Moderate	Moderate	Moderate
Metronidazole	High	Low	Low	Low
Clindamycin	Moderate	High	High	High
Ciprofloxacin	Low	Low	Low	Low

(Modified after Péchère, 1994, and Nord, 1984)

therefore take place in the gut. Imipenem has a rather broad anti-bacterial spectrum with only minor changes in intestinal microflora (Nord, Kager, Philipsson, et al, 1984). When erythromycin is used for extended periods of time it has undesirable effects due to high fecal concentrations. It tends to decrease the numbers of aerobic gram-negative bacilli and anaerobic microflora are also considerably disturbed with new colonization by erythromycin-resistant strains occurring in the oropharynx or colon. Clindamycin therapy results in high concentrations in the large intestine when administered perorally or parenterally, which leads to pronounced changes in aerobic and anaerobic colonic microflora. The end result is that clindamycin-resistant enterococci and enterobacteria proliferate (Nord, Heimdahl, Kager, et al, 1984). Extensive use of tetracyclines results in side effects such as diarrhea and superinfection (Nord, Kager, and Heimdahl, 1984).

4.5 Reservoirs and Antimicrobial Resistance

With respect to nosocomial gram-negative infections, an important factor in determining the frequency of antimicrobial resistance is the presence of a reservoir. A reservoir is an ecological niche in which an infectious agent persists by a cycle of transmission or reproduction, or both. Reservoirs provide an opportunity for the development of resistance either by the exchange of genetic material with other antibiotic-resistant organisms or simply by the persistence of an organism and exposure to the selective pressures that lead to development of resistance (Cohen, 1992). Reservoirs can also provide the opportunity for resistant organisms to persist and be transmitted thus affecting the overall frequency of antibiotic resistance(Brun-Buisson and Legrand, 1994). Greater transmission leads to a higher frequency or prevalence of resistance (Cohen,

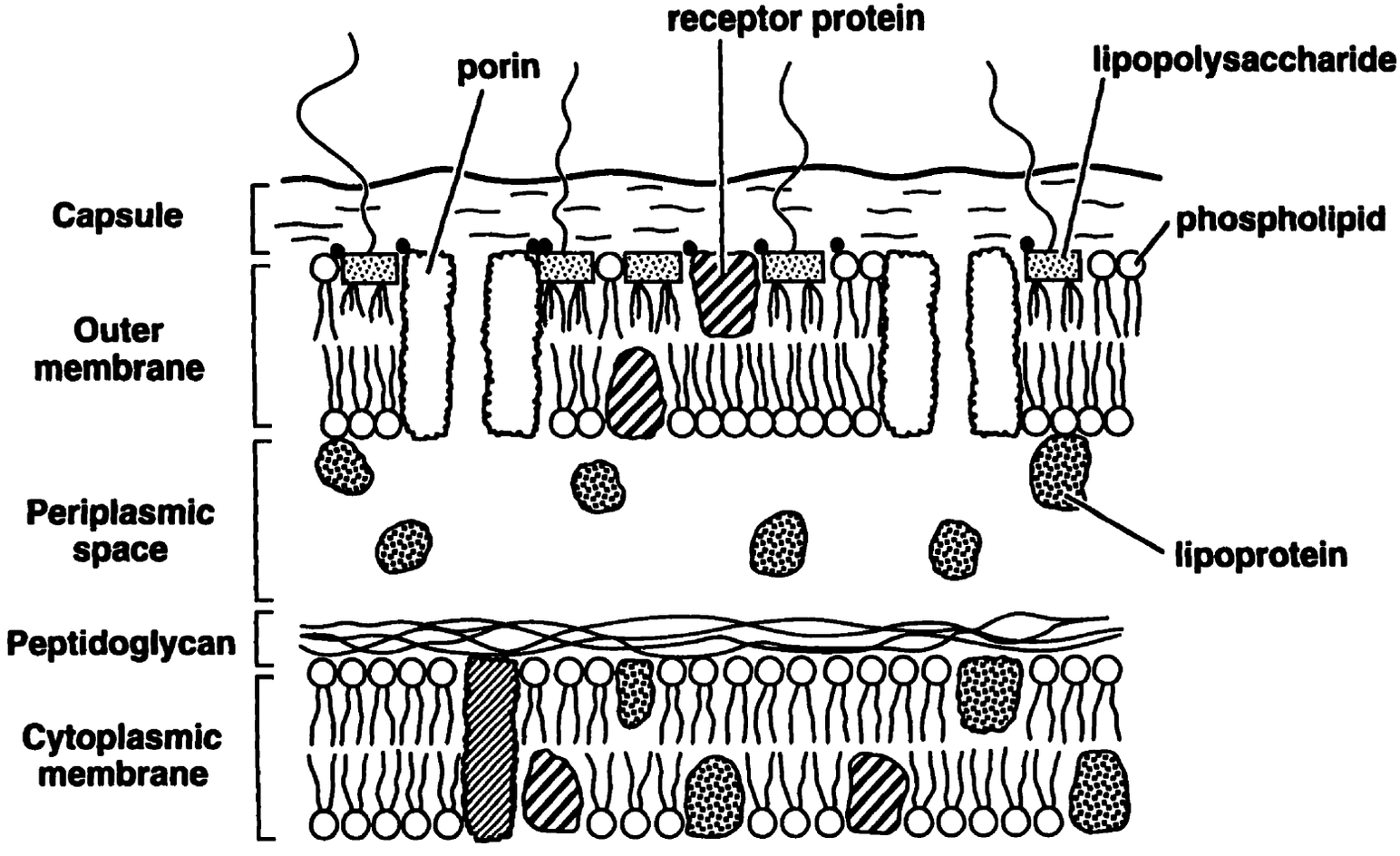
1992). There are multiple reservoirs of resistant bacteria in the hospital environment and the dynamics of bacterial spread need to be examined in order to determine whether resistant organisms originate endogenously (ie.from the patient's own flora) or exogenously (ie. transfer between patients, hospital areas, health care workers, and the environment)(Kropec, Huebner, Riffel et al., 1993). If the reservoir(s) can be identified then this may impact on infection control practices as well as antimicrobial prescribing habits.

4.6 Mechanisms of Antimicrobial Resistance

Since the advent of antimicrobial agents, bacteria have proved to be particularly adept at becoming resistant to each new antibiotic agent discovered (Jacoby and Archer, 1991; Neu, 1984). Resistance has many consequences and a patient infected with a drug-resistant organism is more likely to require extended hospitalization and an increased risk of death. Furthermore, resistance compels the use of more toxic or more expensive alternative drugs (Jacoby and Archer, 1991). The emergence of resistance in bacterial infections is thus a common occurrence. It contributes to clinical failure in a significant proportion of patients and the organisms that are becoming more highly and stably resistant include many of the commonly encountered enteric gram-negative bacilli and *Pseudomonas aeruginosa.*

The gram-negative bacterial cell wall through which antibiotics must pass to reach their sites of action is fairly complex (Figure 4.1). An outer membrane consisting primarily of lipopolysaccharide (LPS) and other proteins is a complex structure that prevents the penetration of many different substances including antibiotics. In addition, many gram-negative species synthesize capsular material which

Figure 4.1 Diagrammatic Representation of the Cell Surface of a Gram-Negative Bacterium



(Modified after Livermore, 1987)

attaches loosely to the outer surface of the outer membrane. The outer membrane contains porins through which bacteria take in nutrients from the environment and excrete waste products. Most antibiotics that are commonly used for gram-negative infections are small enough to pass through these porin channels at a rate dependent on the polarity and charge of the molecules (Barriere, 1992). Once an antibiotic passes through the outer membrane of the bacterial cell wall it enters the periplasmic space between the outer membrane and the cytoplasmic membrane. It is in this space that β-lactamases produced by various gram-negative bacteria are concentrated. Once in the presence of these inactivating enzymes, an antibiotic must be resistant to hydrolysis in order to pass further to the inner membrane and the location of the penicillin-binding proteins (PBPs).

Whilst gram-positive organisms have a cell wall composed mainly of peptidoglycan and teichoic acids, gram-negative organisms have a more complex structure in that the peptidoglycan layer is not in contact with the environment but is bounded on its outer surface by the outer membrane. This peptidoglycan structure affords a barrier to the permeation of many antibiotics, and modification of its composition can increase resistance. In addition, many gram-negative organisms synthesize β-lactamases which are largely retained in the periplasmic space between the cytoplasmic and outer membranes (Livermore, 1987b).

Resistance to antibiotics is a worldwide problem and in no case is the problem of resistance clearer than with regard to β-lactam antibiotics (penicillins, cephalosporins, monobactams, and carbapenems). The widespread prevalence of certain mechanisms of resistance have rendered older drugs in this class obsolete. Resistance may arise by a mutation that reduces target affinity or allows the over-production of a drug-modifying enzyme. More often, resistance genes are carried

on extrachromosomal plasmids that may be transferable from organism to organism by conjugation, transduction, or transformation (Jacoby and Archer, 1991). The genes may even be packaged in units of DNA called transposons that allow them to jump from one DNA site to another, thus further facilitating the spread of resistance. Despite their versatility however, bacteria have a limited number of mechanisms of acquired resistance. These include:

a) Alteration of the drug's target site components
b) Decreased outer membrane permeability
c) Production of inactivating enzymes (Murray, 1991; Sanders, 1992; Murray and Moellering, 1978; Collatz, Gutmann, Williamson et al., 1984; Jacoby and Archer, 1991; Sanders, 1987).

Alterations in the target site include both reduction of receptor affinity and the substitution of an alternative pathway whereas decreased permeability may occur through an active efflux system. Of these three mechanisms, production of inactivating enzymes such as β-lactamases are the most frequently encountered in clinical isolates.

Altered Target Site:

β-lactam antibiotics inhibit the penicillin-binding proteins which are trans- and carboxy- peptidases that catalyze the cross-linking of the cell wall polymer, peptidoglycan. Peptidoglycan plays a vital role in maintaining the strength of the cell wall. β-lactam antibiotics prevent formation of peptide bridges, causing a weakened cell wall to be produced. Resistance arises if the PBPs cease to bind β-lactams, or are protected by permeability barriers, or β-lactamases (Livermore,

1991). The synthesis of new PBPs with low affinity for penicillin is the cause of penicillin resistance in pneumococci, of methicillin resistance in staphylococci, and can cause resistance in some *H.influenzae*, meningococci, and gonococci. There are no well established examples of altered PBPs in the Enterobacteriaceae, but some strains of *Pseudomonas aeruginosa* may owe part of their resistance to altered PBPs (Livermore, 1991; Murray, 1989; Neu, 1984).

Decreased Permeability:

The cell wall structure of gram-negative organisms can shield PBPs from certain antibiotics. Variations in cell wall permeability can modulate resistance to an extent which varies from antibiotic to antibiotic and from strain to strain (Livermore, 1987b). In order for an antibiotic to exert its effect it must be able to pass through the outer membrane of bacteria and reach its target site. Gram-negative bacilli have an outer membrane which serves as an effective permeability barrier. Scattered about and transversing the outer membrane are water filled channels made of porin proteins that facilitate entry of some compounds but not hydrophobic ones. The diameter and number of porin channels vary among different gram-negative species. The presence of the outer membrane also enhances the resistance of organisms that produce β-lactamases by limiting the rate at which the β-lactam is delivered to the enzyme in the periplasmic space. Changes in the porins can cause resistance to various β-lactams, including imipenem, and seem fairly common in *Pseudomonas aeruginosa* and *Enterobacter cloacae.* Permeability mutants also cause resistance to aminoglycosides, chloramphenicol, tetracyclines, and fluoroquinolones (Murray, 1989).

Plasmid-mediated resistance to tetracyclines in gram-negative bacilli is commonly

due to the acquisition of an active efflux system through the inner cytoplasmic membrane. Decreased permeability accounts for some of the resistance of *Pseudomonas aeruginosa* to aminoglycosides. This permeability change may be acquired through exposure in vivo and is not associated with enzymes or transferability. Most of the low level resistance of *Pseudomonas aeruginosa* is due to decreased permeability. High level resistance is generally associated with resistant ribosomes or the presence of inactivating enzymes (Murray and Moellering, 1978; Murray, 1989). Lack of an effective permeability barrier for certain agents may presumably explain why some gram-negative bacilli which produce β-lactamase are capable of hydrolyzing a particular drug in vitro nevertheless remain susceptible to that drug.

Enzyme Inactivation:

Of the three mechanisms by which gram-negative bacteria resist antibiotics, production of β-lactamases is by far the most frequently encountered in clinical isolates. β-lactamases are periplasmic enzymes which are capable of binding and destroying (hydrolyzing) large concentrations of antibiotics. The net effect of the interaction of a β-lactam drug with a β-lactamase is cleaved inactivated antibiotic (Sanders, 1992; Wiedemann, Kliebe, and Kresken, 1989). β-lactamases hydrolyze the β-lactam nucleus of β-lactam antibiotics and although widely distributed in nature, they are usually referred to on the basis of the principal compounds they destroy, hence penicillinases and cephalosporinases (Neu, 1984).

β-lactamases are produced by many gram-negative bacteria and differ in substrate profile, molecular weight, isoelectric point, genetic determination, and susceptibility to inhibition by several inhibitors. For gram-negative bacteria, a

number of classification systems have been proposed but none have been totally satisfactory (Table 4.2). A molecular classification based on amino acid sequences around the active site has been proposed by Bush in an attempt to combine elements of all previous schemes (Bush, 1989a; Bush, 1989b; Bush, 1989c). Using the Bush classification, group I comprises all clavulanate-insensitive chromosomal cephalosporinases. These enzymes include the classical inducible cephalosporinases of *Enterobacter cloacae* and *Pseudomonas aeruginosa*. Group 2 enzymes are sensitive to inhibition by clavulanic acid as well as other inactivators such as sulbactam and tazobactam. This group includes the most prevalent plasmid-mediated β-lactamases among *Escherichia coli* (TEM-1) and *Klebsiella pneumoniae* (SHV-1). Group 3 β-lactamases comprise the metalloenzymes which all require zinc as a cofactor. Group 3 enzymes are usually chromosomally-mediated and hydrolyze imipenem as well as other β-lactam drugs. Group 4 enzymes are rarely encountered penicillinases that are insensitive to clavulanate (Sanders, 1992; Bush, 1989a; Bush, 1989b; Bush, 1989c).β-lactamases are either chromosomally- or plasmid-mediated. The chromosomal β-lactamases of gram-negative bacteria are species-specific and the most important are the group I enzymes which occur in *Pseudomonas aeruginosa* and most of the Enterobacteriaceae (Livermore, 1991). The mode of expression of these group I β-lactamases varies between species and determine their potential to cause resistance. Certain species such as *E.coli* and *Proteus mirabilis* produce β-lactamase **constitutively** (ie. in small basal amounts) whether or not antibiotics are present. These are usually insufficient to protect the bacteria even against drugs such as ampicillin and cephalothin which are hydrolyzed rapidly by the enzymes. Other species such as *Pseudomonas aeruginosa, Enterobacter, Citrobacter, Serratia, Providencia, Proteus,* and *Morganella morganii* have **inducible** expression such that the amount of β-lactamase synthesized relates to the amount of antibiotic present (Livermore,

Table 4.2 Comparison of Various Classification Schemes for β-Lactamases of Gram-Negative Bacteria

Bush Class (1988)	Richard and Sykes (1973)	Sykes and Matthews (1976)	Ambler (1980)	Molecular Class	Enzyme Type	Examples
1	1a, 1b, 1d	A	A	C	Cephalosporinase	Ecl[1], Psa[2], Sma[3]
2a	.....	.....	.....	.....	Penicillinase	Gram-Positives
2b	III	B	C	A	Broad Spectrum	TEM-1,2,SHV-1
2b′	III, IV	B	.....	A	Extended Spectrum	TEM-3,5,SHV-2
2c	V	.....	.....	.....	Carbenicillinase	PSE-1,3,4,
2d	Ic	.....	.....	D	Cloxacillinase	OXA-1,PSE-2
2e	.....	.....	.....	.....	Cephalosporinase	Pvu[4], Bfr[5]
3	.....	B	B	B	Metalloenzyme	SmaI[6], Ahy[7]
4	.....	.....	.....	.....	Penicillinase	Pce[8]

[1] *E.cloacae*
[2] *P.aeruginosa*
[3] *S.marcescens*
[4] *P.vulgaris*
[5] *B.fragilis*
[6] *S.maltophilia*
[7] *A.hydrophila*
[8] *P.cepacia*

1991; Sanders and Sanders, 1988; Livermore, 1987a). Enzyme synthesis is minimal in the absence of antibiotic but increases when a β-lactam drug is added. **Induction** is the transient switching on or elevation of β-lactamase synthesis in response to the addition of an inducer such as a β-lactam drug (Livermore, 1991; Barriere, 1992; Livermore, 1987a).

In gram-negative organisms with inducible β-lactamases, enhanced expression of enzyme can occur by one of two mechanisms. In the first mechanism, there must be exposure of the wild-type organism to an enzyme inducer, usually a stable β-lactam drug such as cefoxitin or imipenem. β-lactamase synthesis commences 1 to 20 minutes after exposure of the bacteria to the antibiotic and usually peaks within 2 hours and levels decline once all the antibiotic has been hydrolyzed or physically removed (Dworzack, Pugsley, Sanders et al., 1987; Sanders, 1987). The ability of various β-lactam antibiotics to induce these enzymes varies greatly depending on the organism, the antibiotic and its concentration, and the length of the induction period. Certain β-lactam antibiotics are potent inducers and include benzyl penicillin, ampicillin, and most first generation cephalosporins (Livermore, 1987a). Second and third generation cephalosporins, ureidopenicillins (pipericillin, mezlocillin, and azlocillin) and monobactams are labile to group I enzymes but are weak inducers. They remain active against β-lactamase inducible species because they fail to induce β-lactamase synthesis and not because they are stable to the enzyme (Livermore, 1991). Amongst the newer β-lactams, imipenem and cefoxitin are strong inducers of group I β-lactamases at low concentrations (Livermore, 1987a).

The second mechanism responsible for elevated levels of group I β-lactamases involves the spontaneous mutation of the wild-type organism to a stably

derepressed state. **Stable derepression** is the permanent hyper production of the enzyme independently of the presence of an antibiotic (Sanders and Sanders, 1988; Neu, Duma, Jones et al., 1992; Sanders, 1987; Livermore, 1987a). β-lactamase inducible species segregate high frequency spontaneous mutants that manufacture group I enzymes continuously at a high level. These mutants are variously termed "constitutive", "stably derepressed", or "enzyme hyper-producer" and are present naturally in inducible populations usually at frequencies of 10^{-7} but occasionally up to 10^{-5}. Because they express β-lactamase independently of induction, the stably-derepressed organisms are more resistant to labile weak inducers (third generation cephalosporins and ureidopenicillins) than are enzyme-inducible strains (Livermore, 1991; Sanders and Sanders, 1988; Barriere, 1992; Sanders, 1987; Livermore, 1987a). When a population of inducible bacteria containing a few derepressed mutants is challenged with a newer cephalosporin, ureidopenicillin, or monobactam, the inducible cells fail to produce enzyme and are killed, whereas the derepressed mutants survive and may overgrow the bacterial population. This process is called **selection** in which enzyme-inducible organisms fail to produce protective quantities of enzyme and are killed, whereas the pre-existing stably derepressed variants are protected by their enzyme and so overrun the population. The typical sequence of events is that a labile weak inducer is administered to a patient who is infected with a β-lactamase inducible organism. Subsequently, stably derepressed mutants are isolated and ultimately these constitute the entire bacterial population in the infected site (Barriere, 1992; Livermore, 1987a). This appears to be a particular problem with *Pseudomonas aeruginosa* and *Enterobacter cloacae* and more rarely with *Citrobacter freundii, Serratia marcescens*, and *Morganella morganii* (Follath, Costa, Thommen et al., 1987). Derepressed mutants of *Pseudomonas aeruginosa* and *Enterobacter cloacae* manufacture five to ten times more enzyme than most other Enterobacteriaceae

explaining their greater resistance and so their greater clinical importance. The clinical significance of constitutive mutants is that they are selected during therapy of individual patients. They can cause clinical failure, and once selected, may become the predominant organism in an ICU setting (Murray, 1992; Burman, Haeggman, Kuistilla et al., 1992)).

Attempts have been made to reduce the frequency of emergence of resistance as well as unsatisfactory clinical results by adding to therapeutic regimens a second antibiotic with activity against organisms that characteristically possess inducible β-lactamases. This has been motivated by the possibility that the second antibiotic agent will inhibit the small subpopulation of stably derepressed mutants that might otherwise be selected by the first antibiotic. The so-called double β-combination (two β-lactam agents) or the addition of an aminoglycoside to a cephalosporin or cephamycin have been most frequently employed (Sanders and Sanders, 1988). The least likelihood of emergence of stably derepressed mutants is achieved by using an antibiotic with the greatest potential in vivo activity versus the infecting organism; that is, an agent that has the lowest MIC relative to the achievable concentration at the site of the infection (Barriere, 1992).

With respect to plasmid-mediated β-lactamases, over 50 types have thus far been isolated. However few are common and one type, TEM-1, predominates. β-lactamases encoded by transmissible plasmids were first observed in Enterobacteriaceae in 1965 and have subsequently increased in frequency and spread to other groups of gram-negative aerobes (Livermore, 1991; Livermore, 1987b). Initially they remained confined to the Enterobacteria, but by 1969 had spread to *Pseudomonas aeruginosa*, and by 1975 to *Haemophilus influenzae* and *Neisseria gonorrhoeae*. They now occur in 25-75% of isolates of many

enterobacteria, although another enzyme, SHV-1 predominates in *Klebsiella* species. In most ampicillin-resistant gram-negative enteric organisms, TEM-1 β-lactamase is the most common plasmid-mediated enzyme (Philippon, Labia, and Jacoby, 1989). Both TEM-1 and SHV-1 belong to the group of relatively unspecialized enzymes hydrolyzing ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and with negligible activity against cefotaxime, ceftazidime or aztreonam (Philippon, Labia, and Jacoby, 1989). PSE-1 and PSE-4 enzymes predominate in *Pseudomonas aeruginosa.* TEM-1, TEM-2, and SHV-1 enzymes are expressed constitutively but the amount of enzyme produced varies between strains, reflecting gene copy number and promoter efficiency. These enzymes hydrolyze and give resistance to ampicillin, carbenicillin, and ticarcillin. They have weaker activity against 1st generation cephalosporins, cefoperazone and the ureidopenicillins (Livermore, 1991). Unfortunately mutants of TEM-1, TEM-2 and SHV-1 are emerging which have from one to four aminoacid changes compared to the parent enzymes, but have a greatly extended substrate range. Unlike TEM-1 the new enzymes give resistance to many 2nd and 3rd generation cephalosporins although not to cephamycins or carbapenems. Like TEM-1 they can be inactivated with inhibitors such as clavulanate. Over 20 TEM and SHV variants have been described. Some, the "CAZ-types" (such as TEM-5), are more active against ceftazidime but less so against cefotaxime (Livermore, 1991). These new TEM and SHV variants are now frequent in France and have also been reported worldwide (Fernandez-Rodriguez, Canton, Perez-Diaz et al., 1992; Jarlier, Nicolas, Fournier et al., 1988). Studies performed in certain French hospitals indicate a rising proportion of *Klebsiella pneumoniae* isolates with these enzymes, from 0.75% in 1985 to 8.5% in 1987 and 11% in 1988 (Livermore, 1991; Philippon, Labia, and Jacoby, 1989). These enzymes have mostly originated in *Klebsiella* species and initial spread was by the transfer of strains from

patient to patient. Subsequently the encoding plasmids have spread to *Escherichia coli, Enterobacter cloacae, Salmonella* species, and *Serratia* species (Philippon, Labia, and Jacoby, 1989; Livermore, 1987b).

Discovery of these enzymes followed introduction of broad-spectrum cephalosporins into clinical use and has been correlated in particular with the extensive use of cefotaxime. Since TEM-1 and SHV-1 are ubiquitous in gram-negative bacteria, mutations leading to extended spectrum activity are likely to occur whenever the use of broad-spectrum cephalosporins and other β-lactams favor such changes (Philippon, Labia, and Jacoby, 1989).

4.7 The Role of Molecular Epidemiological Typing Methods

Until recently hospital infection epidemiology has essentially been based on the study of the phenotypic traits of organisms. Phenotypic techniques detect characteristics expressed by the organisms themselves and bacterial pathogens have been typed using antisera, biochemical profiles, bacteriophage sensitivity, outer membrane protein profiles, monoclonal antibody reactivity, fimbriation, production of bacteriocins, toxins, or enzymes, and antimicrobial resistance patterns (Stull, LiPuma, and Edlind, 1988; Tompkins, 1992; Gerner-Smidt, Tjernberg, and Ursing, 1991; Allardet-Servent, Bouziges, Carles-Nurit et al., 1989). However, problems with typeability, reproducibility, and discriminatory power associated with these techniques have been numerous. The disadvantages of these typing systems are such that they rely on phenotypes that may not be stably expressed, the necessary reagents may not be commercially available, the sensitivity may not be sufficient to distinguish each strain of a species, and the system may be applicable to only one bacterial species (Stull, LiPuma, and Edlind,

1988; Tompkins, 1992; Allardet-Servent, Bouziges, Carles-Nurit et al., 1989).

In order to overcome the difficulties with typeability, reproducibility or discriminatory power associated with many phenotypic techniques, numerous systems using DNA-based methods have been developed (Kostman, Edlind, LiPuma et al., 1992; Dijkshoorn, Aucken, Gerner-Smidt et al., 1993; Maslow, Mulligan, and Arbeit, 1993; Haertl and Bandlow, 1993b; Tenover, 1991; Owen, 1989). These genotypic DNA techniques are often referred to collectively as molecular epidemiology and involve direct DNA-based analyses of chromosomal or extra-chromosomal (Jarvis, 1994; John, Jr., 1989; Seifert, Schulze, Baginski et al., 1994). Typeability refers to obtaining an unambiguous positive result for each isolate analyzed; reproducibility refers to the ability of a technique to yield the same result when the same strain is repeatedly tested; discriminatory power refers to the ability to differentiate among unrelated strains (Maslow, Mulligan, and Arbeit, 1993). The characteristics of the various bacterial typing systems are shown in table 4.3.

A brief description of some of the molecular typing methods that have been frequently used follows:

4.7.1 Plasmid Profile Analysis ("Plasmid Fingerprinting")

The techniques of molecular typing were first applied to the epidemiological study of bacterial infections to trace the spread of gram-negative bacilli causing nosocomial outbreaks of sepsis and urinary tract infections (Tompkins, 1992). Plasmids are extrachromosomal elements composed of double-stranded DNA often found in the cytoplasm of many bacteria (Mayer, 1988). They may contain

Table 4.3 Characteristics of Bacterial Typing Systems

Typing System	Proportion of Strains Typeable	Reproducibility	Discriminatory Power
A. PHENOTYPIC			
Biotyping	All	Fair	Poor
Antibiograms	All	Fair	Poor
Serotyping	Most	Good	Fair
Bacteriophage typing	Most	Fair	Poor
Immunoblotting	All	Excellent	Good
Multilocus enzyme electrophoresis	All	Excellent	Good
B. GENOTYPIC			
Plasmid profile analysis	Most	Fair	Fair
Restriction endo-nuclease analysis	All	Good	Fair
Ribotyping	All	Excellent	Fair
Pulsed-Field Gel Electrophoresis	All	Excellent	Excellent
Polymerase Chain Reaction (PCR)	All	Excellent	Good
AP - PCR	All	Good	Good
Nucleotide Sequence Analysis	All	Excellent	Excellent

(After Maslow et al, 1993)

antibiotic-resistance genes and virulence-factor genes such as enterotoxins and adhesins. Plasmid profile analysis involves the extraction of plasmid DNA followed by the separation of plasmid molecules by agarose-gel electrophoresis. The disadvantages of plasmid profile analysis is that it is not universally applicable since it is limited only to strains that contain plasmids. Furthermore, similar plasmids may be found in different strains of the same bacterial species or even in strains of different species (Bingen, 1994; Stull, LiPuma, and Edlind, 1988; Kropec, Hubner, and Daschner, 1993).

4.7.2 Restriction Endonuclease Analysis of Chromosomal DNA ("Genomic Fingerprinting")

This method primarily examines the genes incorporated into the chromosome and is performed by cleaving ("digesting") the chromosomal DNA at a particular ("restricted") nucleotide recognition sequence. Restriction endonucleases recognize specific sites distributed throughout the chromosome, and the pattern of this distribution is unique for each strain (McGeer, Low, Penner et al., 1990; Maslow, Mulligan, and Arbeit, 1993; Bingen, 1994; Tompkins, 1992). The procedure involves extracting the total chromosomal DNA and digesting it into specific fragments with several restriction endonucleases ("enzymes")(Sader, Pignatari, Leme et al., 1993). The linear fragments can then be analyzed by gel electrophoresis. The major limitation of this technique is the difficulty in interpreting the complex profiles which consist of hundreds of bands which may be unresolved and overlapping (Maslow, Mulligan, and Arbeit, 1993).

4.7.3 Southern Blot Analysis of Chromosomal DNA ("Southern Hybridization Fingerprinting")

After agarose gel electrophoresis, the separated restriction fragments can be transferred onto a nitrocellulose or nylon membrane. This is called a "Southern

Blot". Using a labelled fragment of DNA as a probe, one can detect the restriction fragment(s) containing sequences ("loci") homologous to the probe. Variation in the number and size of these fragments are referred to as "restriction fragment length polymorphisms" (RFLPs) and reflect variations in both the number of loci that are homologous to the probe and the location of restriction sites within or flanking these loci (Maslow, Mulligan, and Arbeit, 1993). The process of hybridization involves denaturation of the double-stranded DNA followed by reannealing of each strand with a complimentary labelled strand called "the probe". The use of a labelled probe to identify a complimentary DNA molecule amid other DNA fragments in a gel is called "Southern Hybridization".

Limiting the number of bands to be analyzed can also be achieved using labelled DNA probes targeted to ribosomal DNA loci ("Ribotyping")(Blanc, Siegrist, Sahli et al., 1993). *Escherichia coli* ribosomal RNA has acquired a wide acceptance because the high degree of conservation of the ubiquitous and polymorphic ribosomal DNA loci in the eubacterial kingdom makes it a universal probe (Bingen, 1994; Stull, LiPuma, and Edlind, 1988). After membrane transfer by Southern blotting, the DNA fragments separated by agarose gel electrophoresis after digestion by restriction endonucleases are hybridized with an rDNA probe. Labelling E.coli rDNA with a nonradioactive system has several advantages over radioactive probes relating to safety, disposal, and stability (Bingen, 1994; Bingen, Denamur, and Elion, 1994). Ribotyping has been applied to the epidemiology of many gram-negative organisms such as *Stenotrophomonas maltophilia, Pseudomonas aeruginosa, E.coli, E.cloacae, Acinetobacter baumannii*, and *Serratia marcescens*(Bingen, Mariani-Kurkdjian, Lambert-Zechovsky et al., 1992; Garaizar, Kaufmann, and Pitt, 1991; Grattard, Pozzetto, Berthelot et al., 1994; Bingen, Denamur, and Elion, 1994; Seifert and Gerner-Smidt, 1995; Gruner, Kropec, Huebner et al., 1993; Poh, Yeo, and Tay, 1992; Gerner-Smidt, 1992; Anderson, Kuhns, Vasil et al., 1991; Bingen, Denamur, Lambert-Zechovsky et al., 1992; Bingen, Denamur, Lambert-Zechovsky et al., 1991).

4.7.4 Pulsed-Field Gel Electrophoresis (PFGE)

Recently, restriction enzymes called low-frequency-cleavage restriction endonucleases ("rare cutter") have been identified that cut chromosomal DNA rarely producing fewer fragments (ie. less than 20 restriction sites)(Maslow, Mulligan, and Arbeit, 1993; Bingen, 1994; Goering, 1993; Allardet-Servent, Bouziges, Carles-Nurit et al., 1989). PFGE provides a highly reproducible restriction profile that typically shows distinct well-resolved fragments representing the entire bacterial genome in a single gel (Maslow, Slutsky & Arbeit, 1993). This technique involves embedding organisms in agarose, lysing the organisms in situ, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently. Slices of agarose containing chromosomal DNA fragments are inserted into the wells of an agarose gel and the restriction fragments are resolved into a pattern of discrete bands in the gel by a contour clamped homogenous electric field (CHEF). This means that fragments are resolved by cyclically altering the orientation of the electric field during electrophoresis. By varying both the direction and duration of the electric field, PFGE allows the separation of DNA molecules well over 1000 kb in length (Bingen, 1994). PFGE has proved to be highly discriminatory and superior to other available molecular techniques (Haertl and Bandlow, 1993a; Struelens, Carlier, Maes et al., 1993; Acar, O'Brien, Goldstein et al., 1993; Gouby, Carles-Nurit, Bouziges et al., 1992).

4.7.5 Polymerase Chain Reaction (PCR)

The essential feature of PCR is the ability to replicate ("amplify") rapidly and exponentially a particular DNA sequence (the "template", typically 0.5 to 2.0 kilobases(kb) in length). The reaction requires a DNA polymerase, a minute amount of template and two small oligonucleotides ("primers", typically 18 - 20 base pairs (bp) in length) corresponding to sequences at opposite ends of the

template (Mullis, 1990). There are many different variations of PCR. Arbitrarily primed PCR for example, employs a single short primer (typically 10 bp in length) whose nucleotide sequence is not directed at a known genetic locus. Such arbitrary primers will result in amplification of one or more unpredictable loci and the PCR will generate a set of fragments, the number and size of which is the basis for typing an isolate (Maslow, Mulligan, and Arbeit, 1993; Bingen, 1994).

In conclusion, molecular typing methods are highly discriminatory for strains that can not be separated by phenotypic methods, and they have improved the understanding of the mechanism of nosocomial acquisition of organisms by allowing distinction between endogenous and exogenous infections. Among exogenous infections it has distinguished between individual and epidemic strains, thus differentiating cross-infection from independent acquisition (Bingen, 1994). Molecular typing techniques are extremely powerful tools when combined with epidemiological findings of case-control or cohort studies. The use of these tools has also enhanced the study of reservoirs of infection and modes of transmission of nosocomial pathogens (Tompkins, 1992).

CHAPTER FIVE

PATIENTS AND METHODS

5.1 Study Design

This study was conducted prospectively on a cohort of patients admitted to the ICU of the Calgary General Hospital. The study population consisted of all patients (male and female) over the age of 16 years admitted between December 1, 1994 and June 30, 1995, to the ICU of the Calgary General Hospital for longer than 72 hours. Patients admitted for less than 72 hours were excluded from the study as insufficient time would have elapsed for them to have developed antimicrobial resistance. Furthermore, drug overdose and high risk surgery patients were also excluded from the study because they were admitted to the ICU for only a short period of time, usually less than 48 hours.

5.2 Sample Size

The prevalence of resistant gram-negative bacilli isolated from the gut of patients in the ICU has been estimated to be about 30% based on findings of other studies conducted in ICUs in Europe and the USA (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991; Garcia-Rodriguez, Sanchez, Bellido et al., 1992). The calculation of sample size in this study was based on this estimate. In this study a 95% confidence interval with a maximum 10% margin of error was allowed. Using the statistical formula for a single population, the following sample size was calculated (Colton, 1974; Rosner, 1986):

$$\bar{x} \pm 1.96 \frac{\sqrt{p(1-p)}}{\sqrt{n}} = \text{error term}$$

where p = prevalence, and n = number of patients

Therefore:

$$1.96 \frac{\sqrt{0.3\ (1.0-0.3)}}{\sqrt{n}} = 0.10$$

Solving for n:

$$\frac{\sqrt{0.21}}{\sqrt{n}} = \frac{0.10}{1.96}$$

$$= 0.0510$$

$$\frac{0.21}{n} = 0.002601$$

$$n = 80.7 \text{ or } 81 \text{ patients}$$

In calculating the required sample size for a given study, it may be necessary to specify the desired values for probabilities of Type I (alpha) and Type II (Beta) errors, the proportion of the baseline population that has the disease under study, and the magnitude of the expected effect. The estimation of the baseline proportions are usually based on previously published reports. The expected magnitude of effect may also be estimated from these sources or, if unavailable, may be taken to represent the minimum effect that the investigators would consider meaningful (Hennekens & Buring, 1987).

5.3 Ethics

Informed consent is usually a mandatory practice in any clinical study requiring the collection of human specimens for research purposes. The nature, aims, risks,

and benefits of any study should normally be explained to a participant. Because most ICU patients are gravely ill on admission to the unit, and almost all are intubated and catheterized and some are comatose, it is often rather difficult to obtain informed consent from such patients. This in no way minimizes the necessity for obtaining consent, but in such cases it is customary to obtain informed consent from family members or relatives. In this study, informed consent from enrolled patients was not obtained for the following reasons:

- The collection of rectal swabs was a routine procedure performed on all ICU patients (except drug overdose and high-risk surgical patients) for the surveillance of multi-resistant organisms. This was in accordance with the infection control policy passed by the infection control committee of the Calgary General Hospital.

- Access to patient records was part of the ongoing hospital active surveillance policy for the tracking of nosocomial pathogens and the development of resistance.

All clinical and laboratory data of individual patients enrolled in this study were kept strictly confidential and securely locked. All results arising from this study that may be published will be in collective form and anonymous, so as to retain confidentiality of all patients.

5.4 Operational Definitions

For the purposes of this study, the following operational definitions were used:

Colonization: Isolation of the same species of bacteria from two or more consecutive cultures from the same body site without clinical signs of infection. ie. organisms are present and/or replicating in the tissues of the host and can be identified by culture in the laboratory.

Infection: Presence and replication of organisms in the tissues of the host which is usually accompanied by a raised white cell count, fever, and/or inflammation.

ICU Diagnosis: Diagnosis based on signs, symptoms and laboratory findings on admission to the ICU.

Resistance: Resistant bacteria are those which are able to survive the effects of antimicrobial action and even multiply in the host. This occurs when penicillin-binding proteins cease to bind β-lactams or are protected by permeability barriers or β-lactamases.

Underlying Disease: Deviation from the normal function of any organ or system of the body that is manifested by a characteristic set of signs and symptoms and whose etiology, pathology, and prognosis may or may not be known.

Immunosuppression: Diminution of the immune response by the administration of immunosuppressive drugs, by irradiation, by malnutrition, and by some disease processes (eg. cancer).

Multiple Organ Systems: Involvement of two or more organ systems either in underlying disease or on admission to the ICU.

Relatedness: Relatedness refers to the goal of strain typing, which is to provide laboratory evidence that strains are both epidemiologically related (ie. derived from a common source) as well as genetically related (ie. indistinguishable from each other by a molecular typing method such as PFGE).

5.5 Data Collection Methods

Several risk factors for colonization and/or infection with resistant strains of bacteria, such as age, underlying illness, duration of hospital stay, prior antibiotics etc., have previously been identified (Holmberg, Solomon, and Blake, 1987; Poole, Griswold, and Muakkassa, 1993). The purpose of collecting clinical data was to include parameters which may indicate dysfunctional organ systems. Using a structured data capture form, data were collected by means of chart reviews. The following data were collected on all patients admitted to this study: age, sex, admission and ICU diagnosis, length of hospitalization, presence of underlying disease, (eg. diabetes), clinical history, presence of intravascular lines, catheters (eg. Foley), tubes such as endotracheal, nasotracheal, nasogastric or chest tubes, use of invasive procedures(eg. tracheostomy) for patient management, surgeries prior to ICU admission and during ICU hospitalization, antimicrobial history and treatment, clinical infections, indices of renal, hepatic and endocrine dysfunction, biochemical and hematological indices, and clinically significant information such as temperature, heart rate, respiratory rate and blood pressure.

5.6 Bacteriological Methods

5.6.1 Specimen Collection

A rectal swab was taken from each patient admitted to this study, immersed in Carey and Blair transport medium, and transported to the laboratory ideally within twelve hours of admission to the ICU. Due to the priorities of patient care, up to 72 hours were allowed for rectal swab collection. On ICU day 10 (or on the day of discharge from the ICU), a second rectal swab was collected and

subjected to the same procedure as described below.

5.6.2 Bacterial Strains

On reception in the laboratory, the specimen was immersed in 0.5 ml of trypticase soy broth (Oxoid, Canada Inc.), vigorously vortexed to disperse the fecal material, and then 10 μl aliquots were inoculated onto five MacConkey agar (Oxoid, Canada Inc.) plates. Two plates contained 2 μg/ml and 16 μg/ml of cefotaxime respectively, and two plates contained 2 μg/ml and 16 μg/ml of gentamicin respectively. A fifth MacConkey agar plate served as a control. The MacConkey agar plates containing the drugs were prepared as follows: Stock solutions of cefotaxime and gentamicin were prepared at concentrations of 1.28 mg/ml. The true concentration of each drug was determined by dividing the desired stock concentration by the potency of the antibiotic.

For example:	Stock concentration desired	= 1.28 mg/ml
	Potency of cefotaxime	= 0.9518 mg/mg
	Therefore 1 mg powder	= 0.9518 mg drug
	Thus 1.28 mg powder	= 1.28 X 0.9518
		= 1.218 mg/ml drug

This is the true concentration of the stock cefotaxime. To obtain a concentration of 2 μg/ml in 250 ml of agar the formula V1 C1 = V2 C2 was used where:

V1 = volume required
V2 = concentration of known solution

C1 = final volume required
C2 = final concentration required

Therefore: V1 X 1218 = 250 X 2
V1 = 0.41 ml

This is the amount of antibiotic solution to be added to 250 ml of agar. This same procedure was also followed for gentamicin.

After inoculation all plates were incubated at 37°C aerobically overnight. Antibiotics (cefotaxime and gentamicin) were added to the agar to prevent overgrowth of normal bowel flora. The number of resistant organisms growing on the plates containing antibiotics were scored (range 1 - 4) in relation to the four quadrants of each plate. From the primary plates representative colonies were streaked out on blood agar in order to obtain pure cultures. Thereafter, isolates were identified using the API 20E bacterial identification system (API Analytab Products, Plainview, NY) according to the manufacturer's instructions. Purified colonies of each resistant organism were frozen at -70°C in brain heart infusion broth (Oxoid, Canada Inc.) containing 20% glycerol for subsequent MIC determinations and molecular typing.

5.6.3 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration is the lowest concentration of an antimicrobial agent needed at the site of infection to inhibit bacterial growth of the infecting organism. It is usually expressed in μg/ml. The following antimicrobial agents were used in MIC determinations:

Penicillins:	ampicillin (Bristol, Myers, Squibb)
	pipericillin (Lederle)
Cephalosporins:	cephalothin (Lilly)
	cefoxitin (Merck)
	cefotaxime (Hoechst Roussel)
	ceftazidime (Lilly)
Aminoglycosides:	gentamicin (Schering)
	tobramycin (Lilly)
	amikacin (Sigma)

Antimicrobial reference powders were obtained directly from the drug manufacturers. The amount of antibiotic powder weighed out was determined by the following formula:

$$\text{Weight (mg)} = \frac{\text{Volume (ml) X Concentration } (\mu g/ml)}{\text{Assay potency}}$$

Antibiotic stock solutions were prepared at concentrations of at least ten times the highest concentration to be tested. The range of dilutions that are normally tested are from 0.25 μg/ml up to 512 μg/ml. This would require making a stock solution of 10240 μg/ml of the antibiotic to be tested. In this study the range was from 0.25 μg/ml to 256 μg/ml. The stock solution of each antibiotic was thus determined to be 5120 μg/ml.

Example calculation: (ampicillin)

$$\text{Potency (on label)} = 911\ \mu g/ml$$
$$= 0.911\ mg/mg$$

	Final volume required	=	10 ml
	Concentration	=	5120 μg/ml
		=	5.120 mg/ml
		=	0.00512 g/ml
Therefore:	Weight of antibiotic	=	$\frac{\text{Volume X Concentration}}{\text{Assay potency}}$
		=	$\frac{10 \text{ X } 0.00512}{0.911}$
		=	0.0562g

This procedure was followed for all antibiotics tested. Powders were weighed directly into 10ml volumetric flasks. All antibiotics were dissolved in sterile distilled water except ampicillin and the cephalosporins, where phosphate buffer, pH 8.0, 0.1M and pH 6.0, 0.1M were used respectively. These represented the stock solutions containing 5120 μg/ml of the antibiotic. A total of twelve 30 ml sterile universal bottles were used for preparing dilutions. The first bottle contained 10 ml of the stock antibiotic solution (5120 μg/ml) whilst the remaining eleven bottles contained 10 ml of sterile distilled water each. The stock antibiotic (10ml) was added aseptically to the first bottle, mixed well, and then 10ml transferred to the next bottle. This procedure was followed until the last bottle where 10ml was discarded after mixing. This process is called "double diluting". The eleven universal bottles thus contained dilutions of the antibiotic to be tested ranging from 2560 μg/ml down to 2.5 μg/ml. After addition of 90ml of Mueller-Hinton agar the final dilutions were 256 μg/ml through 0.25 μg/ml.

Agar Dilution Procedure

The agar dilution method for determining antibiotic susceptibility is a well-established technique and is regarded as the "gold standard". The antimicrobial agent is incorporated into the agar medium with each plate containing a different concentration of the agent. The best medium for routine susceptibility testing is considered to be Mueller-Hinton agar (Oxoid, Canada Inc.) This medium was prepared according to the manufacturer's instructions. Appropriate dilutions of the antimicrobial solution (10 ml) were then added to 90 ml of molten Mueller-Hinton agar that was allowed to equilibrate in a waterbath to 50°C. The agar and antimicrobial solution were mixed thoroughly, poured into a sterile petri dish to a depth of 4 mm and allowed to solidify at room temperature. After solidification plates were stored at 4°C for future use. Drug free plates were prepared from the base medium and used as growth controls (two for each drug tested).

Three to five well-isolated colonies of the same morphological type were selected from an agar plate culture of the organism to be tested. The top of each colony was touched with a sterile wire loop and inoculated into a tube containing 5 ml Brain Heart Infusion broth (Oxoid, Canada Inc.). The broth culture was incubated in a shaking incubator (Labline orbital shakers, Illinois, USA) for two hours at 37°C. The turbidity of the broth culture was then adjusted with sterile Mueller-Hinton broth (Oxoid, Canada Inc.) to obtain a turbidity optically comparable to that of a 0.5 McFarland standard. A photometric device (Vitek Instruments) was used for this procedure. Cultures adjusted this way contained approximately 1 X 10^8 colony-forming units per ml (CFUs/ml). The desired inoculum is 1 X 10^7 CFUs/ml so the 0.5 McFarland adjusted cultures were diluted 1:10 with sterile Mueller-Hinton broth (ie. 100 μl of suspension + 900 μl

Mueller-Hinton broth). The inoculum replicator (Ram head inoculator, Oxoid, Canada, Inc.) deposits approximately 1 μl onto the agar surface giving a final inoculum on the agar of approximately 1 X 10^4 CFUs/ml in a spot of about 5 mm in diameter. An aliquot of each well-mixed suspension was placed in the corresponding well in the replicator inoculum block. Each agar plate was marked for orientation of the inoculum spots. A 1 μl aliquot of the inoculum was then applied to the agar surface by use of the inoculum-replicating device. A growth control plate containing no antibiotics was first inoculated, followed by the plates containing the different antibiotic concentrations (starting with the lowest concentration). A second growth control plate was inoculated last to ensure that there was no contamination or significant antibiotic carryover during the inoculation procedure. All inoculated plates were allowed to stand at room temperature to allow for complete absorption of the inoculum spots into the agar, after which they were inverted and incubated at 37°C for about 20 hours. MICs were recorded as the lowest concentration of antibiotic agent completely inhibiting growth.

5.7 Molecular Epidemiological Methods

The molecular technique used in this study was pulsed field gel electrophoresis (PFGE) which is a variation of agarose gel electrophoresis. PFGE permits the analysis of bacterial DNA fragments over an order of magnitude larger than that of conventional restriction endonuclease analysis. Chromosomal DNA is digested with restriction enzymes that have few restriction sites yielding 5 - 20 fragments ranging from $\sim$ 10 kb to 800 kb in length. Enzymes used in PFGE recognize specific 8-base sequences or selected 6-base sequences depending on the mole percent guanine and cytosine (G+C) content of the species under investigation.

One of the most common approaches is contour clamped homogenous electric field (CHEF) which was used in this study.

Resistant gram-negative bacteria that were stored at -70°C were grown overnight in tubes containing 5 ml brain heart infusion broth (Oxoid, Canada Inc.) at 37°C in a shaking incubator (Labline orbital shaker, Illinois, USA). Cells were pelleted by centrifugation for 20 minutes at 4000 rpm and then resuspended in 2 ml of SE buffer (10 mM Tris-HCl, pH 7.6; 1M NaCl). At this point a plug mold was assembled and chilled at 4°C for one hour prior to use. A 700 μl aliquot of PIV cell suspension was then mixed with 700 μl of molten agarose (1.6% low-melt agarose, Imbed LM agarose, FMC Bioproducts, USA), the mixture dispensed into the plug mold wells (two wells per culture suspension), and then allowed to solidify on a level surface in the refrigerator for 15 minutes. The agarose blocks ("gel plugs") were then transferred into 10 ml of EC lysis solution (6 mM Tris-HCl, 1 M NaCl, 100mM EDTA, 0.5% Brij-58, 0.2% Sodium deoxycholate, 0.5% Sodium lauryl sarcosine, 10 mg lysozyme, 20 μg/ml RNAse) and incubated overnight at 37°C in a shaking incubator. Following this the EC lysis solution was poured off carefully and the tube refilled with 10 ml ESP solution (0.5 M EDTA, pH 9 - 9.5, 1% sodium lauryl sarcosine, 50 μg/ml proteinase K) and incubated overnight at 50°C with gentle shaking. The ESP solution was then gently decanted, each tube filled with about 15 ml of TE buffer, and placed in a rotating rack at 37°C. The buffer was changed every 30 minutes until at least two liters had been used. The gel plugs were finally equilibrated with 10 ml TE buffer and kept at 4°C overnight for restriction endonuclease digestion later. The following day, the TE buffer was gently decanted from the tube and the gel plug placed onto a sterile glass slide. A sterile coverslip was used to cut a 5 mm wedge gel slice which was then transferred to a microfuge tube. The gel slice was then

immersed in 100 μl of enzyme buffer for two hours at 4°C. Thereafter the buffer was carefully aspirated from the tube leaving the gel slice behind. The following constituents were then added to the gel slice in the microfuge tube:

Distilled water	200 μl
10X reaction buffer	20 μl
Restriction endonuclease	2 μl
(*Sma*I or *Xba*I, New England Biolabs Inc., Canada))	

The tube was mixed gently by "flicking" and kept at room temperature overnight for complete enzyme digestion (when *Xba*I was used tubes were kept at 37°C). The following day the reaction solution was carefully aspirated from the microfuge tube and the latter placed in a waterbath at 65°C for 20 minutes to melt the gel plugs. A gel casting tray was assembled using "Fastlane" agarose (1%)(FMC Bioproducts, USA) made up with 0.5X TBE buffer. Each DNA sample was then loaded into the wells and sealed with molten 1% agarose. Electrophoresis was performed in a CHEF-DR II apparatus with a hexagonal electrode array (Bio-Rad, USA). PFGE was carried out at 9°C for 27 hours at 200 volts. After PFGE was completed, the agarose gel was placed in ethidium bromide solution (25 mg/ml) for 15 minutes for DNA staining, and then destained with several changes of distilled water for about 90 minutes. Thereafter the agarose gel was placed on a transilluminator and results recorded using a polaroid gel documentation system (Fisher Scientific, Canada).

5.8 Statistical Methods and Calculations

The prevalence and incidence rates of gram-negative resistance in the ICU

population were calculated as follows: The prevalence rate on admission was defined as the number of existing cases of a disease divided by the total population at a point in time. In this study the numerator was all patients with some antimicrobial resistance on admission to the ICU. Resistance in this context was defined as those gram-negative bacteria with MICs of ≥ 64 μg/ml for cefotaxime and ≥ 8 μg/ml for gentamicin. The denominator was the total number of patients admitted to the ICU.

$$\text{Prevalence} = \frac{\text{Number of existing cases of resistance on admission}}{\text{Total number of cases admitted to study (measured at a point in time)}}$$

The incidence rate was defined as the number of new cases of a disease divided by the population at risk measured over a period of time. However, the time during which the outcome (development of resistance) was observed was not uniform for each patient, thus the incidence density was the appropriate measure of incidence in this study. The numerator was therefore all patients who developed resistance by day ten of their ICU stay (or on discharge if this was before day ten) less those patients with resistance on admission(ie. new cases). The denominator on the other hand was the total person-days of observation.

$$\text{Incidence Density} = \frac{\text{Number of new cases of antimicrobial resistance}}{\text{Total person-days of observation}}$$

In this prospective cohort study, the association between potential risk factors and the development of antibiotic resistance was evaluated by determination of the relative risk (RR). Relative risk compares the probability of an outcome among

individuals who have a specific characteristic or who have been exposed to a given risk factor, to the probability of that outcome among individuals who lack the characteristic or who have not been exposed. Thus relative risk is the ratio of the incidence of the outcome among exposed individuals to the incidence among non-exposed individuals. The analysis of differences between those patients who developed antimicrobial resistance and those who did not was performed using the student's *t*-test. The chi-square test was used to determine a statistically significant association between two variables. For small frequency values, Fisher's exact test was used.

CHAPTER SIX

RESULTS AND DISCUSSION

6.1 Summary Data

6.1.1 Demographics

A total of 89 patients admitted to the ICU of the Calgary General Hospital were enrolled in this study which was conducted over a seven-month period from December 1, 1994 to June 30, 1995. There were 38 (42.7%) females and 51 (57.3%) males. The mean age for males was 47.2 years and 57.9 years for females ($p = 0.02$). The frequency distribution by age category is shown in figure 6.1 which indicates that the greater number of patients occurred in the age group 66 years and older (36.0%), followed by those in the 19 to 35 year old age group (23.6%). The youngest patient was 16 years old and the oldest 86 years old. The majority of patients were Caucasian (83.1%). Approximately half of the patients (47/89=52.8%) were admitted to the ICU from the Emergency Room, whilst 42 (47.2%) were admitted from nursing units in the hospital.

Clinical diagnoses were classified on the basis of ten separate organ systems: respiratory, cardiovascular, gastrointestinal, musculoskeletal, neuropsychiatric, metabolic, hematological, renal, hepatic, and immunological (Carrico, Meakins, Marshall et al., 1986; Marshall, Christou, and Meakins, 1993). Table 6.1 shows the number of patients with each type of clinical diagnoses. The three leading diagnoses were respiratory illness (38/89=42.7%), musculoskeletal related conditions (24/89=27.0%), and neuropsychiatric diagnoses (10/89=11.2%).

Figure 6.1 Frequency distribution by age category

of 89 patients admitted to the ICU

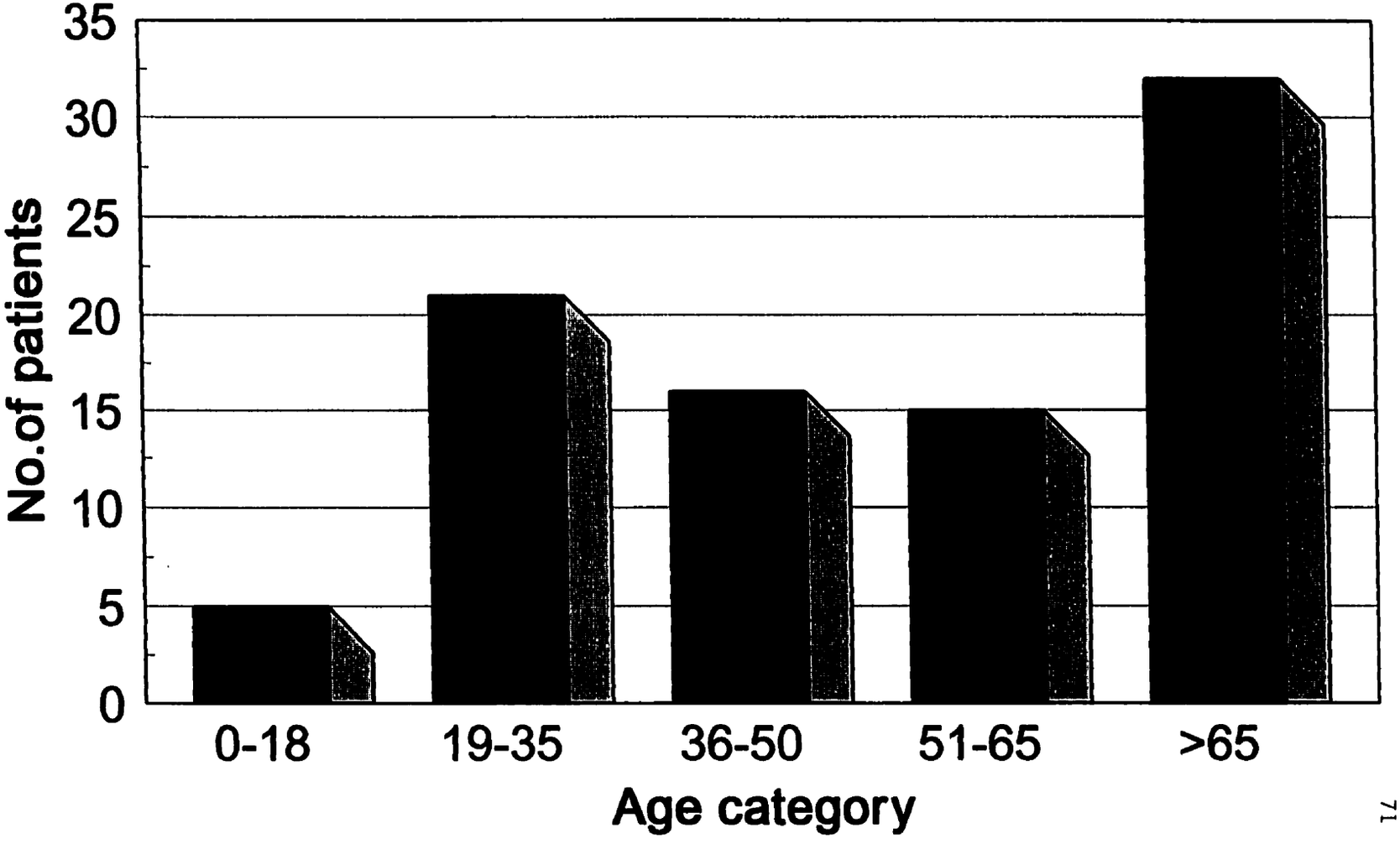


Table 6.1 Classification of ICU Clinical Diagnoses by Organ System

Organ System	Number of patients	%
Respiratory	38	43
Musculoskeletal	24	27
Neuropsychiatric	10	11
Gastrointestinal	8	9
Cardiovascular	7	8
Renal	1	1
Hepatic	1	1
Metabolic	0	0
Hematological	0	0
Immunological	0	0
Total =	89	100%

With respect to length of stay, 30 of 89 (33.7%) patients were in the ICU for up to six days, 18 of 89 (20.2%) for up to 9 days, and 41 of 89 (46.1%) patients were in the ICU for at least 10 days or longer. The mean length of stay was 7.9 days (minimum stay 4 days, maximum stay ≥10 days). The mortality rate in this study was 23.6% (21 of 89 patients).

6.1.2 Clinical History

Underlying diseases were also classified by ten separate organ systems. Nearly two thirds of the patients (55/89=61.8%) had multiple organ system involvement on admission (Table 6.2). The most common underlying illnesses were smoking (32.6%), alcohol abuse (24.7%), and hypertension (22.5%) (Table 6.3).

6.1.3 Invasive Devices

Invasive devices were considered to be intravascular catheters such as arterial, triple lumen, and Swan/Cordis lines, Foley catheters, and tubes such as endotracheal, nasotracheal, nasogastric, and chest tubes. Of 89 patients, 82 (92.1%) had one or more arterial lines during their ICU stay, 44 (49.4%) had triple lumens, and 50 (56.2%) had Swan/Cordis lines. All 89 patients had a Foley catheter in place whilst 86 (96.6%) were intubated. Eighty-five (96%) patients had nasogastric tubes and 29 (33%) had chest tubes (Table 6.4).

6.1.4 Invasive Procedures

Invasive procedures were regarded as the following: tracheostomy, bronchoscopy, thoracentesis, paracentesis, spinal tap, bone marrow, and dialysis. Additional

Table 6.2 Organ System Involvement of 89 Admitted Patients

Number of organ systems	Number of patients	%
0	18	20
1	16	18
2	14	16
3	20	22
4	16	18
5	4	5
6	1	1

Table 6.3 Underlying Diseases of 89 Patients Admitted to the ICU

Organ System	Underlying illness	Number of patients	%
1.Respiratory	Smoking	29	32.6
	COPD[1]	15	16.9
	Emphysema	3	3.4
	Asthma	3	3.4
	Previous TB[2]	2	2.2
2. Musculoskeletal	Osteoarthritis	7	7.9
	Osteoporosis	2	2.2
3. Neuropsychiatric	Depression	6	6.7
	CVA[3]	3	3.4
	Schizophrenia	2	2.2
4. Gastrointestinal	Cholelithiasis	2	2.2
5. Cardiovascular	Hypertension	20	22.5
	CHF[4]	11	12.4
	CAD[5]	7	7.9
6. Renal	Nephrolithiasis	4	4.5
7. Hepatic	Alcohol abuse	22	24.7
	Drug abuse	3	3.4
	Liver cirrhosis	3	3.4
8. Metabolic	Diabetes	20	22.5
	Obesity	7	7.9
	Hypothyroidism	2	2.2
9. Hematological	Anemia	6	6.7
10. Immunological	Immunosuppression	4	4.5
	HIV[6]	1	1.1

[1] COPD=chronic obstructive pulmonary disease, [2] TB=tuberculosis,

[3] CVA=cerebrovascular accident, [4] CHF=congestive heart failure,

[5] CAD=coronary artery disease, [6] HIV=human immunodeficiency virus

Table 6.4 Invasive Devices such as IV Catheters, Foley Catheters, and Tubes

Invasive Device	No. of patients			Mean no.of days±SD
	≤ 4 days	5-9 days	≥ 10 days	
1. IV Catheters				
1st arterial	22	56	4	6.01±2.23
2nd arterial	19	7	1	4.11±2.17
3rd arterial	1	0	0	
1st triple lumen	18	25	1	5.18±2.11
2nd triple lumen	5	3	1	4.44±2.70
3rd triple lumen	1	0	0	
1st swan/cordis	18	31	1	5.70±1.99
2nd swan/cordis	3	4	1	5.25±3.24
3rd swan/cordis	0	0	0	
2. Foley Catheters	12	40	37	7.57±2.41
3. Tubes				
Endotracheal	24	35	27	6.79±2.76
Nasogastric	18	37	30	7.02±2.62
Chest (1)	14	12	3	5.24±2.50
Chest (2)	1	5	1	6.86±2.04
Chest (3)	1	5	1	6.86±2.04

procedures such as angiograms, pyelograms, cystoscopy, endoscopy, enterostomy, esophagoscopy, plasmapheresis, peritoneal lavage, and sigmoidoscopy were also performed (Table 6.5). Several patients underwent more than one invasive procedure: bronchoscopy (5), dialysis (2), thoracentesis (1), and biopsy (1).

6.1.5 Surgeries

Concerning surgeries, 26 of 89 (29.2%) patients had surgery prior to ICU admission, whilst 23 (25.8%) had surgery during their stay in the ICU.

6.1.6 Bacterial Isolates

Numerous bacteria were isolated from various body sites of 89 patients (other than from the rectum on admission and discharge). There were 66 gram-positive isolates, with *Staphylococcus aureus* (19/66=28.8%) being the most common isolate, followed by *Streptococcus pneumoniae* (17/66=25.8%), and *Enterococcus* (13/66=19.7%). There were 59 gram-negative isolates with non-typeable *Haemophilus influenzae* (17/59=28.8%) being the most frequent isolate, followed by *Escherichia coli* (15/59=25.4%), and other members of the Enterobacteriaceae. There were 35 isolates of *Candida albicans.* The distribution of the various isolates and their sites of isolation are shown in table 6.6.

6.1.7 Antimicrobials

Prior to hospitalization (up to four weeks), only 6 (6.7%) patients received antimicrobial therapy. Four received β-lactam antibiotics, one tetracycline and one could not recall the specific antibiotic taken. Prior to ICU admission, 29

Table 6.5 Invasive Procedures Performed on 89 ICU Patients

Procedure	Number of patients	%
Bronchoscopy	16	18
Tracheostomy	13	14
Spinal Tap	7	8
Thoracentesis	5	6
Dialysis	5	6
Biopsy	5	6
Bone marrow	1	1
Other procedures[1]	16	18
No procedures	21	23
Total =	89	100

[1] Angiograms, pyelograms, cystoscopy, endoscopy, enterostomy, esophagoscopy, plasmapheresis, peritoneal lavage, sigmoidoscopy

Table 6.6 Distribution of Species of Bacteria (n) Isolated from 89 ICU Patients

Isolate	Site of isolation					
	Respiratory	Blood	GU[1]	Body fluids	Wounds	Other[2]
1.Aerobic GNR[3]						
E.cloacae	3	0	0	1	0	0
E.coli	6	1	4	0	3	1
P.aeruginosa	2	0	1	1	0	0
K.oxytoca	0	0	0	0	0	1
S.maltophilia	1	0	0	0	0	0
A.lwoffii	0	1	0	0	0	0
A.baumannii	1	1	0	0	2	0
S.marcescens	3	0	0	0	0	0
P.mirabilis	0	0	0	0	1	0
P.penneri	1	0	0	0	0	0
K.pneumoniae	2	0	2	0	0	0
E.aerogenes	1	0	0	0	0	0
H.influenzae	15	2	0	0	0	0
2.Aerobic GPC[4]						
S.aureus	17	0	1	0	0	0
S.pneumoniae	11	3	0	3	0	0
Other Streps	6	1	0	1	0	0
Enterococcus	0	0	5	2	4	2
Staph.species	0	3	1	1	1	1
3.Yeasts						
C.albicans	24	0	4	6	0	1

[1]GU= genitourinary, [2] Other= catheter tips, tissue, biopsy specimens, [3] GNR= gram-negative rods, [4]GPC = Gram-positive cocci

Table 6.7 Antimicrobials Administered to Patients on the Floor (pre-ICU)

Antibiotic Class	No.of patients	Dose (mg[1] or g[2])	Dose interval (hours)	Route	Mean doses per patient
1. PENICILLINS					
Ampicillin	5	1-2g	6	IV[3]	5.2
Pipericillin	2	3-4g	4-6	IV	9.0
Pip/Tazo	2	3.375-4.5g	6-8	IV	8.0
2. CEPHALOSPORINS					
Cefoxitin	2	1-1.5g	8	IV	2.0
Cefazolin	14	1-2g	8	IV/PO[4]	4.5
Cefuroxime	9	750mg-1.5g	8	IV	2.8
Cefotaxime	2	1g	12	IV	1.5
Ceftriaxone	2	1-2g	12-24	IV	2.5
3. AMINOGLYCOSIDES					
Gentamicin	5	300-500mg	24	IV	1.6
Tobramycin	2	350-400mg	24	IV	1.5
Amikacin	1	500mg	12	IV	7.0
4. MACROLIDES					
Erythromycin	8	500mg-1g	6	IV/PO	3.4
5. FLUOROQUINOLONES					
Ciprofloxacin	1	500mg	12	IV	10.0
6. TETRACYCLINES					
Doxycycline	1	100mg	8	PO	12.0
7. LINCOMYCINS					
Clindamycin	2	600mg	8	IV	1.1
8. IMIDAZOLES					
Metronidazole	12	500mg	8	IV/PO	5.1
Ketoconazole	1	200mg	12	PO	8.0
9. TMP/SMX					
Septra	2	800mg	12	PO	5.0

[1] mg=milligrams, [2] g=grams, [3] IV=intravenous, [4] PO=per os

Table 6.8 Antimicrobials Administered to Patients in the ICU

Antibiotic class	No.of patients	Dose (mg[1] or g[2])	Dose interval (hours)	Route	Mean doses per patient
1. PENICILLINS					
Ampicillin	18	1-2g	4-8	IV[3]	13.1
Pipericillin	7	2-5g	4-12	IV	17.6
Penicillin G	4	2-5mU	4-6	IV	26.5
Cloxacillin	4	500mg-2g	4-6	IV	13.5
Ticarcillin	1	3.1g	4	IV	32.0
Amoxycillin	1	500mg	stat	PO[4]	1.0
Pip/Tazobactam	7	3.375-4.5g	6-8	IV	12.9
2. CEPHALOSPORINS					
Cefazolin	32	1-2g	6-12	IV	13.1
Cefuroxime	25	750mg-1.5g	8-12	IV	9.9
Cefotaxime	17	1-2g	8-24	IV	11.7
Ceftriaxone	7	1-2g	12-24	IV	4.0
Cefpirome	4	2g	12	IV	9.8
Ceftazidime	1	2g	24	IV	2.0
3. AMINOGLYCOSIDES					
Gentamicin	32	80-700mg	12-24	IV	4.0
Tobramycin	3	250-300mg	24	IV	2.0
Amikacin	1	500mg	12	IV	20.0
4. MACROLIDES					
Erythromycin	10	500mg-1g	6-8	IV	21.5
5. FLUOROQUINOLONES					
Ciprofloxacin	18	250-750mg	12-24	IV/PO/NG[5]	5.7
6. TETRACYCLINES					
Doxycycline	3	100mg	12	IV	9.2

Table 6.8 (continued)

Antibiotic class	No.of patients	Dose (mg or g)	Dose interval (hours)	Route	Mean doses per patient
7. LINCOMYCINS					
Clindamycin	6	500-600mg	8	IV	9.2
8. IMIDAZOLES					
Metronidazole	55	500mg	8-12	IV/PO/NG	12.9
Fluconazole	4	200-400mg	24	IV/PO	2.8
Ketoconazole	1	200mg	12	PO	4.0
9. TMP/SMX					
Septra	5	450mg	6-12	IV/PO/NG	11.8
10. OTHER					
Chloramphenicol	1	1g	6	IV	9.0
Vancomycin	2	500mg	6-24	IV	3.5
Imipenem	3	500mg	6-12	IV	19.3
Rifampin	5	600mg	12-24	PO/NG	10.0
Tazocin	1	3.375g	8	IV	15.0
Amphotericin B	2	45-55mg	24	IV	4.0
Acyclovir	2	300-400mg	6-12	IV/NG	3.0

[1] mg = milligrams

[2] g = grams

[3] IV = intravenous

[4] PO = per os

[5] NG = nasogastric

(32.6%) patients received antimicrobials on the floor whilst 84 (94.4%) received antimicrobials in the ICU (Tables 6.7 and 6.8).

6.1.8 Laboratory Parameters

6.1.8.1 Liver Function Tests

With respect to protein and albumin tests, no patient ever had a value above normal during their entire ICU stay. Of 89 patients, 23 (25.8%) had albumin and protein tests performed on admission and 17 (73.9%) had protein values below 60 g/l (normal range 60-84 g/l) whilst 19 (82.6%)had albumin values below 35 g/l (normal range 35-50 g/l). Concerning liver enzyme tests, 20 of 89 (22.5%) patients had an alkaline phosphatase (ALP) test performed on admission, and 23 (25.8%) an alanine transaminase (ALT) and an aspartate transaminase (AST). For ALP, 7 of 20 (35.0%) patients had values above 125 U/L (normal range 35-125 U/L); for ALT, 12 of 23 (52.2%) patients had values above 50 U/L (normal range 5-50 U/L)and for AST, 16 of 23 (69.6%) patients had values above 50 U/L (normal range 5-50 U/L). Total bilirubin values were elevated in 12 of 21 (57.1%) patients on admission (normal range 2-22 μmol/L).

6.1.8.2 Renal Function Tests

With respect to blood urea, 86 of 89 (96.6%) patients had a test performed on admission, and 32 of 86 (37.2%) had elevated values (normal range 2.1-7.5 mmol/L). For creatinine, 28 of 86 (32.6%) patients had values above 130 μmol/L (normal range 60-130μmol/L).

6.1.8.3 Metabolic Tests

On admission 87 of 89 (97.8%) patients had glucose tests performed, and of 87, 80 (90.0%) had elevated levels (normal range 3.6-6.1 mmol/L).

6.1.8.4 Hematological Parameters

Hemoglobin tests were performed on 86 of 89 (96.6%) patients on admission and 55 of 86 (64.0%) had values below the lower limit of normal (normal range 120-160g/L). Of 89 patients, 85 (95.5%) had white cell counts performed on admission and 60 of 85 (70.6%) had raised values (normal range 4-10 X 10^9/L). range 50.3, mean 15.4 X 10^9/L). The white cell differential test was performed on 72 of 89 (80.9%) patients on admission and 54 of 72 (75.0%) had a left shift of the neutrophils.

6.1.8.5 Coagulation Parameters

Of 89 patients, 72 (80.9%) had an INR and PTT on admission and all had INR values within normal limits (normal range <2.0) whilst 23 of 72 (31.9%)had an elevated partial thromboplastin time (PTT)(normal range 24-36 seconds). Concerning platelet levels, 84 of 89 (94.4%) patients had a platelet test performed on admission and 26 of 84 (31.0%) had values below normal (normal range 150-400 X 10^{12}/L). Fibrinogen levels were determined in only 11 of 89 (12.4%) patients and 2 of 11 (18.2%) had raised values (normal range 2.05-4.74g/L).

6.1.8.6 Respiratory Parameters

Arterial blood gases were obtained from 87 of 89 (97.8%) patients admitted to the ICU and 42 of 87 (48.3%)had pH values below the lower limit of normal (normal range 7.35-7.45). The pO_2 levels of 34 of 87 (39.1%) patients were lower than normal (normal range 75-90mm Hg), and the pCO_2 levels of 38 of 87 (43.7%) patients were decreased (normal range 35-45).

6.1.8.7 Vital Functions

Of 89 patients, 87 (97.8%) had their temperature recorded on admission, and 13 of 87 (14.9%)were febrile (temperature ≥38.5°C). The mean overall temperature was 37.8°C (range 5.2, minimum 35.5, maximum 40.7°C). The heart rate was elevated in 77 of 87 (88.5%) patients on admission (normal range 70-80 bpm), and 79 of 87 (90.8%) patients had an increased respiration rate (normal range 10-14 ipm). The blood pressure of only 4 of 89 (4.5%) patients was elevated (> 160/110) on admission, whereas 57 of 89 (64.0%) had a low pressure (< 100/50). Normal pressure (120/80) was recorded in 28 of 89 (31.5%) patients (minimum 110, maximum 140).

6.2 Characterization of Resistant Organisms

6.2.1 Rectal Swabs

A total of 178 rectal swabs were collected from patients (89 on admission and 89 on discharge or after ten days of admission) and processed in the laboratory as described in the methods section (see page 59). It was desirable that a rectal swab be collected within twelve hours of admission of an enrolled patient but this was not always possible primarily due to the priority of giving life support to

critically ill patients. Of 89 patients, 37(42%) had rectal swabs collected within twelve hours of admission, 27 (30%) within 12 to 24 hours, 8 (9%) within 24 to 36 hours, 2 (2%) within 36 to 48 hours, 10 (11%) within 48 to 60 hours, and 5 (6%) within 60 to 72 hours. Rectal swabs were collected by available nursing staff.

6.2.2 Phenotypic Typing

From the primary inoculation plates (2μg/ml and 16μg/ml of cefotaxime and gentamicin respectively in MacConkey agar), representative colonies were streaked out onto blood agar for purity. Biotyping of these isolates was performed. A total of 102 different gram-negative isolates were obtained from the rectal swabs of 58 of 89 (65.2%)(Table 6.9).

6.2.3 Minimum Inhibitory Concentration (Agar Dilution Technique)

Purified colonies of gram-negative isolates recovered from the primary isolation plates which were inoculated into brain heart infusion broth with 20% glycerol and frozen at -70°C were tested against the following antimicrobials to determine minimum inhibitory concentrations: ampicillin, pipericillin, cephalothin, cefoxitin, cefotaxime, ceftazidime, gentamicin, tobramycin, and amikacin. A total of 102 organisms were tested, 78 isolated from the 2 μg/ml plates and 24 from the 16 μg/ml plates.

The MIC_{50} and MIC_{90} (ie. the MICs at which 50% and 90% of isolates are inhibited) and the breakpoints recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1995) are shown in tables 6.10 and 6.11.

Table 6.9 Gram-Negative Bacteria Recovered from the Primary Isolation Media

Isolate	Number of Isolates							
	2 μg/ml CTX[1]		2 μg/ml GEN[2]		16 μg/ml CTX		16 μg/ml GEN	
	A[3]	D[4]	A	D	A	D	A	D
P.aeruginosa	8	1	4	4	2	1	0	0
E.coli	0	1	22	13	0	0	1	0
E.cloacae	1	3	0	0	2	9	0	1
A.baumannii	0	5	0	3	0	0	0	4
S.maltophilia	0	2	0	0	0	0	0	0
P.mirabilis	0	0	1	0	0	0	1	0
P.vulgaris	0	0	0	1	0	0	0	0
C.freundii	2	1	1	0	1	1	0	0
C.amalonaticus	0	0	0	1	0	0	0	0
K.pneumoniae	1	0	1	1	0	0	0	0
K.oxytoca	0	1	0	0	0	0	1	0
Total =	12	14	29	23	5	11	3	5
Grand Total = 102								

[1]CTX = Cefotaxime

[2]GEN = Gentamicin

[3]A = Admission

[4]D = Discharge

Table 6.10 **In Vitro Susceptibilities of 78 Gram-Negative Isolates (recovered from 2 μg/ml plates) to βeta-Lactam and Aminoglycoside Agents**

Antimicrobial	*E.coli* (n=36)						*P.aeruginosa* (n=17)					
	Range	MIC_{50}[1]	MIC_{90}[2]	% S[3]	% I	% R	Range	MIC_{50}	MIC_{90}	% S	% I	% R
Ampicillin	≤0.25-≥256	4	128	78	0	22	0.5-≥256	≥256	≥256	1	0	94
Pipericillin	≤0.25-16	2	4	100	0	0	0.5-16	4	4	100	0	0
Cephalothin	0.5-16	4	8	89	11	0	16-≥256	≥256	≥256	0	6	94
Cefoxitin	0.5-16	4	8	97	3	0	16-≥256	≥256	≥256	0	6	94
Cefotaxime	≤0.25-0.5	≤0.25	≤0.25	100	0	0	1-32	8	32	53	47	0
Ceftazidime	≤0.25-1	≤0.25	0.5	100	0	0	0.5-4	1	2	100	0	0
Gentamicin	≤0.25-16	1	2	97	0	3	0.5-64	1	2	94	0	6
Tobramycin	≤0.25-4	1	4	100	0	0	≤0.25-128	0.5	0.5	94	0	6
Amikacin	0.5-16	2	16	100	0	0	1-128	2	4	94	0	6

[1] 50%, MIC at which 50% of isolates are inhibited

[2] 90%, MIC at which 90% of isolates are inhibited

[3] Breakpoints are those of the National Committee for Clinical Laboratory Standards (1995)

S, susceptible; I, intermediate, R, resistant

Table 6.10 (continued)

Antimicrobial	*A.baumannii* (n=8)						*E.cloacae* (n=4)					
	Range	MIC_{50}[1]	MIC_{90}[2]	% S[3]	% I	% R	Range	MIC_{50}	MIC_{90}	% S	% I	% R
Ampicillin	4-≥256	16	32	12.5	62.5	25	≥256	≥256	≥256	0	0	100
Pipericillin	1-16	8	16	100	0	0	8-64	8	64	75	25	0
Cephalothin	2-≥256	≥256	≥256	12.5	0	87.5	≥256	≥256	≥256	0	0	100
Cefoxitin	2-128	128	128	12.5	0	87.5	32-≥256	64	≥256	0	0	100
Cefotaxime	0.5-8	8	8	100	0	0	2-8	4	8	100	0	0
Ceftazidime	≤0.25-8	8	8	100	0	0	8-64	32	64	25	0	75
Gentamicin	0.5-32	32	32	25	0	75	≤0.25-0.5	≤0.25	0.5	100	0	0
Tobramycin	0.5-8	4	8	37.5	50	12.5	≤0.5	0.5	0.5	100	0	0
Amikacin	0.5-16	16	16	100	0	0	1-2	1	2	100	0	0

[1] 50%, MIC at which 50% of isolates are inhibited

[2] 90%, MIC at which 90% of isolates are inhibited

[3] Breakpoints are those of the National Committee of Clinical Laboratory Standards (1995)

S, susceptible; I, intermediate; R, resistant

Table 6.10 (continued)

Antimicrobial	*C.freundii* (n=4)						Other GNB[4] (n=9)					
	Range	MIC_{50}[1]	MIC_{90}[2]	% S[3]	% I	% R	Range	MIC_{50}	MIC_{90}	% S	% I	% R
Ampicillin	≥256	≥256	≥256	25	0	75	4-≥256	64	≥256	11	11	78
Pipericillin	2-≥256	8	≥256	50	0	50	0.5-≥256	16	64	67	22	11
Cephalothin	8-≥256	≥256	≥256	25	0	75	2-≥256	16	≥256	33	22	45
Cefoxitin	2-≥256	≥256	≥256	25	0	75	2-256	4	256	67	0	33
Cefotaxime	≤0.25-64	2	64	75	0	25	≤0.25-64	≤0.25	64	78	0	22
Ceftazidime	≤0.25-≥256	64	≥256	25	0	75	≤0.25-16	≤0.25	4	89	11	0
Gentamicin	≤0.25-4	≤0.25	4	100	0	0	≤0.25-256	0.5	128	67	11	22
Tobramycin	≤0.25-4	0.5	4	100	0	0	0.5-256	2	128	78	0	22
Amikacin	1-16	1	16	100	0	0	1-256	2	256	78	0	22

[1] 50%, MIC at which 50% of isolates are inhibited

[2] 90%, MIC at which 90% of isolates are inhibited

[3] Breakpoints are those of the National Committee for Clinical Laboratory Standards (1995)

[4] GNB, gram-negative bacilli; *K.pneumoniae* (3), *S.maltophilia* (2); *K.oxytoca* (1)
C.amalonaticus (1); *P.mirabilis* (1); *P.vulgaris* (1)

Table 6.11 In Vitro Susceptibilities of 24 Gram-Negative Isolates (recovered from 16 μg/ml plates) to βeta-Lactam and Aminoglycoside Agents

Antimicrobial	*E.cloacae* (n=12)						*A.baumannii* (n=4)					
	Range	MIC_{50}[1]	MIC_{90}[2]	% S[3]	% I	% R	Range	MIC_{50}	MIC_{90}	% S	% I	% R
Ampicillin	≥256	≥256	≥256	0	0	100	1-32	1	32	50	25	25
Pipericillin	4-≥256	64	128	42	42	16	≤0.25-16	≤0.25	16	100	0	0
Cephalothin	≥256	≥256	≥256	0	0	100	≤0.25-≥256	2	≥256	50	0	50
Cefoxitin	64-≥256	≥256	≥256	0	0	100	1-128	1	128	50	0	50
Cefotaxime	1-4	4	4	100	0	0	≤0.25-16	≤0.25	16	75	25	0
Ceftazidime	1-128	64	128	8	0	92	≤0.25-8	≤0.25	8	100	0	0
Gentamicin	≤0.25-0.5	≤0.25	0.5	100	0	0	≤0.25-32	≤0.25	32	50	0	50
Tobramycin	≤0.25-0.5	0.5	0.5	100	0	0	≤0.25-8	≤0.25	8	100	0	0
Amikacin	0.5-4	1	4	100	0	0	≤0.25-16	≤0.25	16	50	50	0

[1] 50%, MIC at which 50% of isolates are inhibited

[2] 90%, MIC at which 90% of isolates are inhibited

[3] Breakpoints are those of the National Committee for Clinical Laboratory Standards (1995)

S, susceptible; I, intermediate; R, resistant.

Table 6.11 (continued)

Antimicrobial	*P.aeruginosa* (n=3)						Other GNB[4] (n=5)					
	Range	MIC_{50}[1]	MIC_{90}[2]	% S[3]	% I	% R	Range	MIC_{50}	MIC_{90}	% S	% I	% R
Ampicillin	≥256	≥256	≥256	0	0	100	≥256	≥256	≥256	0	0	100
Pipericillin	4-128	16	128	67	0	33	8-≥256	16	64	60	0	40*
Cephalothin	≥256	≥256	≥256	0	0	100	4-≥256	32	≥256	20	20	60*
Cefoxitin	≥256	≥256	≥256	0	0	100	2-≥256	8	≥256	60	0	40*
Cefotaxime	16-≥256	128	≥256	0	33	67	≤0.25-64	≤0.25	64	80	0	20*
Ceftazidime	2-32	8	32	67	0	33	≤0.25-64	≤0.25	64	60	0	40*
Gentamicin	≤0.25-1	1	1	100	0	0	≤0.25-16	8	16	60	20	20**
Tobramycin	≤0.25-0.5	0.5	0.5	100	0	0	0.5-4	0.5	4	100	0	0
Amikacin	≤0.25-4	4	4	100	0	0	1-4	4	4	100	0	0

[1] 50%, MIC at which 50% of isolates are inhibited

[2] 90%, MIC at which 90% of isolates are inhibited

[3] Breakpoints are those of the National Committee for Clinical Laboratory Standards (1995)

S, susceptible; I, intermediate; R, resistant

[4] GNB, gram-negative bacilli; *C.freundii* (2); *K.oxytoca* (1); *E.coli* (1); *P.mirabilis* (1)

**C.freundii*; ***E.coli*

Wherever two identical organisms of the same biotype were recovered from the same concentration of either cefotaxime or gentamicin, then only one of these was tested for minimum inhibitory concentration.

Table 6.10 shows the in vitro susceptibilities of 78 gram-negative isolates (recovered from the 2 μg/ml screen plates) to β-lactam and aminoglycoside agents. Susceptibility percentages for all agents tested against *E.coli* were 89% or higher, except ampicillin which was 78%. The most active agents against *P.aeruginosa* were pipericillin, ceftazidime, gentamicin, tobramycin, and amikacin (susceptibility 94% or higher). Isolates tested against ampicillin, cephalothin, and cefoxitin were 94% resistant. *A.baumannii* was fully susceptible to pipericillin, cefotaxime, ceftazidime and amikacin but poorly susceptible to ampicillin, cephalothin, cefoxitin, gentamicin, and tobramycin. Cefotaxime, gentamicin, tobramycin, and amikacin (all 100%) were the most active agents against *E.cloacae*, whereas ampicillin, cephalothin, and cefoxitin had no activity. *C.freundii* isolates were fully susceptible to gentamicin, tobramycin, and amikacin, whereas resistance to the other agents ranged from 25 to 75%. For other gram-negative isolates, susceptibilities ranged from 11 to 89% whereas resistance ranged from 11 to 78%.

Table 6.11 shows the in vitro susceptibilities of gram-negative isolates (recovered from the 16 μg/ml screen plates) to β-lactam and aminoglycoside agents. All *E.cloacae* isolates were fully susceptible to cefotaxime, gentamicin, tobramycin, and amikacin, and fully resistant to ampicillin, cephalothin, and cefoxitin. Resistance percentages for pipericillin and ceftazidime were 16 and 92% respectively. Pipericillin, ceftazidime, and tobramycin (100%) were the most effective agents against *A.baumannii,* whereas resistance to the other agents ranged from 25 to

50%. *P.aeruginosa* isolates were fully susceptible to aminoglycoside agents and fully resistant to ampicillin, cephalothin, and cefoxitin. Pipericillin and ceftazidime (67%) showed the least activity. For other gram-negative isolates, susceptibilities ranged from 20 to 100%.

For organisms recovered from the 2 μg/ml screen plates, it is clear that ampicillin, cephalothin, and cefoxitin had very little activity against them (MICs ≥256), whereas pipericillin, cefotaxime, ceftazidime gentamicin, tobramycin and amikacin were the most active agents (MICs ≤0.25-2). For organisms recovered from the 16 μg/ml screen plates, ampicillin, cephalothin, and cefoxitin once again were the least active agents, whilst pipericillin, cefotaxime, and ceftazidime were less active, and gentamicin, tobramycin, and amikacin were the most active.

The acceptable quality control ranges recommended by the NCCLS for reference strains that were used in the determination of MICs in this study are shown in table 6.12.

For isolates recovered from the 2 μg/ml screen plates, MICs should have been ≥2. For the gentamicin plates however, 14 isolates had MICs of ≤0.25, 4 had MICs of 0.5, and 18 had MICs of 1. For the cefotaxime plates, only 2 isolates had MICs of ≤0.25, 1 had an MIC of 0.5 and 2 had MICs of 1. Clearly the majority of isolates that had MICs of less than the potency of the antimicrobial in the MacConkey screen plates were recovered from plates containing gentamicin. There are three possible reasons for this phenomenon:

i) The potency of the gentamicin used in the screen plates may have been less than that stipulated by the manufacturer possibly due to aging.

Table 6.12 Quality Control Ranges for MICs (μg/ml)for ATCC[1] Reference Strains Compared with RGNB[2] Study Results

Reference Strain	Antimicrobial								
	AMP	PIP	CEN	CFX	CTX	CAZ	GEN	TOB	AMK
P.aeruginosa (ATCC 27853)	-	1-4	-	-	4-16	1-4	0.5-2	0.25-1	1-4
RGNB study	-	2	-	-	8	1	1	0.5	2
E.coli (ATCC 25972)	2-8	1-4	4-16	1-4	0.06-0.25	0.06-0.5	0.25-1	0.25-1	0.5-4
RGNB study	4	2	8	8*	≤0.25	≤0.25	≤0.25	0.5	2

[1] ATCC, American Type Culture Collection

[2] RGNB, Resistant Gram-Negative Bacilli

* Cefoxitin was the only antimicrobial that gave an MIC outside the range recommended by the NCCLS, and by only one dilution. This was probably due to a dilution factor.

("antibiotic effect"). The MICs were performed using a different gentamicin batch and may account for this discrepancy.

ii) The effect and potency of gentamicin in MacConkey agar is not known and it is possible that one of the ingredients in the agar could have affected it ("media effect")

iii) Most of the isolates were within one or two dilutions of 2 μg/ml which may occur in the doubling dilution technique especially when concentrations are low ("dilution effect").

For isolates recovered from the 16 μg/ml screen plates MICs should have been $\geq$16. For cefotaxime, 12 of 24 isolates tested had MICs of $\leq$16 and for gentamicin 5 of 24 had MICs of $\leq$16. In the section describing the mode of action of β-lactamases (see page 44), it was stated that certain gram-negative bacilli (eg. *P.aeruginosa, E.cloacae*, etc) have inducible expression of β-lactamase such that the amount synthesized relates to the amount of anti-microbial present. Induction is the transient switching on of β-lactamase synthesis in response to the addition of an inducer such as a β-lactam drug. Of the 22 patients from whom 24 isolates were recovered, 20 were on β-lactam drugs prior to ICU admission, and during ICU stay. At the time the admission rectal swab was collected, high levels of β-lactamase were being produced by some gram-negative gut flora which resulted in their growth on the 16 μg/ml screen plates. These isolates were subcultured on to blood agar plates for purification and then kept at -70°C for several months before MIC testing was performed. When the isolates were subcultured again on to blood agar just prior to MIC testing, this was in the absence of any inducer. No β-lactamase was therefore produced and this would explain the low MICs recorded. In order to verify that this is indeed what happened, an induction assay using cefotaxime was performed.

Briefly, 3 *P.aeruginosa*, 11 *E.cloacae*, and 2 *C.freundii* isolates from the first MIC assay that had shown low MICs were selected for the induction assay. Two sets of MICs were performed firstly by growing the isolates at 37°C for 18 hours in BHI broth containing 0.5 μg/ml of cefotaxime (sub-inhibitory concentration), and secondly by simultaneously growing the same isolates at 37°C for 18 hours in BHI broth without cefotaxime. A small aliquot from each of the latter broths was then transferred to a fresh BHI broth containing 0.5 μg/ml of cefotaxime and incubated at 37°C for 4 hours only. MIC assays were then performed on both sets of isolates. The results are shown in table 6.13.

These results clearly show that in the majority of gram-negative isolates, there was a transient "switching on" of β-lactamase in the presence of an inducer (even in the presence of a weak inducer such as cefotaxime). This is especially true of *E.cloacae* as all the isolates tested show remarkable increases in the MIC values of the 2nd and 3rd assays when compared with the 1st assay.

6.2.4 Genotypic Typing

6.2.4.1 Pulsed-Field Gel Electrophoresis

Purified colonies of gram-negative isolates were typed by pulsed-field gel electrophoresis according to the method described on page 65. Isolates that were obtained from other body sites as part of routine submission of specimens to the microbiology laboratory were included in the analysis. These isolates were cultured from specimens obtained from bronchoalveolar lavage, intra-abdominal fluid, sputum, blood, wounds, and central lines. Results are shown in figures 6.2 to 6.7

Table 6.13 MICs After an Induction Assay Using Cefotaxime as an Inducer of β-Lactamase

Patient No.	Isolate	MIC 1st assay[1] (μg/ml)	MIC 2nd assay[2] (μg/ml)	MIC 3rd assay[3] (μg/ml)
007	*P.aeruginosa*	16	128	256
008	*E.cloacae*	4	256	256
017	*E.cloacae*	2	256	256
018	*E.cloacae*	2	64	16
025	*C.freundii*	2	32	32
030	*C.freundii*	64	16	32
031	*E.cloacae*	4	≥256	≥256
039	*E.cloacae*	4	64	128
041	*P.aeruginosa*	128	128	128
052	*E.cloacae*	2	64	128
053	*E.cloacae*	1	32	16
067	*P.aeruginosa*	≥256	≥256	≥256
067	*E.cloacae*	2	256	128
077	*E.cloacae*	4	64	32
078	*E.cloacae*	4	256	256
083	*E.cloacae*	4	64	64

[1] MIC 1st assay, initial assay performed on all isolates

[2] MIC 2nd assay, assay performed on isolates grown overnight in BHI broth containing 0.5 μg/ml cefotaxime

[3] MIC 3rd assay, assay performed on isolates grown overnight in BHI broth without cefotaxime, then for 4 hours in broth with cefotaxime.

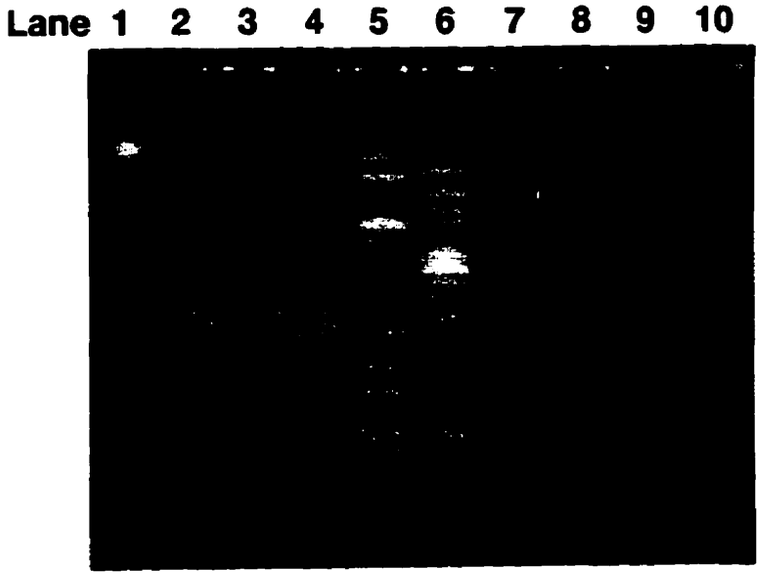


Figure 6.2 PFGE patterns of chromosomal DNA restriction fragments for *Enterobacter cloacae* digested with *Xba*I. Lane 1, MW marker; lane 2 and 3, rectal isolate and BAL isolate respectively (both the same patient); lane 4 , rectal isolate (different patient); lane 6, *E.coli* control, lanes 5 (rectal isolate) and 7 to 10 (IAF isolates) all from same patient. MW=molecular weight, BAL=bronchoalveolar lavage, IAF=intra-abdominal fluid.

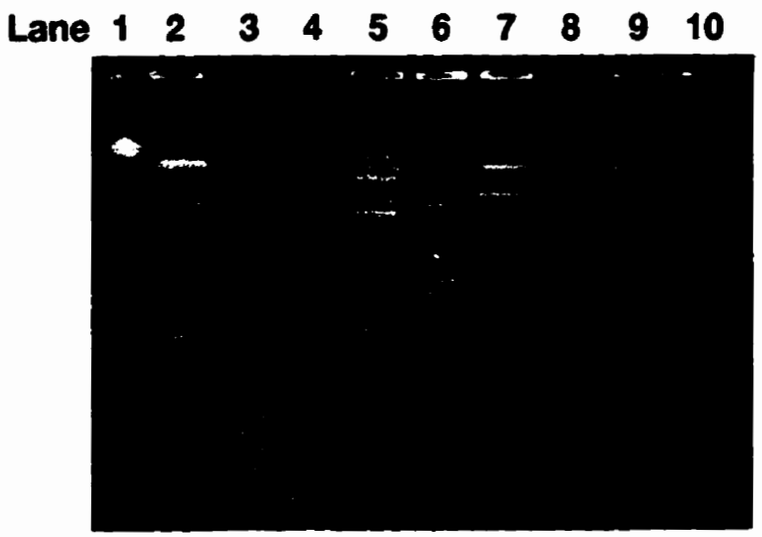


Figure 6.3 PFGE patterns of chromosomal DNA restriction fragments for *Enterobacter cloacae* digested with *Xba*I. Lane 1, MW marker; lanes 2 to 10, rectal isolates (all different patients).

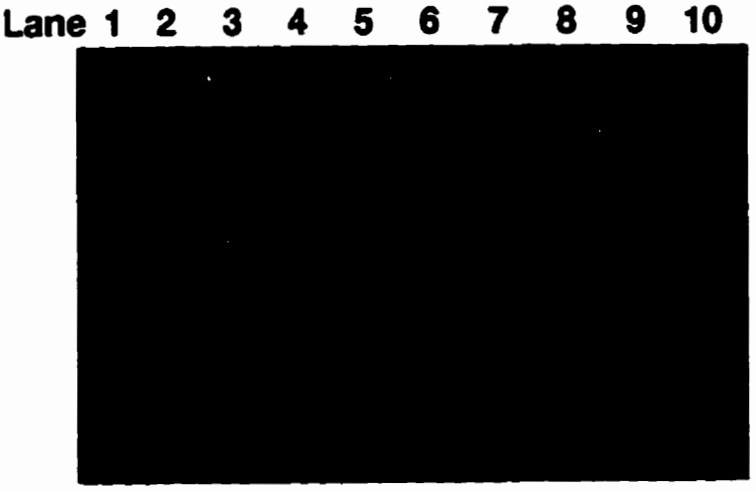


Figure 6.4 PFGE patterns of chromosomal DNA restriction fragments for *Pseudomonas aeruginosa* digested with *Spe*I. Lane 1, MW marker; lane 2, rectal isolate; lane 3, penis (wound); lane 4, sputum; lane 5, blood; lane 6, central line (lanes 2-6 represent isolates from one patient); lanes 7 and 8, rectal isolates from one patient; lanes 9 and 10, rectal isolate and sputum respectively, from the same patient.

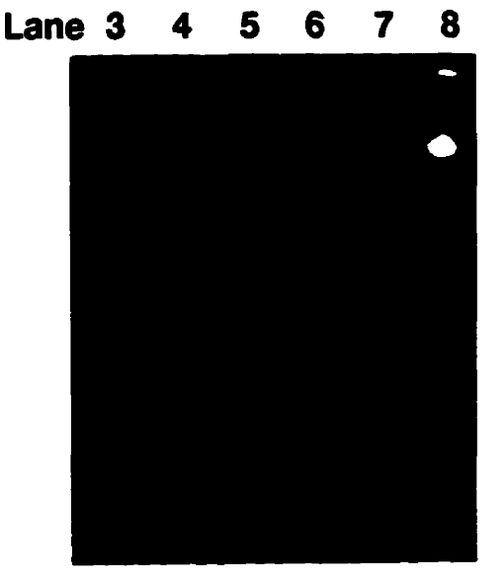


Figure 6.5 PFGE patterns of chromosomal DNA restriction fragments for *Acinetobacter baumannii* digested with *Spe*I. Lanes 1 and 2, empty (not loaded); lane 3, rectal isolate from one patient, lanes 4 and 5, rectal and wound isolate respectively, from the same patient; lanes 6 and 7, rectal isolates from different patients; lane 8, MW marker; lanes 9 and 10, empty (not loaded).

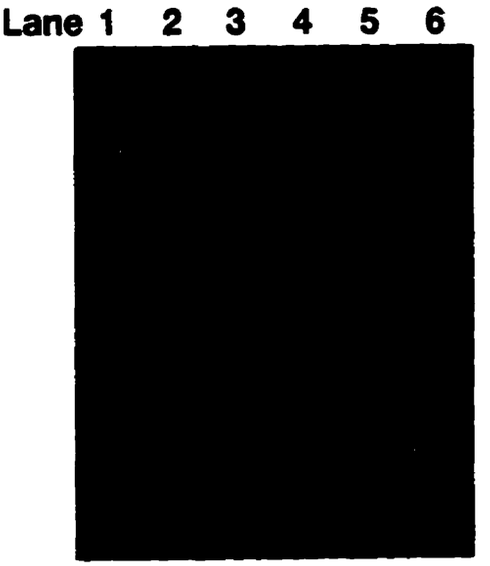


Figure 6.6 PFGE patterns of chromosomal DNA restriction fragments for *Acinetobacter baumannii* digested with *Sma*I. Lane 1, MW marker; lane 2, rectal isolate from one patient; lanes 3 and 4, rectal and wound isolates respectively, from the same patient; lanes 5 and 6, rectal isolates from different patients; lanes 7 to 10, empty (not loaded).

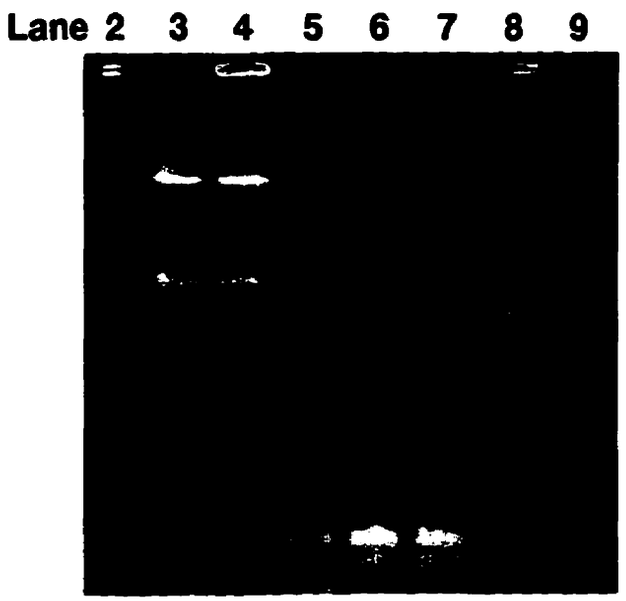


Figure 6.7 PFGE patterns of chromosomal DNA restriction fragments for *Escherichia coli, Proteus mirabilis,* and *Citrobacter freundii* digested with *Xba*I. Lane 1, empty (not loaded); lane 2, MW marker; lane 3 and 4, *E.coli* rectal isolates from the same patient; lanes 5 and 6, *Proteus mirabilis* rectal isolates from the same patient; lane 7, *Proteus mirabilis* wound isolate from the same patient as lanes 5-6; lanes 8 and 9, *Citrobacter freundii* rectal isolates from different patients; lane 10, empty (not loaded).

6.2.4.2 Interpretation of PFGE Patterns

Figure 6.2 shows the PFGE patterns of chromosomal DNA fragments for *Enterobacter cloacae* digested with *Xba*I. Lane 2 shows a rectal isolate and lane 3 a bronchoalveolar lavage isolate from the same patient. They are genetically indistinguishable as their restriction patterns have the same number of bands and the corresponding bands are the same size. The two isolates are thus considered to represent the same strain (Tenover, Arbeit, Goering et al., 1995). Lane 4 shows a rectal isolate from a different patient with at least seven band differences from the isolates in lanes two and three. If lane 4 had shown an identical banding pattern to lanes two and three then patient to patient or some other form of horizontal transmission may have occurred. Lane 5 (rectal isolate) and lanes 7 to 10 (intra-abdominal fluid) represent one patient, but whilst the intra-abdominal isolates are genetically indistinguishable, they differ somewhat from the rectal isolate. This indicates that the patient was infected/colonized with two genetically distinct strains of *Enterobacter cloacae*. The intra-abdominal isolates were collected from the patient on different successive days.

Figure 6.3 shows a continuation of the PFGE patterns of chromosomal DNA fragments for *Enterobacter cloacae* digested with *Xba*I. Lanes 2 to 10 (rectal isolates) show PFGE patterns that are unrelated. Lanes three and four do not show any bands at all, with a lot of DNA smeared in low molecular weight areas. This could have been due to extensive shearing of DNA prior to cutting, or star activity which is cleavage by a restriction enzyme at a nucleic acid sequence different from its recognition sequence as a result of altered incubation conditions such as excess NaCl present in the reaction.

Figure 6.4 shows the PFGE patterns of chromosomal DNA fragments for *Pseudomonas aeruginosa* digested with *Spe*I. Lanes 2 to 6 represent one patient with lane 2 being a rectal isolate, lane 3 a wound, lane 4 sputum, lane 5 blood, and lane 6 an isolate from a central line. All of these isolates show essentially the same banding patterns with one exception: lanes 3 to 6 appear to have lost 2 bands when one compares them with lane 2. Such a difference may be explained by possible plasmid DNA, a point mutation, or insertion or deletion of DNA (Tenover, Arbeit, Goering et al., 1995). Lanes 7 and 8 (rectal isolates) represent the same patient with lane 7 showing only very faint bands possibly due to insufficient DNA. Lanes 9 and 10 represent one patient, with lane 10 showing no evidence of any DNA fragments. The isolates in lanes 7, 8, and 9 are genetically different from the isolates in lanes 2 to 6.

Figures 6.5 and 6.6 show the PFGE patterns of chromosomal DNA fragments for *Acinetobacter baumannii* digested first with *Spe*I and then with *Sma*I. These isolates came from different patients and are genetically indistinguishable from each other. The patients represented by lanes 4 to 8 were all admitted to the ICU within 3 weeks of each other. The reason for digestion with a second restriction endonuclease (*Sma*I) was to confirm that these isolates were indeed indistinguishable. If digestion with the second enzyme had shown different banding patterns amongst the isolates then they would have been classified as unrelated. Their relatedness, however, makes this a strong case for exogenous acquisition or horizontal transmission either from patient to patient or from care giver to patient, or from the environment to the patient (Hartstein, Rashad, Liebler et al., 1988). It is well known that *Acinetobacter* species have the propensity to colonize normal skin more frequently than any other gram-negative organisms. The carriage rate is much higher in hospitalized patients

and colonization plays an important role in subsequent contamination of the hands of hospital staff during patient contact, thereby contributing to the spread of the organism (Dijkshoorn, van Dalen, van Ooyen et al., 1993; Buxton, Anderson, Werdegar et al., 1978; Bergogne-Berezin and Towner, 1996).

In an outbreak of *Acinetobacter baumannii* that occurred in the ICU after the present study was completed, it was found that the isolates from the outbreak were genetically identical to the isolates from the study. Subsequent investigation found that these organisms were being transmitted to patients via a suction container holder that was not being properly cleaned and disinfected. In this present study this latter mechanism was the most likely mode of spread from patient to patient. The goal of molecular typing is to provide evidence that epidemiologically related isolates are also genetically related and thus represent the same strain (Tenover, Arbeit, Goering et al., 1995). This would appear to be the case with the *Acinetobacter* isolates in this study. In saying this however, it should be noted that by chance alone some epidemiologically unrelated isolates may have similar or indistinguishable genotypes, particularly if there is limited genetic diversity within a species (Tenover, Arbeit, Goering et al., 1995). This could have been the case with these isolates and it is therefore not possible to state with absolute certainty whether they were acquired exogenously or endogenously.

Figure 6.7 shows the PFGE patterns of chromosomal DNA fragments for *Escherichia coli, Proteus mirabilis,* and *Citrobacter freundii* digested with *Xba*I. Lanes 3 and 4 are rectal isolates of *E.coli* from the same patient, whilst lanes 5 and 6 (rectal isolates) and lane 7 (wound) represent *P.mirabilis* from the same patient. Lanes 8 and 9 are rectal isolates of *C.freundii* from different patients. The

banding patterns for the *E.coli* and *P.mirabilis* isolates are identical which indicate endogenous acquisition by these patients.

6.3 Potential Risk Factors for Gram-Negative Colonization/Resistance

6.3.1 Patients Colonized on Admission

Of 89 patients admitted to this study, 3 (3.4%) were colonized with resistant gram-negative bacilli on admission to the ICU. These isolates were *C.freundii, Koxytoca*, and *E.cloacae* respectively. Some characteristics of these patients are shown in table 6.14. It is not possible to establish with any degree of certainty the reasons why these patients were colonized with resistant gram-negative bacilli on admission to the ICU. What may be said however, is that there are some plausible reasons why this occurred bearing in mind that plausibility is neither necessary nor sufficient evidence to establish a cause-and-effect relationship.

It is well known that patients who are elderly, have underlying disease conditions, who are immunocompromised and who have been exposed to antimicrobials are at risk for colonization by multi-resistant organisms as well as other serious nosocomial infections (Holmberg, Solomon, and Blake, 1987). All three patients were elderly and immunocompromised and two of the three received antimicrobials prior to ICU admission. It is plausible therefore that these factors played a role in the colonization by resistant gram-negative bacilli of these patients.

Table 6.14 Characteristics of Patients Colonized with Gram-Negative Bacilli on Admission to the ICU

Variable	Patient 1 (*C.freundii*)	Patient 2 (*K.oxytoca*)	Patient 3 (*E.cloacae*)
Age	86	74	64
Underlying illness	previous TB[1] COPD[2] smoker	diabetic hypertension	diabetic COPD smoker
Preadmission antibiotics	none	unknown	cefuroxime
Pre-ICU antibiotics	cefotaxime metronidazole erythromycin	none	none

[1] TB =tuberculosis

[2] COPD =chronic obstructive pulmonary disease

6.3.2 Patients Colonized on Admission and Discharge

There were 5 (5.6%) patients that were colonized with resistant gram-negative bacilli on admission as well as discharge from the ICU. These isolates were *E.coli, E.cloacae, P.aeruginosa,* and *P.mirabilis* respectively (see table 6.15). The same organism was recovered from four patients on admission and discharge,whereas one patient had a different organism on admission and a different organism on discharge *(P.aeruginosa* and *E.cloacae*). As with the patients who were colonized on admission only, those colonized on admission as well as discharge were all elderly, and had serious underlying illnesses which may have played a role in the acquisition of resistant organisms.

6.3.3 Patients Colonized on Discharge

Fifteen (16.9%) patients were colonized on discharge, and 66 (74.1%) were not colonized. The ICU population thus fell into two distinct groups: those who were colonized with resistant gram-negative bacilli and those who were not colonized. To compare colonized and non-colonized patient characteristics and potential risk factors for colonization/resistance, relative risks and student's *t*-tests with probability values for categorical and continuous variables were calculated. Patients who were colonized on admission only and those colonized on admission and discharge were not included in this analysis.

The potential risk factors for gram-negative colonization/resistance for categorical variables are shown in table 6.16. Patients colonized with gram-negative bacilli were more likely than non-colonized patients to have had surgery prior to ICU admission (p=0.06). No other significant statistical differences

Table 6.15 **Characteristics of Patients Colonized with Gram-Negative Bacilli on Admission to, and Discharge from the ICU**

Variable	Patient 1 *E.coli*	Patient 2 *E.cloacae*	Patient 3 *P.aeruginosa*	Patient 4 *P.aeruginosa* *E.cloacae*	Patient 5 *P.mirabilis*
Age	76	59	72	65	55
Underlying illness	anemia jaundice pneumonia	COPD[1] smoker asthma	diabetic CA[2] lung melanoma	diabetic hypertensio n smoker	diabetic TB[3] hypertension
Preadmission antibiotics	unknown	none	none	none	none
Pre-ICU antibiotics	cefuroxime TMP/SMX[4]	none	none	TMP/SMX	ampicillin metronidazole gentamicin

[1]COPD=chronic obstructive pulmonary disease

[2]CA=carcinoma

[3]TB=Tuberculosis

[4]TMP/SMX=trimethoprim/sulphamethoxazole

Table 6.16 Potential Risk Factors for Gram-Negative Colonization/Resistance

Categorical Variables:

Risk factor	Colonized patients (n=15)	Non-colonized patients (n=66)	RR	CI 95%	p
Sex:male	11 (73%)	36 (55%)	2	0.69-5.72	0.30
Route of admission	8 (53%) floor	28 (42%) floor	1.4	0.57-3.57	0.63
Diabetic	2 (13%)	18 (27%)	0.5	0.12-1.90	0.34*
Smoker	6 (40%)	19 (29%)	1.5	0.60-3.74	0.54*
Alcohol abuse	5 (33%)	17 (26%)	1.3	0.52-3.49	0.54*
Hypertension	3 (20%)	16 (24%)	0.8	0.26-2.50	1.0*
Smoking related illness	3 (20%)	11 (17%)	1.2	0.39-3.69	0.72*
Tracheostomy	4 (27%)	10 (15%)	1.7	0.65-4.68	0.28*
Bronchoscopy	4 (27%)	13 (20%)	1.4	0.50-3.77	0.51*
Invasive procedures	8 (53%)	30 (45%)	1	0.42-2.60	0.84
Pre-ICU surgery	8 (53%)	18 (27%)	2.4	0.98-5.95	0.06*
In-ICU surgery	7 (47%)	17 (26%)	2.1	0.85-5.09	0.12*

* Fishers Exact Test

existed between colonized and non-colonized patients with respect to gender, route of admission, whether diabetic, a smoker, an alcohol abuser, hypertensive, smoking related illnesses, tracheostomy, bronchoscopy or any other invasive procedure. This is perhaps not a surprising finding given the fact that the sample size in this study was not large and the number of colonized patients was small. The effect of the sample size is seen in the width of the confidence intervals. If the study sample had been large this would have been reflected in narrower confidence intervals. However the wide intervals suggest that the data are compatible with a true increased risk for colonization but the sample size was simply not large enough to have had statistical power to exclude chance as a likely explanation for the findings (Hennekens & Buring, 1987).

The potential risk factors for gram-negative colonization/resistance for continuous variables are shown in table 6.17. Patients colonized with gram-negative bacilli were more likely than non-colonized patients to have had a chest tube in place during their ICU stay ($p=0.008$). No other significant statistical differences existed between colonized and non-colonized patients with respect to age, length of stay and other invasive devices such as IV and Foley catheters, and endotracheal and nasogastric tubes. Here again the same reason cited for categorical variables applies.

The potential risk factors for gram-negative colonization/resistance by antimicrobial use are shown in table 6.18. Antimicrobial agents were administered to all patients in this study and the particular use of β-lactam drugs was significantly associated with gram-negative colonization. Patients colonized with gram-negative bacilli were more likely than non-colonized patients to have received a higher mean number of antibiotic doses ($p=0.02$), to

Table 6.17 **Potential Risk Factors for Gram-Negative Colonization/Resistance**

Continuous Variables

Risk factor	Colonized patients(n=15)	Non-colonized patients(n=66)	RR	CI 95%	p
Age (mean years)	51.7	49.7	-	-	0.74
Length of stay (mean days)	8.3	7.7	-	-	0.36
Chest tube (mean days)	3.7	1.5	-	-	0.008
IV catheter (mean days)	14.3	13.6	-	-	0.76
Foley catheter (mean days)	7.9	7.4	-	-	0.47
Endotracheal tube (mean days)	6.4	6.7	-	-	0.70
Nasogastric tube (mean days)	7.6	6.4	-	-	0.17

Table 6.18 **Potential Risk Factors for Gram-Negative Colonization/resistance**

Receipt of Antimicrobials

Risk Factor	Colonized patients (n=15)	Non-colonized patients (n=66)	RR	CI 95%	p
Abx[1] prior to admission	7 (47%)	20 (30%)	1.8	0.71-4.32	0.36
Mean abx per patient	3.8	3.4	-	-	0.49
Mean doses per patient	47.3	30.5	-	-	0.02
Receipt of metro.[2]	14 (93%).	35 (53%)	9.1	1.26-66.17	0.01
Receipt of metro. as empiric Rx[3]	12 (80%)	24 (36%)	5	1.53-16.38	0.01
Receipt of cephs.[4]	11 (73%)	44 (67%)	1.3	0.46-3.70	0.76
Receipt of cephs. as empiric Rx	8 (53%)	23 (35%)	1.8	0.74-4.58	0.30
Receipt of cefazolin and metronidazole	8 (53%)	14 (21%)	3.1	1.26-7.45	0.02*
Receipt of 1st or 2nd gen.[5]cephs. (mean doses)	11.9	6.1	-	-	0.02
Receipt of any ceph.+ metronidazole	12 (80%)	29 (44%)	3.9	1.19-12.8	0.02*

[1]abx=antibiotics, [2] metro=metronidazole, [3] Rx=therapy, [4]cephs=cephalosporins

[5] gen=generation, * Fishers Exact Test

have received metronidazole as empiric therapy as well as during their ICU stay (p=0.005 and 0.009 respectively), to have received a higher mean number of doses of 1st or 2nd generation cephalosporins (p=0.02), and to have received a combination of any cephalosporin and metronidazole (p=0.02). No statistical differences were noted between colonized and non-colonized patients with respect to antimicrobials received prior to admission, mean number of antimicrobials per patient, and receipt of cephalosporins in general.

6.4 Measures of Disease Frequency

6.4.1 Prevalence

The prevalence rate (ie. colonization with resistant organisms on admission to the ICU)was defined as the number of existing patients with gram-negative colonization/resistance divided by the total number of patients admitted measured at a point in time (see page 67). There were eight patients in this study who were colonized with gram-negative bacilli on admission to the ICU. Therefore the prevalence rate of gram-negative colonization/resistance in this study population was 8/89 = 9.0%. This is comparable to rates reported from Europe and the USA although the latter were multi-center studies with large sample sizes (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991).

6.4.2 Incidence

The incidence rate (ie. colonization with resistant organisms at least 3 days after admission) was defined as the number of new cases of colonization/resistance divided by the population at risk measured over a period of time. However, as

has been pointed out in section 5.8, the length of time in days during which the outcome (=colonization/resistance) was observed was not the same for all patients, and the appropriate measure of incidence is therefore the incidence density. The incidence density is the number of new cases of colonization/resistance less the population colonized on admission, divided by the total person-days of observation (ie. to calculate incidence density one would only utilize the new cases of colonization on discharge less those cases colonized on admission, and the denominator would be the total number of patient days for all 89 patients). New cases of colonization refer to cases not prevously colonized on admission but colonized on discharge.

Calculation of Incidence Density:

Number of patients colonized on admission only	=	3
Number of patients colonized on admission+discharge	=	5
Number of new cases of colonization/resistance	=	15
Number of patients not colonized	=	66
Population at risk (ie. all patients admitted to study)	=	89
Total person-days contributed by entire cohort	=	701

Incidence Density= $\frac{\text{No. of new cases of colonization/resistance - admission cases}}{\text{total person-time of observation}}$

$= \frac{16 - 7^{*}}{701\text{(days)}}$

= 13 cases/1000 person days of observation

* Of 5 patients colonized on admission and discharge, 4 had the same organism on discharge, one had a different organism on discharge, hence there were 16 patients in total who were new cases on discharge. The number of admission cases would then be 3+4=7.

The risk of acquiring a resistant gram-negative organism for patients not previously colonized is determined by the number of patients colonized on discharge (9), divided by the number of patients not colonized on discharge (80). Thus:

$$RR = \frac{9}{80} = 11.3\%$$

Similarly, the risk of acquiring a resistant gram-negative organism for those patients who were previously colonized is determined by the number of patients colonized on admission (1), divided by the number of patients colonized on discharge (8).
Thus:

$$RR = \frac{1}{8} = 12.5\%$$

The risk of acquiring a resistant gram-negative organism in the ICU is determined by the risk of acquisition for those previously colonized (12.5%) divided by the risk of acquisition for those not previously colonized (11.3%). Thus:

$$RR = \frac{12.5}{11.3}$$

$$= 1.11$$

The risk of acquiring a resistant gram-negative organism in the ICU is therefore 1.11. Because of small numbers and the fact that there is nothing with which to compare this figure, it is not known whether it has any significance or not.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction

Since the early 1940s when sulphonamides and penicillin first became available to treat infections, many different antibiotics have been discovered in nature or synthesized. There was a drastic decline in the number of infectious diseases largely because of antimicrobials but these same diseases are unfortunately reemerging mainly because the agents that cause them have developed resistance to antimicrobials (Gaynes, 1995). The development of resistance has become an acute problem facing hospitals worldwide because of the rapid emergence and dissemination of nosocomially acquired resistant organisms within the hospital environment.

This study was undertaken to determine the prevalence and incidence of resistant nosocomial gram-negative bacilli in an ICU population, to characterize these resistant organisms by phenotypic and genotypic typing methods, and to ascertain whether there was an association between the development of resistance and underlying disease, exposure to antimicrobials, invasive devices and invasive procedures.

7.2 Main Findings

7.2.1 Measures of Disease Frequency

In this study the prevalence rate of colonization with resistant gram-negative bacilli was nearly 10%. This is in keeping with findings reported from Europe and the USA (Snydman, 1991; Verbist, 1991; Shah, Asanger, and Kahan, 1991; Ismaeel, 1993) and although the sample size in this study was small, it nevertheless highlights the fact that antimicrobial resistance is a worldwide phenomenon and in the community extensive use of antimicrobial agents may have resulted in the selection of organisms which are resistant to many commonly used antimicrobials.

The incidence density was calculated to be 13 cases per 1000 person days of observation. As no other reports in the literature were found, there is nothing with which to compare this figure and it is therefore not known whether it is low or high.

7.2.2 Inducible Resistance

In this study population patients who were colonized with resistant gram-negative bacilli were more likely to have received cephalosporins as part of their antimicrobial therapy. This is especially true of first and second generation cephalosporins, but even cefotaxime (third generation weak inducer) was able to induce gram-negative isolates (mainly *P.aeruginosa* and *E.cloacae*) to synthesize excessive amounts of β-lactamases. One of the problems that arises from the administration of labile weak inducers (third generation cephalosporins) is that

stably-derepressed mutants may eventually constitute the entire bacterial population in an infected site. A further problem is posed by the accumulation of stably-derepressed organisms in the hospital microflora. The clinical use of labile weak inducers could lead to a rise in their frequency and therefore monotherapy would be inadvisable for serious infections caused by species where there is a strong chance of stably-derepressed selection (ie. those organisms where enzyme expression is normally inducible). Antimicrobial therapy should therefore be carefully monitored in order to rapidly detect the appearance of a derepressed population of organisms and the regimen changed appropriately (Livermore, 1987).

7.2.3 Acquisition of Resistant Organisms

Pulsed-field gel electrophoresis patterns of chromosomal DNA fragments of gram-negative isolates showed that patients enrolled in this study acquired resistant organisms from an endogenous source (with the exception of *Acinetobacter baumannii*). When organisms are endogenous to the patient, barrier infection control measures have been less successful in containing the problem (Olson, Weinstein, Nathan et al., 1984). In the case of *Acinetobacter baumannii* isolates, it seems probable that these organisms were acquired from an exogenous source, most likely a suction container holder that was not being properly cleaned and disinfected. From an infection control point of view, barrier isolation precautions have been fairly successful in reducing spread where hospitalized patients have acquired resistant organisms from an exogenous source (Tenover and McGowan, 1996).

7.2.4 Risk Factors for Colonization/Resistance

Concerning invasive procedures and devices, only chest tubes were a likely risk factor ($p=0.008$) for colonization with gram-negative bacilli and their use may provide a portal of entry for resistant nosocomial organisms. Surgery prior to ICU admission was nearly significant ($p=0.06$). It is well known that patients who are admitted to the ICU after surgery have their gastric pHs controlled to prevent upper GI bleeding and this practice has been associated with up to a fourfold greater risk of stomach colonization with gram-negative bacilli (Flynn, Weinstein, Kabins, 1988). No other significant statistical differences between colonized and non-colonized patients were noted. The main reasons for this are probably the small sample size, and limitation to a single institution. In order to overcome these problems it is likely that a multicenter study would have to be performed to provide enough observations to allow adequate conclusions to be made.

7.2.5 Antimicrobial Usage

A significant finding in this study was the association of β-lactam antimicrobials with gram-negative colonization/resistance. It is likely that excessive anti-microbial prescribing contributes heavily to this problem, for as antimicrobial resistance becomes more prevalent there is increasing reliance on the newest and most potent broad-spectrum agents for prophylaxis and treatment of infections in high-risk, critically ill patients. The excessive use of metronidazole in this ICU population for anaerobic coverage was significantly associated with an increased risk of gram-negative colonization/resistance and is a practice that may have to be reviewed. It is likely that the administration of metronidazole decimates the

population of anaerobic microflora which provide important colonization resistance in the gastrointestinal tract. Similarly, the use of cephalosporins was also associated with an increased risk of gram-negative colonization/resistance due to their propensity to induce gram-negative organisms to synthesize β-lactamases. Excessive cephalosporin usage may therefore be a predisposing factor for the selection of certain gram-negative bacilli such as *E.cloacae* and *P.aeruginosa*.

7.3 Control of Antimicrobial-Resistant Organisms in Hospitals

Since the introduction of sulphonamides in the 1930s, the rapidity with which microorganisms have developed resistance to antimicrobial agents has been phenomenal. The laws of evolution dictate that microorganisms will eventually develop resistance to nearly every antimicrobial known to man (ASM, 1995). It is therefore painfully obvious that the current resistance problems are not just going to go away and if present practice prevails the trends in antibiotic resistance seen particularly in the last decade will continue into the 21st century. Unless currently effective antimicrobials can be successfully preserved and the transmission of resistant organisms curtailed, then the post-antimicrobial era may be rapidly approaching in which infectious disease wards housing untreatable conditions will again be seen (Cohen, 1992; Jacoby and Archer, 1991).

Are there any solutions to the current resistance problems? The answer is "yes, no, maybe" (Neu, 1993). Several proposals have been suggested:

7.3.1 Novel Antimicrobial Development

Faced with the gradual erosion in the efficacy of even the newest antimicrobials,

there has been increasing reliance on the ability of the pharmaceutical industry to develop novel agents (Goldmann, Weinstein, Wenzel et al., 1996). However, since the introduction of quinolones in the 1980s no new class of antimicrobials has been introduced. The costs of discovering, developing, testing and approving antimicrobials are enormous and with the rapid emergence of resistance, the incentive to develop new agents diminishes as their projected lifespan declines. This is not to say that no new antimicrobials will be introduced but it should be realized that the problem of antibiotic resistance in hospitals will not be solved solely by the repetitive introduction of new antimicrobials (Gaynes, 1995). Those who are looking for the "magic bullet" will be disappointed.

7.3.2 Efficacious Vaccine Production

Health efforts need to be focused on preventing transmission and infection as antimicrobial resistance increases, rather than on treating illness once it has occurred. The development of vaccines to prevent diseases that are difficult to treat would be a very effective mechanism for dealing with the emergence of antimicrobial resistance (Cohen, 1992). As with new antimicrobial development however, the enormous cost of developing vaccines does not make this a viable option for dealing with current resistance problems at this present time.

7.3.3 Appropriate Antibiotic Stewardship

Despite numerous published guidelines for appropriate antimicrobial use and antimicrobial restriction policies in many hospitals, excessive and inappropriate antimicrobial prescription continues. As antimicrobial resistance is largely the result of selective pressure, improving antimicrobial use should be the cornerstone

in dealing with multiresistant organisms (Neu, Duma, Jones et al., 1992; Weinstein, 1991; Chow, Fine, Shlaes et al., 1991; Sanders and Sanders, 1985; McGowan, Jr. and Gerding, 1996). Appropriate antibiotic stewardship includes not only the limitation of use of inappropriate agents, but also cyclic antimicrobial use, optimized pharmacokinetic dosing, and synergistic combination therapy.

7.3.3.1 Cyclic Antimicrobial Use

Also called rotational antimicrobial use, this has been suggested as a way of relieving the selective pressure that the continued use of certain antimicrobials have in selecting out a population of resistant organisms. An example of this approach for the treatment of *P.aeruginosa* would be the use of say ceftazidime and tobramycin for a limited period of time, then change the regimen to pipericillin and gentamicin etc (Ballow and Schentag, 1992). In this way antibiotics would not be given an opportunity to select out resistant populations of bacteria. Unfortunately there are few data to determine the impact of such an approach. Furthermore, the antimicrobial agents to be cycled, the duration of the cycles, and the preferred order in which agents are cycled are unknown and a large multicenter study would have to be undertaken to determine these factors (McGowan, Jr. and Gerding, 1996).

7.3.3.2 Optimized Pharmacokinetic Dosing

Optimized pharmacokinetic dosing essentially refers to the aminoglycosides and it utilizes innovative dosing regimens which replace traditional dosing methods if they are shown to provide improved clinical response with less toxicity. Dose-

related toxicities of the aminoglycosides include nephrotoxicity and ototoxicity (Miyagawa, 1993). Aminoglycosides exhibit concentration-dependent killing and therefore as the peak concentration of aminoglycoside to MIC concentration is increased, the rate and extent of killing are also increased. Furthermore, the post-antibiotic effect of aminoglycosides translates into dosing regimens with prolonged dosing intervals (ie. every 24 hours versus every 8 hours). Optimized pharmacokinetic dosing therefore serves to balance the use of high levels of antibiotic while limiting toxicity at the same time. The end result may be a decrease in the emergence of antimicrobial-resistant subpopulations, the appearance of which is a distinct possibility when only subtherapeutic levels are achieved.

7.3.3.3 Synergistic Combination Therapy

Combination therapy (eg. an aminoglycoside plus a third generation cephalosporin for gram-negative infections) is often used to broaden the antibacterial spectrum, to enhance antimicrobial therapy, and to reduce the emergence of resistant organisms. This is of particular importance in the ICU where treatment of infections is often initiated before any pathogens are identified (Craig, 1988; Salacata and Chow, 1993). Antimicrobial combinations may act synergistically to enhance the killing of multiresistant organisms by taking advantage of different mechanisms of action thus facilitating penetration of the drugs (Kosmidis and Koratzanis, 1986). The effect is therefore more than the sum of two agents alone. Synergism may also allow the use of lower, less toxic doses of antimicrobials.

7.3.4 Focused Microbiological Surveillance

Areas of high antimicrobial use such as ICUs are at increased risk of developing different forms of resistance in response to the selective pressure of β-lactam antimicrobials (Bryce and Smith, 1995). The customary way of detecting trends in antimicrobial susceptibility patterns is by hospital antibiograms derived from cumulative information from all clinical units. However, this may lead to an underestimate of resistance in high use areas such as ICUs. Because the true epidemiological picture in the ICU may be obscured by the "dilution" of this smaller group in a much larger hospital population, early detection and control of resistant organisms may be achieved by focused surveillance (Bryce and Smith, 1995). Focused microbiological surveillance data can be used to restrict antimicrobial therapy in the ICU as well as encourage sound antimicrobial stewardship. Monitoring of high antimicrobial use areas such as ICUs may lead to early identification of changes in resistance patterns containment measures, and restricted antimicrobial policies.

7.3.5 Infection Control Compliance

It has been stated that a major factor in the rapid emergence of resistance is the intense selective pressure of antimicrobial use and abuse. An equally important factor is the inconsistent application of basic infection control techniques by hospital personnel, which largely accounts for the dissemination of resistant strains in the hospital environment (Goldmann, Weinstein, Wenzel et al., 1996; Solomkin, 1996). To address this dissemination problem, barrier isolation techniques have been used to try and interrupt transmission of resistant organisms and the main emphasis in such efforts has been on proper handwashing and the

use of specially tailored isolation precautions (McGowan, Jr., 1983). This is especially true where the risk of transmission of resistant organisms is greatest, namely in overcrowded, understaffed units where the sickest and most vulnerable patients are concentrated and where the normal respiratory and gastrointestinal flora of these patients are rapidly replaced by the nosocomial pathogens circulating in the unit (McGowan, Jr., 1983). A further problem in preventing transmission is that patients with resistant pathogens may be difficult to spot because very few of those colonized actually develop clinical infection.

7.4 Strengths and Weaknesses of Study

7.4.1 Strengths

The main strength of this study was the fact that it was conducted prospectively on a cohort of patients admitted to the ICU. Prospective cohort studies are generally thought to provide the most definitive information about disease etiology and the most direct measurement of the risk of disease development (Hennekens and Buring, 1987). Furthermore, this study was an objective one and has provided good and useful information in an area where there is much myth, speculation and little data. Finally, a strength of this study is also the fact that it is Canadian, providing Canadian data where little or none exist.

7.4.2 Weaknesses

Some of the limitations of this study include the fact that it was performed at only one institution and the small sample size. Few significant statistical differences between colonized and non-colonized were found and a multicenter

study with a larger sample size may provide enough observations to make more definite conclusions. Concerning the collection of rectal swabs, it was desirable that these be collected from patients within twelve hours of admission, but due to the priorities of life support in the ICU this was not always possible. This was a practical reality which had to be faced but in retrospect, it would be prudent in future studies to employ someone to specifically perform this task. Finally, this study can never be repeated at the present location because of its pending closure, and this is also a limitation.

What then should be done as the 21st century rapidly approaches? At the very least there should be strict adherence to essential infection control practices (Daschner, 1985; Crombach, Dijkshoorn, van Noort-Klaassen et al., 1989; Weinstein, 1991). In addition, the use of simple barrier precautions to prevent colonization and infection warrants further study. For example, more effective approaches to disinfecting hands as well as the effects of cutaneous antiseptics are needed. Many health care workers are blissfully unaware of even the most basic infection control practices, such as handwashing, which continues to be done inconsistently. Effective ways to educate hospital personnel concerning infection control practices, especially handwashing, aseptic use of devices, and antimicrobial therapy, and to apply these measures would have immediate beneficial effects (Maki, 1989).

In conclusion it is clear that there are no easy solutions to the increasing problem of antimicrobial resistance. The implications of a post-antimicrobial era are too frightening to even contemplate and it is therefore imperative that the entire healthcare delivery system see the antimicrobial resistance problem as a critical one and collectively address it as such. When this happens a multifaceted solution may go a long way to solve a multifaceted problem.

Diseases desperate grown
By desperate appliances are relieved
Or not at all

William Shakespeare
Hamlet

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