THE UNIVERSITY OF CALGARY

"Comparison of Cefotaxime and Ceftriaxone on the Selection of Third-Generation Cephalosporin-Resistant Gram-Negative Bacilli in Gut Microflora of Hospitalized Patients"

by

Felicia Laing

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Comparison of Cefotaxime and Ceftriaxone on the Selection of Third-Generation Cephalosporin-Resistant Gram-Negative Bacilli in Gut Microflora of Hospitalized Patients" submitted by Felicia Laing in partial fulfillment of the requirements for the degree of Master of Science.

Supervisor, T.J. Louie
Department of Microbiology and Infectious Diseases

E.A. Henderson
Department of Community Health Sciences

D.E. Woods
Department of Microbiology and Infectious Diseases

External Examiner, K.E. Sanderson
Department of Biological Sciences

Date April 30/1996

ABSTRACT

Cefotaxime and ceftriaxone are therapeutically equivalent third-generation cephalosporins (TGC) which have low and high gut elimination, respectively. **Objective:** To evaluate the gut ecological consequences of low versus high intestinal concentrations of antibiotics on the acquisition of TGC-resistant Gram-negative bacilli (GNB). **Results:** Resistant GNB were selected from fecal microflora of 3.8 times more cefotaxime- than ceftriaxone-patients after 3 treatment-days. Cefotaxime-patients were 9.2 and 1.5 times more likely than untreated and cefazolin controls to harbour resistant GNB. Type 1 cephalosporinase-producing species were the most frequently isolated resistant GNB. In vitro susceptibilities showed cross-resistance with other TGCs, extended-spectrum penicillins, and ß-lactamase inhibitors but not to fluoroquinolones nor carbapenems. Genotypic analysis indicated that resistant strains were selected from patients' endogenous flora. **Conclusions:** Low fecal antibiotic concentrations select TGC-resistant organisms in gut microflora. Cefotaxime should be prescribed judiciously and avoided in neutropenic patients.

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Finally, I am grateful to my family for their love and support.

DEDICATION

For his unwavering love, patience, and support, I dedicate this work to my husband, Andrew

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
ANOVA	analysis of variance
API	analytic profile index
ATCC	American Type Culture Collection
BA	blood agar
BHI	brain heart infusion
BUN	blood urea nitrogen
BVC	Bow Valley Centre
CFU	colony forming units
CGH	Calgary General Hospital
cm	centimetres
COPD	chronic obstructive pulmonary disease
CRO	ceftriaxone
CTX	cefotaxime
CZ	cefazolin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FOX	cefoxitin
g	grams

LIST OF ABBREVIATIONS, Continued:

ABBREVIATION	EXPLANATION
GNB	Gram-negative bacilli
ID	infectious disease
I.V.	intravenous(ly)
L	litre
MIC	minimum inhibitory concentration
kg	kilograms
M	molar
Mac	MacConkey agar
Mac_2	MacConkey agar containing 2 µg/mL antibiotic
Mac_{16}	MacConkey agar containing 16 µg/mL antibiotic
mg	milligrams
mL	millilitres
mm	millimetres
mM	millimoles
µM	micromoles
NaCl	sodium chloride
NCCLS	National Committee for Clinical Laboratory Standards

LIST OF ABBREVIATIONS, Continued:

ABBREVIATION	EXPLANATION
PFGE	pulsed-field gel electrophoresis
PID	pelvic inflammatory disease
PLC	Peter Lougheed Centre
q	quaque (every)
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
TBE	Tris boric acid-EDTA
TE	Tris-EDTA
Tris-HCl	Tris hydrochloric acid
TS	tryptic soy
U	units
vol	volume
wt	weight
µ	microns
µg	micrograms
χ^2	chi-square

1.0 INTRODUCTION

Third-generation cephalosporins have often been selected as standard therapy for serious hospital-acquired infections and this practice has been associated with a corresponding increase in therapeutic resistance to them.

Resistance was primarily mediated by chromosomal cephalosporinases and developed in common nosocomial (hospital-acquired) Gram-negative pathogens that included several genera within the family *Enterobacteriaceae*, *Pseudomonas* species and *Acinetobacter* species (Sanders and Sanders, 1988). The development of antibiotic resistance affected not only the ability to treat the infection, but also the cost and duration of treatment.

Emergence of resistance was associated with antibiotic use and abuse, selection of resistance phenotypes and resistance genes, persistence or dissemination of resistant organisms, and presence of bacterial reservoirs (Cohen, 1992). Currently, the size and nature of a colonized colonic reservoir is unknown and its clinical significance is uncertain. Antimicrobial agents that cause significant disturbances in the normal gut microflora have selected for Gram-negative pathogens that display resistance to the antimicrobial or cross-resistance to others of different drug classes and thus the bacteria have become multiresistant (Follath *et al.*, 1987; Livermore, 1991).

The intestinal anaerobes and facultative aerobes are thought to confer "colonization resistance" to a host and thus limit colonization of the gut lumen by *Enterobacteriaceae* and *Pseudomonas* species (Vollaard and Clasener, 1994).

Antimicrobial agents that disrupt colonization resistance may permit the overgrowth of resistant species.

High intraintestinal concentrations of antibiotics may disrupt colonization resistance but if the antibiotic levels were high enough to exceed the minimum inhibitory concentration of that antibiotic in resistant Gram-negative bacilli (GNB), the emergence of resistant GNB may be suppressed. Thus, low intraintestinal concentrations of antibiotic would favour the selection of resistant species by killing susceptible species.

There are few data on the selection of third-generation cephalosporin-resistant GNB in the intestinal microflora of individuals receiving third-generation cephalosporins with differential biliary elimination. Cefotaxime is a third-generation cephalosporin that is primarily excreted in the urine (Jones and Thornsberry, 1982) while ceftriaxone is excreted in substantial amounts in the bile (Bergan, 1987). Ceftriaxone may impair the colonization resistance of the gut microflora and allow for the overgrowth of resistant GNB while subinhibitory levels of cefotaxime in the gut may favour the selection and colonization of resistant GNB.

In both scenarios, the development of resistance increases the risk of acquiring a nosocomial infection (Prevot *et al.*, 1986; Shah *et al.*, 1991). Knowledge of the causes of resistance development would lead to opportunities to prevent the establishment and spread of antibiotic resistant strains, both endogenously and exogenously.

2.0 LITERATURE REVIEW

2.1 A Reservoir for Resistant Bacteria

One of the more readily-assumed causes of clinical failure is antibiotic resistance, whether it develops in the strain causing the infection, is selected for from a resistant subpopulation, or is induced by the antibiotic used (Norrby, 1991). Members of the indigenous flora have become major pathogens in nosocomial infections, and it is recognized that the indigenous gut flora is a potential source of antibiotic-resistant GNB in hospitalized patients (Rose and Schreier, 1968).

The bowel represents, by far, the main human reservoir for bacteria. The intestine contains 10^9 to 10^{11} bacteria/g stool and is populated mostly by obligately anaerobic bacteria, *Escherichia coli*, and *Enterococcus* species (Tancrède, 1992). The skin, the oropharynx, and the vagina can be considered as additional reservoirs of lesser importance in terms of absolute numbers of bacteria and bacterial concentrations they contain (Hawkey, 1986; Péchère, 1994). Selection of resistant bacteria can occur in any of these reservoirs during antibiotic therapy, but the intestine is the dominant niche for resistance selection (Péchère, 1994).

Resistant bacteria were initially encountered in urban hospitals but have been detected as causes of community-acquired infections (Levy *et al.*, 1988) and as colonizers of the human and animal oropharynx and gut (Guggenbichler and Kofler, 1984; Hirsh *et al.*, 1980; Nord and Heimdahl, 1986). Patients with community-acquired infections that were admitted to hospital had usually been

exposed to at least one course of antibiotic therapy. Other patient populations have been described that contain antibiotic-resistant organisms in the gut flora.

Among the normal non-pathogenic flora of ambulatory and hospitalized individuals, resistance to single and multiple antibiotics have been shown (Ismaeel, 1993; McGowan, 1983). In patients not receiving antibiotics, carriage of resistant lactose-fermenting bacteria was found to be as high as 49.4% (ampicillin-resistance), whereas healthy individuals harboured between 3.7 - 40.8% antibiotic resistant strains (Ismaeel, 1993). Over 60% of stool samples from more than 300 healthy volunteers in Boston contained $\geq$ 10% of the total aerobic Gram-negative flora resistant to one of ampicillin, tetracycline, streptomycin, or kanamycin (Levy, 1986). These reports indicate that the human intestine, even from individuals not ingesting antibiotics, is a reservoir of resistant bacteria. Disturbing the ecological balance of the normal gut microflora upsets the equilibrium between indigenous microflora and host.

The administration of antimicrobial agents is the most common and significant cause of disturbances in the normal microflora (Nord *et al.*, 1984a). The degree of disturbance is dependent on the intrinsic activity of the antibacterial agent against the indigenous bacteria of the intestine as well as on the pharmacokinetic characteristics of the antibacterial agent, which determines the concentration of the agent in the gut (van Ogtrop *et al.*, 1991).

Substantial changes in the intestinal microflora that accompany injudicious use of antimicrobials may result in a number of potentially adverse effects. One is the overgrowth of already-present microorganisms such as yeasts, which may produce systemic infection in immunosuppressed patients,

and of *Clostridium difficile*, which may lead to colitis (Mulligan *et al.*, 1984; Rose and Schreier, 1968).

A second potential consequence is a reduction in "colonization resistance". Colonization resistance is a concept whereby anaerobes and likely, *E. coli*, of the normal flora prevent the establishment of exogenous strains (as opposed to the overgrowth of already-present strains) in the fecal microflora (van der Waaij *et al.*, 1971). The physiological basis for colonization resistance has been attributed to competition for nutrients, competition for attachment sites, production of bacteriocins, and production of volatile fatty acids by anaerobic flora that may inhibit members of the *Enterobacteriaceae* (Freter *et al.*, 1983; Louie *et al.*, 1985; Nord *et al.*, 1984a).

A third effect is the selection of antibiotic resistance among members of the normal fecal flora (Livermore, 1991; Nord *et al.*, 1984a). In a population of GNB, certain species are capable of producing constitutive levels of enzyme that destroy or inhibit the action of ß-lactam antibiotics. Subinhibitory concentrations of antibiotics that kill susceptible cells will also select resistant mutants that are able to survive (Livermore, 1991). In vivo, these resistant strains can overgrow to dominate the microflora.

2.2 Host Factors Associated With Antibiotic Resistance

Impairment of the indigenous bacteria is contributory, but several host factors have been associated with colonization, infection or superinfection by resistant organisms. Immunocompromised patients receiving prolonged courses

of antibiotics are at particular risk of developing resistance in GNB during treatment with broad-spectrum antibiotics (Chow *et al.*, 1991; Follath *et al.*, 1987). Resistant organisms may emerge more rapidly as a result of the routine use of cephalosporins than of other antibiotics (Sanders and Sanders, 1985).

Diabetes mellitus, malignancy, and cardiovascular disease were common underlying illnesses associated with emergence of resistance in patients with *Enterobacter* bacteremia (Chow *et al.*, 1991). Positive cultures were not observed until after abdominal surgery and occurred in patients receiving third-generation cephalosporins. There have also been reports of emergence of resistance during therapy to the extended-spectrum penicillins, carbenicillin and piperacillin (Letendre *et* al., 1988; Gribble *et al.*, 1983). As well, prior exposure to third-generation cephalosporins and extended-spectrum penicillins was associated with the isolation of organisms resistant to these antibiotics (Jacobson *et al.*, 1995; Tancrède *et al.*, 1984).

2.3 Third-Generation Cephalosporins

The clinical importance of ß-lactam antimicrobials is two-fold: they are the most widely used antibacterial drugs, and all exhibit a selection pressure on bacterial populations that lead to the emergence of resistant variants (Wiedemann, 1986). ß-lactam antibiotics include the penicillins, cephalosporins, cephamycins, oxacephems (moxalactam), carbapenems and monobactams. All have a common ß-lactam ring. They are bactericidal agents because they block

the synthesis and growth of the bacterial cell wall by binding to penicillin-binding proteins (PBPs) on the cytoplasmic membrane (Bryan and Godfrey, 1991).

The third-generation cephalosporins were developed in response to a significant increase in the proportion of infections that were no longer treatable with "traditional" antibiotics, such as ampicillin or the first-generation cephalosporins, because of antibiotic resistance. Third-generation cephalosporins were designated as broad spectrum cephalosporins because they have acceptable activity against most Gram-positive organisms, remarkable potency against all species of *Enterobacteriaceae*, and in some cases, activity against *Pseudomonas aeruginosa* (Thornsberry, 1985).

Cephalosporins are derivatives of 7-aminocephalosporanic acid (7-ACA) which is a bicyclic structure comprised of the 4-membered lactam ring attached to a six-membered dihydrothiazine ring (Donowitz and Mandell, 1988). The cephalosporin nucleus provides many sites for manipulation that have allowed for the evolution of antimicrobial agents with increased potency, improved spectrum, and/or pharmacokinetic advantages. Modifications at the 3-position and 7ß-side chain moieties of 7-ACA increased the serum half-lives and ß-lactamase stability, respectively of the second-generation cephalosporins as compared to the first-generation agents (Price and McGregor, 1984). Modifications to include an aminothiazole group at the 7ß position conferred higher intrinsic activity than previous cephalosporins and the combination of a methoxyimino moiety added ß-lactamase resistance to extend the intrinsic antibacterial activity of third-generation cephalosporins (Price and McGregor, 1984).

The safety, clinical efficacy and favourable pharmacokinetics made the third-generation cephalosporins the preferred antibiotic in many clinical situations (Klein and Cunha, 1995). Increased membrane penetration capacities, low affinity for ß-lactamases, high affinity for PBPs, and high intrinsic activity characterized these new agents as promising tools in the fight against ß-lactam-resistant microorganisms (García-Rodríguez *et al.*, 1992). The third-generation cephalosporins include cefotaxime, ceftizoxime, cefoperazone, ceftriaxone and ceftazidime.

2.3.1 Antibacterial activity of cefotaxime and ceftriaxone

Third-generation cephalosporins are frequently used for empirical treatment. For serious and difficult-to treat Gram-negative infections, third-generation cephalosporins were the drugs of choice because of a low frequency of adverse effects, high degree of clinical and microbiological activity, and tissue penetration (Donowitz, 1989; Garber *et al.*, 1992). Cefotaxime was the initial third-generation cephalosporin to be used on a widespread basis in clinical practice (Patel *et al.*, 1995). Even today, it provides good empiric therapy against susceptible organisms in immunocompromised patients and is of proven efficacy in Gram-negative meningitis, urinary tract infections, acute osteomyelitis or septic arthritis (Klein and Cunha, 1995).

Cefotaxime exhibits in vitro and in vivo activity against members of the *Enterobacteriaceae* family including *Enterobacter* species, *E. coli*, *Klebsiella* species, *Providencia* species and *Proteus* species. However, it lacks activity

against *Pseudomonas* species (Patel *et al.*, 1995). Cefotaxime, also, has good Gram-positive activity against methicillin-sensitive *Staphylococcus aureus*, and multidrug-resistant *Streptococcus pneumoniae* (Patel *et al.*, 1995). It shows moderate activity against anaerobes and lacks activity against *Enterococci* species (Donowitz, 1989).

Ceftriaxone displays a similar antibacterial spectrum and potency and stability to ß-lactamases but has different pharmacokinetic properties than cefotaxime which are dictated by variations in the C3 position (Bergan, 1987; Price and McGregor, 1984). It has excellent activity against *S. pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* and penetrates cerebrospinal fluid in high levels (Cherubin *et al.*, 1989; Klein and Cunha, 1995). Ceftriaxone has become an agent of choice for treating uncomplicated urethral, anorectal, or pharyngeal gonorrhea including cases caused by penicillin-resistant strains (Donowitz, 1989).

When used as single agents, cefotaxime and ceftriaxone have produced overall response rates of 80-90% in the treatment of pneumonias, bacteremias, urinary tract infections, intraabdominal infections, and infections of the skin and soft tissue (Donowitz, 1989). Comparative trials have shown no differences in efficacy among cefotaxime or ceftriaxone (Garber, *et al.*, 1992; Mandell *et al.*, 1989; Smith *et al.*, 1989).

2.3.2 Metabolism and excretion of cefotaxime and ceftriaxone

The elimination half-life of cefotaxime is approximately one hour. It is rapidly metabolized in the liver to desacetyl-cefotaxime and generally retains antimicrobial activity that is eightfold lower than that of the parent compound, depending on the pathogen involved (Jones and Thornsberry, 1982; Oizumi *et al.*, 1988). Desacetyl-cefotaxime inhibits 90% of the strains from the *Enterobacteriaceae* family but is less active than cefotaxime against *Streptococcus* species, *Staphylococcus* species, and *Pseudomonas* species (Neu, 1982; Oizumi *et al.*, 1988).

Excretion of cefotaxime is mainly via the kidneys by glomerular filtration and tubular secretion (Wise *et al.*, 1981). Slightly more than 80% of a radiolabelled dose of cefotaxime is found in urine: about 50-60% as unchanged parent drug, 20-25% as desacetyl-cefotaxime and 20-25% as opened ß-lactam ring lactone metabolites, M_2 and M_3 (Jones and Thornsberry, 1982). In patients with severely impaired renal function, the serum half-life is prolonged, mainly in the form of desacetyl-cefotaxime (Jones *et al.*, 1982). Less than 2% of cefotaxime is recovered in bile (Bergan, 1987).

The serum half-life of ceftriaxone is 7.2 hours (Bergan, 1987). Ceftriaxone is not metabolized significantly and it is characterized by a dual mechanism of excretion with 30-67% of ceftriaxone secreted in the bile and 33-67% secreted in the urine (Bergan, 1987; Christ, 1991). In patients with mild to moderate impairment of biliary excretion, compensatory renal excretion occurs and with mild to moderate renal function impairment, biliary excretion is

enhanced (Blumer, 1991). Metabolic products recovered after biliary excretion of ceftriaxone are microbiologically inactive.

2.3.3 Effects of cefotaxime and ceftriaxone on fecal flora

Various studies have investigated the ecological impact of cefotaxime and ceftriaxone on the intestinal microflora (Arvidsson *et al.*, 1982; Lambert-Zechovsky *et al.*, 1985; Nilsson-Ehle *et al.*, 1985; van der Waaij, 1983). Often a small number of subjects were examined, the subjects were healthy volunteers, and/or the selection of resistant species was not examined. Levy *et al.* (1988) reported that the number of antibiotic-resistant bacteria in feces increased after patients had received antibiotics. Resistance to third-generation cephalosporins was not examined in that study.

As well, conflicting results have been reported in the occurrence of resistance development. Treatment with third-generation cephalosporins have resulted in the isolation of resistant enterobacteria in patients treated with cefotaxime (Tancrède *et al.*, 1984) but not in patients treated with ceftriaxone (Hoepelman *et al.*, 1988). In 6 healthy volunteers, cefotaxime impaired colonization resistance and allowed colonization by a challenge strain of *Enterobacter cloacae* (Vollaard *et al.*, 1990). Secondary colonization in one patient occurred with a resistant *Acinetobacter calcoaceticus* strain.

Michéa-Hamzehpour *et al.* (1988) reported more pronounced effects on the fecal microflora with ceftriaxone than with cefotaxime but comparisons were made with only a prophylactic dose of a cephalosporin. Resistance did not

develop after the single dose of cefotaxime but appeared after the single dose of ceftriaxone.

Guggenbichler and Kofler (1984) compared the effects of cefoperazone (75% biliary excretion), ceftriaxone (n=9) and cefotaxime (n=6) on the aerobic fecal flora of 20 children undergoing therapy for serious bacterial infections. A comparison of pretreatment stool specimens with specimens obtained during therapy showed eradication of susceptible GNB after the first dose of cefoperazone and ceftriaxone. After 7 days of therapy with ceftriaxone, GNB reappeared that were resistant to cefotaxime, ceftriaxone, cefoperazone, cefotiam and mezlocillin by the disk diffusion method. The number of patients colonized was not stated. Conversely, cefotaxime therapy had only a moderate influence on the fecal flora and microorganisms initially identified, persisted throughout therapy, with their original susceptibility patterns.

The effect of high biliary excretion on the fecal microflora has also been reported in adults treated with ceftriaxone (Arvidsson *et al.*, 1982; Bodey *et al.*, 1983). During the first 3-4 days of treatment, the number of aerobic GNB decreased or disappeared, and the anaerobic flora was also suppressed depending on the concentrations of ceftriaxone found in stools (Arvidsson *et al.*, 1982; de Vries-Hospers *et al.*, 1991).

In stool specimens where no ceftriaxone was found, no effect was seen on the number of anaerobic bacteria if ß-lactamase activity was detected (Arvidsson *et al.*, 1982). Subjects showing a decrease in the number of anaerobic organisms had measurable concentrations of ceftriaxone in their stool ranging from 28 to 75 mg/kg of stool and no detectable ß-lactamase activity. In

6 volunteers, concentrations of ceftriaxone reached levels as high as 1600 mg/kg (de Vries-Hospers *et al.*, 1991). No comparative studies examining cefotaxime levels in stool have been made.

2.4 Mechanisms of Resistance to Third-Generation Cephalosporins

ß-lactamases nearly always seem to be involved as the main factor in the development of resistance in GNB that cause nosocomial infections (Mulgrave, 1991). Resistance to cephalosporins and other ß-lactams is mostly a result of selection of preexisting mutant organisms that produce large amounts of ß-lactamase constitutively (Sanders and Sanders, 1988). Some of these organisms also have decreased expression of the outer membrane proteins.

Two major porins of *E. coli*, OmpC and OmpF, and presumably analogous structures in other *Enterobacteriaceae* (Kanenko *et al.*, 1984), are usually associated with altered permeability to antimicrobial agents. Decreased expression of OmpF, the channel through which cephalosporins cross the outer cell wall of GNB, coupled with increased expression of OmpC, a channel through which the cephalosporins do not readily pass (Neu, 1992) result in inhibition of the entry of the cephalosporins or of penicillins. High affinity of the ß-lactamase for the cephalosporins coupled with a decreased amount of drug coming into the periplasmic space, where the ß-lactamase resides, results in destruction of the cephalosporin (Neu, 1992).

ß-lactamases catalyze the hydrolysis of the ß-lactam ring and so destroy antibiotic activity. This resistance is related to the large numbers of ß-lactamase

molecules that accumulate in the periplasmic space of the organism and the high affinity of these enzymes for many drugs (Sanders and Sanders, 1988). Genes mediating the production of ß-lactamases can either be located on the chromosome of the bacterial cell, on plasmids or on transposons (Wiedemann, 1986). Genetic changes in each location can lead to increased resistance to ß-lactam antibiotics.

2.4.1 Chromosomal ß-lactamases

P. aeruginosa, A. calcoaceticus and most enterobacteria (except *Salmonella* sp. and *Klebsiella* sp.) produce chromosomally-mediated ß-lactamases. These enzymes are classified as Type 1 ß-lactamases or cephalosporinases because they prefer cephalosporin substrates, although they can inactivate penicillins as well (Bush, 1989; Sanders and Sanders, 1988). In wild-type organisms not previously exposed to antibiotics, the production of cephalosporinase is usually inducible - little enzyme is produced, but the amount increases markedly after exposure to many ß-lactams (Lindberg and Normark, 1986). When low quantities of enzyme are made, they are sufficient to cause resistance to early-generation cephalosporins, but when high quantities are produced, resistance is also seen to third-generation cephalosporins and the cephamycins (Lindberg and Normark, 1986).

Enhanced expression of chromosomal ß-lactamases can occur by mechanisms that affect the genes governing enzyme expression. A family of related cephalosporinase genes, referred to as *ampC*, share significant

homology among various species (Lindberg and Normark, 1987; Galleni *et al.*, 1988). Induction is governed by the *amp*R regulatory gene, which encodes a DNA-binding protein that functions as an activator for *amp*C transcription (Lindberg *et al.*, 1988). High-level constitutive ß-lactamase production involves spontaneous mutation in the *amp*D gene of the wild-type organism. This mutation occurs in 10^{-5} to 10^{-7} wild type cells and the increased levels of enzyme persist even in the absence of an inducer. In enterobacteria, the *ampD* gene encodes a cytosolic protein which acts as a repressor of ß-lactamase expression (Honoré *et al.*, 1989; Jacobs, *et al.*, 1995). A transmembrane protein, encoded by *amp*G, is required for ß-lactamase production both upon induction by ß-lactams or subsequent to mutational loss of *ampD* (Korfmann and Sanders, 1989; Lindquist *et al.*, 1993).

In GNB that possess chromosomal cephalosporinases, selection of mutants that are stably derepressed is more likely to occur than induction of wild type cells to transient high levels of enzyme (Lindberg *et al.*, 1988; Sanders and Sanders, 1983). The species known to possess this type of enzyme and against which third-generation cephalosporins would normally be active include *Enterobacter* species, *Serratia* species, *Citrobacter freundii*, *Acinetobacter* species, *Aeromonas* species, *Proteus vulgaris*, *Providencia* species, *P. aeruginosa*, and *Morganella morganii* (Bush, 1989). The ß-lactamase inhibitors, clavulanate, sulbactam, and tazobactam do not inhibit the cephalosporinases found in these species (Bush, 1989; Jacoby and Medeiros, 1991).

The clinical significance of constitutive mutants is that they are selected during therapy of individual patients, they can cause clinical failure and once

selected, they may become the prominent organism, particularly in an intensive care unit setting (Murray, 1992). Extensive use of ß-lactamase-stable cephalosporins in hospitals have generated selective pressure that caused the appearance of numerous *Enterobacter* bacteremias (Chow *et al.*, 1991).

2.4.2 Plasmid-mediated ß-lactamases

The most common plasmid-mediated ß-lactamases encountered in *Enterobacteriaceae* are TEM-1, TEM-2 and SHV-1 enzymes, which have weak activity against first-generation cephalosporins (Medeiros, 1993). Extended-spectrum ß-lactamases (ESBLs) are mutant enzymes which derive from TEM-1, -2 and SHV and confer resistance to cefotaxime, ceftazidime, other broad-spectrum cephalosporins and aztreonam. ß-lactamase inhibitors such as clavulanate, sulbactam and tazobactam inhibit most of the ESBLs. The plasmid-encoded MIR-1 enzyme, however, is not inhibited by clavulanate (Papanicolaou *et al.*, 1990).

Most of the isolates that produce ESBLs have come from hospitalized patients and have frequently caused nosocomial outbreaks, primarily due to *Klebsiella pneumoniae* strains. When clinical isolates were studied in France, it was noted that 30% of *K. pneumoniae* in surgical intensive care units (ICUs) and 18.3% in medical ICUs had extended spectrum ß-lactamases (Sirot *et al.*, 1992). ESBLs are found much less frequently in other species of *Enterobacteriaceae* (Liu *et al.*, 1992; Sirot *et al.*, 1992). In Canada, only one isolate of a *Klebsiella*

species resistant to third-generation cephalosporins was detected (Toye *et al.*, 1993).

2.5 Clinical Significance

In the last two decades the medical community has become increasingly aware of antibiotic resistant organisms in the hospital setting. Failure of a patient to respond to antibacterial intervention increases patient morbidity and mortality (Neu, 1993; Norrby, 1991).

The clinical importance of cephalosporinase-producing organisms has increased as use of the third-generation cephalosporins has increased. Emergence of resistance, that has been seen during therapy with third-generation cephalosporins increased, from 7% to 24% between 1982 and 1989 in the U.S. (Schaberg *et al.*, 1991) and from 6.4% to 21% in France between 1980 and 1985 (Jarlier *et al.*, 1988). The rise in resistance has affected the ecology of organisms in the hospital environment (Berkowitz and Metchock, 1995; Mulgrave, 1991).

Enterobacter species have replaced *Klebsiella pneumoniae* as the third leading cause of Gram-negative nosocomial infections in the United States behind *E. coli* and *P. aeruginosa* (Sanders and Sanders, 1992). *Enterobacter cloacae* is resistant to many agents and has been a problem, particularly in institutions where third-generation cephalosporins are extensively used (Chow *et al.*, 1991; Johnson and Ramphal, 1990). In Canada 16.3% to 23.7% of *E. cloacae* were resistant to third-generation cephalosporins (Toye *et al.*, 1993). *E.*

cloacae provides a particularly difficult problem in infection control and will continue to be a problem as it has the potential to become resistant to fourth-generation cephalosporins, carbapenems and fluoroquinolones (Neu, 1993).

Intestinal colonization by resistant strains capable of elaborating the broad-spectrum cephalosporinases provides an important hospital reservoir of potentially pathogenic microorganisms. These organisms are commonly associated with nosocomial infections so that their selection can subsequently lead to spread of resistance by way of medical equipment or the hands of medical staff (Poilane *et al.*, 1993). The emergence of resistance in bacterial species commonly associated with nosocomial infections, thus, poses a serious public health problem (Holmberg *et al.*, 1987).

3.0 STUDY PURPOSE

3.1 Hypothesis

Given the high and low gut elimination of ceftriaxone and cefotaxime, respectively, these antimicrobials should have different effects on the gut flora. An investigation of the influence exerted by these two cephalosporins on the intestinal microflora should show differences in the development of antibiotic resistance in the microflora of patients on these antibiotics.

If colonization resistance was a factor in the selection of resistant GNB, the ecology of the bowel microflora of patients who received ceftriaxone should be disrupted to a greater extent because of higher elimination in the intestine compared to cefotaxime. If greater changes were more likely to occur in the microflora of patients receiving ceftriaxone, it was expected that resistance in the bowel flora developed in a higher proportion of patients who received ceftriaxone.

Alternately, it was just as possible that higher intraintestinal levels of ceftriaxone prevented the development of resistance, since minimum inhibitory concentrations were better obtained, when compared to cefotaxime. The higher intraintestinal levels of ceftriaxone therefore, suppressed the emergence of resistant clones.

The following objectives were developed to examine the aspects of antibiotic resistance that resulted from the possible differential impact of cefotaxime and ceftriaxone.

3.2 Objectives

1. The first objective was to evaluate the gut ecological consequences of low versus high intestinal concentrations of antibiotics on the acquisition of third-generation cephalosporin-resistant GNB.

2. The second objective was to investigate the extent of cross-resistance of cephalosporin-resistant organisms isolated from patients receiving either cefotaxime or ceftriaxone.

3. The third objective was to examine the risk factors for the acquisition of antibiotic-resistant GNB in patients who received cefotaxime or ceftriaxone therapy.

4.0 MATERIALS AND METHODS

4.1 Institution and Antibiotic Policy

Between September 1993 and November 1995, the Calgary General Hospital (CGH) was a 600-bed tertiary care hospital consisting of two sites, the Bow Valley Centre (BVC) and Peter Lougheed Centre (PLC). It housed an intensive care, intensive coronary care, neonatal intensive care, and neurological/neurosurgical, orthopedic, medical teaching and surgical units.

The antibiotic policy at CGH was determined by the Pharmacy and Therapeutics Committee. Cefazolin and cephalexin were the only first-generation cephalosporins on the hospital formulary. The second- and third-generation cephalosporins on formulary were cefuroxime, cefoxitin, cefotaxime (CTX), ceftriaxone (CRO) and ceftazidime. Cefoxitin was restricted to indications for pelvic inflammatory disease (PID) and to patients intolerant to the combination of cefazolin and metronidazole. CRO and ceftazidime were restricted and had to be approved for use by the infectious disease (ID) service. CTX was not restricted.

4.2 Study design

The study design was a randomized ecological trial with a quasi-experimental design (Cook and Campbell, 1979). Four groups of patients were

included to examine the impact of third-generation cephalosporins on the gastrointestinal flora:

1) patients receiving CTX;

2) patients receiving CRO;

3) patients receiving cefazolin;

4) untreated controls.

The first two groups were case groups that consisted of patients randomized to receive treatment with CTX or CRO, as part of the randomized clinical trial. The cefazolin and untreated groups served as "treatment" and "negative" controls, respectively. The study had a quasi-experimental design because fecal specimen collection did not always follow the required schedule as outlined in section 4.5.2.

4.3 Recruitment

To access the case and control populations, a line list of patients started on CTX or cefazolin was generated by the hospital pharmacy on a daily basis and included patients at both hospital sites. Patients admitted to the BVC and PLC were assessed for enrollment into the study according to criteria described below. Patients eligible for the case groups, consisted of those diagnosed or suspected of having a GNB infection treatable with CTX or CRO.

Allocation of treatment to one or the other cephalosporin was determined by a random list of numbers, generated by a sequential number code with an overall equal ratio of each antimicrobial agent. This number code was changed

to a ratio of 2:1 CRO:CTX toward the end of the study to increase the number of subjects receiving CRO. Patients who were already receiving CTX were randomized to continue receiving 1 gram CTX intravenously (I.V.) every (q) 8 hours or, alternatively, to receive 1 gram CRO q 24 hours. This was the norm unless a different dosing schedule was indicated because of poor renal function or because of infections involving the central nervous system which required higher doses for penetration of the blood brain barrier.

Patients who received more than 6 doses of CTX prior to randomization to CRO therapy were excluded. Enrolled patients were followed continuously from initiation of treatment to the day of final stool specimen collection. This was the "surveillance period" although treatment may have continued beyond this period. When possible, stool specimens were collected during an earlier part of the surveillance period (Day 1-4) as well as later (Day 5+).

4.3.1 Patient inclusion criteria for the case groups

1). Patients who had given informed consent for enrollment into the study.

2). Non-intensive care patients.

3). An infection treatable with a third-generation cephalosporin.

4) Age $\geq$ 18 years.

4.3.2 Patient exclusion criteria for the case groups

1). Known hypersensitivity to penicillins or cephalosporins.

2). Concentration of serum creatinine >265 μM/L. An approximate assessment of renal function was most easily obtained by the measurement of the concentration of creatinine in the serum. At the CGH, the reference range of "normal" adult renal function for serum creatinine concentration was 60-130 μM/L. A concentration of >265 μM/L served as an indicator of severe renal impairment which also indicated impairment of renal excretion of CTX.

3). Patients known to have organisms resistant to a third-generation cephalosporin.

4). Granulocytopenic patients with an absolute granulocyte count of $<0.5x10^9$/L.

5) Prophylaxis indication only.

6) Prior therapy with fluoroquinolones such as ciprofloxacin within 72 hours of initiating treatment with CTX or CRO.

7) Concurrent therapy with ciprofloxacin and CTX or CRO.

4.3.3 Patient inclusion/exclusion criteria for control groups

Patients eligible for the treatment control group consisted of those who received cefazolin for any indication other than prophylaxis and received treatment for 3 days (24 doses) or more. Treatment controls received cefazolin as prescribed by their physicians, most commonly at a dose of 1g I.V. q 8 hours.

The surveillance period included the day the first stool specimen was collected to the day of last stool specimen collection or discontinuation of treatment, whichever occurred first. These patients were excluded from the study if they were treated with ciprofloxacin concomitantly.

Patients who were not being treated for infections were considered eligible for the untreated control group if they were on the same unit as the case group patients. The surveillance period included the day of enrollment into the study to the day of discharge from the hospital or the first day the patient received a dose of antibiotic, whichever occurred first. Untreated controls who received any antibiotics in the six months prior to hospitalization were excluded from the study.

4.4 Sample size

The sample size of patients being treated with each antibiotic under study was calculated using a standard formula for an unmatched case-control study (Epi Info Version 5.01B) (Fleiss, 1981). A total of 142 patients were required to be randomized for CTX and CRO therapy. To detect a 10% difference in the proportion of CTX or CRO patients who develop resistance, 71 patients were needed in each of the case groups. This sample size provided a 95% confidence that the observed resistance was true at the 0.05 level of significance and that there was only a 20% chance (power = 0.80) that any differences were not detected.

4.5 Data and Specimen Collection

4.5.1 Data Collection

Data were collected from patients' medical charts and from interviews with patients. Information obtained included patient demographics, underlying illnesses, indication for treatment with cephalosporin (when documented), laboratory values such as creatinine and blood urea nitrogen (BUN) as measures of renal function, culture and sensitivity results of organisms isolated from the patient, prior antimicrobial use and concomitant antimicrobial therapy. Underlying disease was categorized according to Andreoli *et al.* (1993) and the International Classification of Diseases (ICD-9) codes given in the discharge coding summary of a patient's chart.

Prior use of antibiotics was obtained from records of previous inpatient and outpatient visits and the memory of the patients up to 6 months prior to current therapy with CTX, CRO or cefazolin. Patient interviews and chart reviews of admissions in the 6 months prior to hospitalization were also performed for untreated controls to assess prior antibiotic use.

4.5.2 Collection and processing of specimens for microbiological studies

Patient stool specimens were collected within 72 hours of the first dose of cephalosporin therapy for baseline measurements of resistance, again at 5 to 7 days, then weekly after first administration of drug. When possible, this

schedule was followed for all 4 groups of patients. Specimens were also collected on patients at various times during their hospital or treatment course whether or not an initial specimen had been collected.

Specimens were collected in sterile plastic containers and were screened for resistant Gram-negative organisms. Whenever necessary, stool flora were sampled by obtaining a rectal swab in patients incontinent of stool. Stool and rectal swab specimens were immediately placed in a tube containing 500 µl of brain heart infusion (BHI) (Oxoid CM) + 10% glycerol and vortexed vigorously. In this manner, fecal specimens were preserved and were stored at -70°C after screening for resistance.

The fecal specimens were screened for organisms resistant to third-generation cephalosporins by inoculating the following media:

i) blood agar base with 5% sheep's blood (BA) (growth control);

ii) MacConkey (Mac) agar (growth control);

iii) Mac + 16 µg/mL (Mac_{16}) CTX or CRO and

iv) Mac + 2 µg/mL (Mac_2) CTX or CRO.

Representatives of each morphological type were selected for isolation from the screen plates and identified to the species level by API 20E (bioMérieux, Inc., St. Laurent, PQ.) or the VITEK system (bioMérieux). All isolates were frozen at -70°C in skim milk-glycerol.

For enumeration of colonies of resistant GNB, the first 17 fecal specimens were assessed by serially diluting the fecal specimens. One gram of specimen was mixed in 9 mL of phosphate buffered saline and serial tenfold dilutions were

made up to 10^{-8}. One hundred microlitres of each dilution was inoculated onto BA, Mac, Mac_2 CTX, Mac_{16} CTX, Mac_2 CRO and Mac_{16} CRO.

At the same time, quantification of the 17 specimens was also assessed by a quadrant growth method to determine if results would correlate with those determined by the serial dilution method. Using the specimens above which were set up for the serial dilution method, a sterile cotton swab was inserted in the stool and rotated to ensure good representation of the specimen. The soiled swab was vortexed in 500 µl of BHI broth containing 10% glycerol, and one calibrated loop (10 µl) of this suspension was inoculated on BA, Mac, Mac_2 CTX, Mac_{16} CTX, Mac_2 CRO and Mac_{16} CRO then streaked with a sterile wire loop on each quadrant of the agar plate. The results suggested that:

1) Growth on all four quadrants (4^+) was indicative of 10^8 to 10^{10} coliform colony forming units/g stool;
2) Growth on 3 quadrants (3^+) was indicative of 10^6 to 10^7 CFU/g stool;
3) Growth on 2 quadrants (2^+) was indicative of 10^4 to 10^5 CFU/g stool;
4) Growth on the first quadrant (1^+) was indicative of 10^2 to 10^3 CFU/g stool.

Subsequent stool and rectal swab specimens were screened and quantitated using this quadrant growth method.

4.6 Antibiotic Susceptibility Testing

The stability of cephalosporin-resistance (growth on Mac_{16} CTX or Mac_{16} CRO) was tested by sequential subculture (at least 4 passages on BA or Mac) of

resistant strains, then determination of in vitro susceptibilities using the agar dilution method (NCCLS, 1993a). Several susceptible isolates (growth on Mac_2 CTX or Mac_2 CRO) were also tested, and minimum inhibitory concentrations (MICs) for the following antimicrobials were determined: CTX (Hoffman-La Roche), CRO (Hoescht-Roussel), ceftazidime (Eli Lilly), cefazolin, ticarcillin and ticarcillin/clavulanate (Smith-Kline Beecham), piperacillin and piperacillin/tazobactam (Lederle Cyanamid), imipenem (Merck-Sharpe Dohme) tobramycin (Eli Lilly), gentamicin (Schering), clinafloxacin (Parke-Davis) ciprofloxacin (Miles), and ofloxacin (R.W. Johnson Pharmaceutical Research Institute). Antibiotic susceptibilities were determined on Mueller-Hinton (Oxoid CM337) media with antibiotic concentrations ranging from 0.25 to 512 µg/mL for the third-generation cephalosporins and other ß-lactams except imipenem. The fluoroquinolones, aminoglycosides and imipenem were tested at concentrations from 0.015 to 32 µg/mL. Drug combinations that included a ß-lactamase inhibitor contained fixed concentrations (4 µg/mL) of tazobactam or clavulanate.

Dilutions of 2-hour broth cultures were inoculated onto antibiotic-containing Mueller-Hinton agar with a multi-prong replicator to yield a final inoculum of 10^4 CFUs per spot. The MIC was defined as the lowest antibiotic concentration at which no more than one colony was present after 18 to 20 hours of incubation at 37°C. Reference strains from the American Type Culture Collection were *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *E. coli* ATCC 25922.

4.7 ß-Lactamase Assay

Constitutive ß-lactamase production was detected using the chromogenic cephalosporin, nitrocefin (Doern and Tubert, 1987; Montgomery *et al.*, 1979). This substrate undergoes a colour change (from yellow to purple) upon hydrolysis of the ß-lactam ring. A colour reaction, therefore, indicates the presence of ß-lactamase.

After serial passage on BA or Mac, isolates were grown overnight on Mueller-Hinton agar at 37°C. Three to four CFUs were applied onto Whatman filter paper permeated with 50 mg/mL of a commercial nitrocefin reagent (Cefinase; Becton-Dickinson Microbiology Systems, Cockeysville, MD). Colour reactions were recorded 15 minutes after application. *S. aureus* ATCC 29213 was used as the ß-lactamase-positive control and *H. influenzae* ATCC 10211 as the negative control.

Bacterial isolates from which constitutive ß-lactamase was not detected were examined for inducible production of ß-lactamase by the disk approximation test (Sanders and Sanders, 1979). Preparation of the inoculum for performing disk diffusion tests were followed according to NCCLS recommendations (1993b). Strains were grown in tryptic soy (TS) broth (BBL, Cockeysville, Md.) at 35°C for 2 hours then diluted to a 0.5 McFarland standard with TS broth. Commercially prepared 30 mg cefoxitin disks (Merck, Sharpe & Dohme) were placed on inoculated Mueller-Hinton agar next to a disk containing 30 mg CTX or CRO. The distance between the disks was equivalent to the radius of the zone of inhibition produced by the CTX or CRO disks when tested alone. Induction of CTX- or CRO-inactivating ß-lactamase was presumed to

have occurred if the radius of the zone of inhibition between the CTX/CRO and cefoxitin disks was decreased by at least 4 mm in comparison to the radius of the zone of inhibition on the far side of the CTX/CRO disk.

4.8 Fecal Concentrations of Cefotaxime and Ceftriaxone

Fecal samples (≥5 g) were retained only from patients receiving single therapy with CTX or CRO for measurement of fecal drug concentrations. Drug concentrations were determined microbiologically with the indicator strain, *Clostridium perfringens* (ATCC 13124) (Louie *et al*, 1976) which has an MIC of 0.06 - 0.25 µg/mL for CTX (NCCLS, 1990).

Five to seven colonies of *C. perfringens* were inoculated into 5 mL of BHI broth under CO_2 followed by incubation at 37°C for 6 hours. One millilitre of whole defibrinated sheep blood (5% vol./vol.) and 0.6 mL of $1x10^6$ bacterial culture were added to 18.4 mL of cooled molten agar, mixed, and poured onto plastic petri dishes (50 x 15 mm). The seeded medium was allowed to harden on a level bench, and 4-mm diameter wells (maximum of 25/plate) were aspirated with a well cutter.

Standard concentrations of CTX and CRO were diluted in distilled water to give final concentrations ranging from 0.125 to 64 µg/mL. Each dilution was added to wells in volumes of 25 mL each. Stool samples were prepared by mixing 2 grams of stool with 2 mL of sterile distilled water then centrifuged at 3500 rpm (MSE Centaur2, Johns Scientific Inc.). The supernatant was collected then centrifuged at 14,000 rpm (eppendorf Centrifuge 5415C). Twenty-five

microlitres of several dilutions of stool supernatant were inoculated to wells of the seeded TS plates. Standards were prepared in duplicate and stool samples in triplicate per plate. Distilled water was used as the control. The plates were incubated at 37°C overnight in an anaerobic glove box (Forma Scientific, Marietta, OH).

Zones of inhibition of growth for each drug concentration were averaged. Drug concentrations were logarithmically transformed (y-axis) and plotted against average zone sizes (x-axis) for linear regression analysis. The actual concentrations of CTX or CRO in stool samples were calculated from the linear regression line based on the equation: $Y = ß_0 + ß_1X$ where $ß_0$ is the y-intercept of the line, $ß_1$ is the slope, X is the averaged zone of growth inhibition and Y is the calculated log of the drug concentration in the stool specimen (Kleinbaum and Kupper, 1978).

4.9 Genotyping By Pulsed-Field Gel Electrophoresis

Genomic DNA was prepared by a modification of a previously described method (Laing *et al.*, 1995). Isolates were grown for 18 hours in 5 mL of BHI broth (Becton Dickinson, Cockeyville, MD) at 37°C. The cells were harvested and suspended in 10 mL of warmed (42°C) buffer (100 mM EDTA, 20 mM NaCl, 10 mM Tris-HCl, [pH 7.2]). 0.5 mL of this suspension was mixed with 0.5 mL of 1.6% low-melting-temperature agarose (Imbed LMP Agarose; New England Biolabs, Beverly, Mass.), pipetted into a plug mold (Bio-Rad Laboratories, Richmond, CA), and allowed to solidify for 30 minutes at 4°C. Plugs were

placed in 10 mL of fresh, cold lysis solution (100 mM EDTA [pH 8.0], 50 mM NaCl, 10 mM Tris-HCl [pH 7.2], 0.2% [wt/vol] Na-deoxycholate, 0.5% [wt/vol] *N*-lauroylsarcosine, 1 mg of lysozyme per mL of lysis solution), and the mixture was incubated for 2 hours at 37°C with shaking. This solution was replaced with 10 mL of proteinase K solution (100 mM EDTA [pH 8.0], 0.2% [wt/vol] Na-deoxycholate, 1% [wt/vol] *N*-lauroylsarcosine, 1 mg proteinase K per mL of proteinase K solution), mixed well, and incubated for 14 hours at 42°C with gentle shaking. The plugs were washed at least five times with TE buffer (50 mM EDTA, 20 mM Tris-HCl [pH 8.0]) and were stored at 4°C.

A 5-mm slice of a DNA plug was incubated with 16 U of *Xba*I or *Not*I for *Enterobacter* sp. and *Citrobacter* sp. or 20 U of *Spe*I for *P. aeruginosa* in a reaction volume of 0.2 mL at 37°C. All enzymes were from New England Biolabs (Beverly, Mass.). The digested samples were equilibrated in 0.5X TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA) before being melted and loaded onto 1% agarose gels (FastLane agarose; FMC, Rockland, Maine) in 0.5X TBE buffer. Electrophoresis was performed with a contour-clamped homogeneous electric field apparatus (CHEF mapper; Bio-Rad) at 14°C. Initial and final switch times were 5 and 50 seconds, respectively, following *Xba*I digestion, 3 and 15 seconds, respectively, following *Not*I digestion, and 5 and 45 seconds, respectively, following *Spe*I digestion. Parameters also included a linear ramping factor and a run time of 20 hours at 6.0 volts/cm. Bacteriophage lambda concatemers (Mid-range; New England Biolabs) were run as molecular weight standards. Gels were visualized with UV light after staining with ethidium bromide (10 mg/mL). Isolates were differentiated by visual inspection of PFGE

patterns on the agarose gels. They were considered clonally similar if their PFGE patterns had 3 or less different DNA bands between isolates and corresponding bands were the same apparent size (Tenover *et al.*, 1995).

4.10 Statistical Calculations

Patient information was entered into a database (FoxPro 2.5) for analysis. The Student's *t*-test compared the means of two groups and analysis of variance (ANOVA) compared the means of three or more groups for continuous variables analysis (InStat GraphPad Software, version 2.01, San Diego, CA). For categorical data, either a two-tailed Fisher's exact statistic (two groups) or a two-tailed χ^2 test (multiple groups) (InStat) was employed. A χ^2 statistic was stated where this test was performed.

5.0 RESULTS

Between September 1993 and November 1995, 321 patients were included for investigation of third-generation cephalosporin-resistant GNB in the intestinal microflora.

5.1 Characteristics of Patient Population

5.1.1 Age and sex

The mean ages of the study population are shown in Table 1. Analysis of variance indicated no difference in age between all groups (p=0.9885). The proportion of males and females differed significantly among the groups (χ^2=9.1366, p=0.0275) although the ratio (2.1:1) was the same in the CTX-treated and CRO-treated groups (Table 1).

5.1.2 Underlying disease

Patients received treatment with CTX, CRO or cefazolin mostly for pneumonia (n=48), sepsis and bacteremia (n=34), mixed cellulitis (n=29) with Gram-positive or anaerobic organisms and GNB, surgical wound infections (n=23), central nervous system infections (n=18) including meningitis, abdominal and pelvic infections (n=18), abscesses (n=16) including intra-abdominal abscesses, peritonitis (n=10), urinary tract infections (n=8), fever of unknown origin (n=8), bone and joint infections (n=5) and as part of empiric therapy where no indication was documented (n=11).

Table 1. Characteristics of untreated patients and patients who were treated with cefotaxime, ceftriaxone, and cefazolin.

	Untreated	Cefazolin	Cefotaxime (CTX)	Ceftriaxone (CRO)	p-Value
I. Total Patients	93	84	81	63	
A. Mean age ± standard deviation (years)	62.7 ± 15.9	56.9 ± 19.6	59.5 ± 19.3	52.7 ± 21.8	0.9855[1]
B. No. of Males/Females (%)	45/48 (48/52)	50/34 (60/40)	55/26 (68/32)	43/20 (68/32)	0.0275[2]
C. Mean no. underlying diseases ± SD	1.7 ± 0.9	1.4 ± 0.7	1.7 ± 0.9	1.6 ± 0.9	0.0828[1]

[1]ANOVA

[2]χ^2=9.1366

The mean number of underlying diseases per patient (Table 1) was similar among all groups (p=0.0828, ANOVA). When the types of underlying disease that occurred in CTX (n=81) and CRO patients (n=63) were compared (Table 2) no significant differences between the two groups were found (p>0.05, Fisher's exact test).

The most common underlying disease categories in patients treated with the third-generation cephalosporins (N=144), were respiratory (n=34/144; 24%) and cardiovascular (n=32/144; 22%) illnesses (Table 2). These included chronic obstructive pulmonary disease (COPD n=23/34), pneumonia (n=4/34), asthma (n=2/34), silicosis, bronchitis, recurrent otitis media, pleural effusions and pulmonary hypertension, congestive heart failure (n=9/32), coronary arterial disease (n=7/32), chronic atrial fibrillation (n=3/32), ischemic heart disease (n=2/32), peripheral vascular disease (n=2/32), cerebral vascular accident (n=2/32), pulmonary heart disease (cor pulmonale) (n=2/32), cardiomegaly, severe biventricular failure, cardiomyopathy, hypertension, and hypotension.

Alcoholics comprised 16% (n=23/144) of the patients who received third-generation cephalosporins. Patients with underlying endocrinologic disease (n=23/144; 16%) were mostly type II diabetics (non-insulin dependent diabetes mellitus) (n=15/23), while 4 diabetics were insulin-dependent, and 4 others had thyroid disorders. Patients with gastrointestinal disease (n=22/144; 15%) had liver disease (n=7/22), including cirrhosis (n=3/22) and hepatitis secondary to alcoholism (n=1/22), peptic ulcer disease (n=3/22), noninfectious gastroenteritis or colitis (n=3/22), appendicitis (n=2/22), gastrointestinal hypomobility, benign neoplasm of the colon, bowel dysfunctions and pancreatic pseudocyst.

Table 2. Comparison of underlying diseases in patients receiving cefotaxime and patients receiving ceftriaxone.

	Cefotaxime (CTX)	Ceftriaxone (CRO)	p-Value
Total patients	81	63	
Underlying disease (%)			
Alcohol	13 (16.0)	10 (15.9)	1.0000
Cardiovascular	21 (25.9)	11 (17.5)	0.3124
Endocrinologic	14 (17.3)	9 (14.3)	0.6549
Gastrointestinal	13 (16.0)	9 (14.3)	0.8192
Genitourinary	11 (13.6)	6 (9.5)	0.6043
Hematologic	4 (4.9)	2 (3.2)	0.6959
Malignancy	13 (16.0)	7 (11.1)	0.4712
Musculoskeletal	6 (7.4)	7 (11.1)	0.5606
Neurologic	5 (6.2)	4 (6.3)	1.0000
Other	5[2] (6.2)	10[3] (15.9)	0.0964
Respiratory	18 (22.2)	16 (25.4)	0.6955
Skin & Soft Tissue	4 (4.9)	3 (4.8)	1.0000
Trauma	2 (2.5)	4 (6.3)	0.4044

[1]Fisher's exact statistic
[2]Head & Neck n=2; Immunologic n=1; Metabolic n=2
[3]Head & Neck n=4; Immunologic n=4; Metabolic n=1; Mixed Connective Tissue n=1

Malignancies (n=20/144; 14%) included lymphomas (n=4/20), renal cell cancer brain tumor and cancers of the pancreas (n=2/20), breast (n=2/20), prostate, (n=2/20), squamous cell cancer (n=2/20), adenocarcinoma, pituitary tumor and cervix, bowel, liver, and larynx.

5.1.3 Prior antibiotic therapy

Previous courses of antibiotic therapy is summarized in Table 3. Results from the 6-month period did not differ between CTX and CRO patients (p=0.6430; Fisher's exact test).

The number of CTX-treated patients who received antibiotics in the 2-week period before study enrollment was not significantly different from the number of CRO-treated patients with previous therapy (p=0.1949). Thirty patients (37.0%) received a mean of 2.2 different antibiotics prior to treatment with CTX. For patients treated with CRO, 31 (49.2%) received a mean of 2.0 different antibiotics previously. Metronidazole was the most frequently prescribed antibiotic in the two weeks prior to treatment with either CTX (n=12) or CRO (n=12). Days of treatment with prior antibiotics did not differ significantly between CTX and CRO patients for any of the antibiotic classes (Table 3).

5.1.4 Concomitant therapy

At the time of stool collection, 64 (79%) CTX patients and 47 (74.6%) CRO patients were concurrently receiving other antibiotics (p=0.6711; Fisher's exact test) (Table 4). Antianaerobic agents were prescribed the most alongside

Table 3. Comparison of prior antibiotic use in patients receiving cefotaxime and patients receiving ceftriaxone.

	Cefotaxime (CTX)	Ceftriaxone (CRO)	p-Value[1]
Total patients	81	63	
No. of patients with prior antibiotics (%)			
≤6 months prior to therapy	42 (51.8)	36 (57.1)	0.6430[2]
≤2 weeks prior to therapy	30 (37.0)	31 (49.2)	0.1949[2]
Mean no. prior antibiotics/patient[3]	2.2 ± 1.5	2.0 ± 1.3	0.5769
Prior antibiotic days/patient[3] (Mean)			
ß-lactam/Cephalosporin	91/23 (4.0)	57/25 (2.3)	0.0838
Antianaerobe	47/12 (3.9)	36/14 (2.6)	0.3445
Aminoglycoside	29/8 (3.6)	5/2 (2.5)	0.6442
Macrolide/Azalide	9/3 (3.0)	16/5 (3.2)	0.9000
Other antibiotics	6/3 (2.3)[4]	13/3 (4.3)[5]	0.2791

[1]Student's *t*-test statistic unless otherwise stated

[2]Fisher's exact statistic

[3]Antibiotic use ≤2 weeks prior to study therapy with CTX or CRO

[4]Trimethoprim-Sulfamethoxazole (n=2); Ciprofloxacin (n=1)

[5]Trimethoprim-Sulfamethoxazole (n=2); Doxycycline (n=1)

Table 4. Comparison of concomitant antibiotic use in patients receiving cefotaxime and patients receiving ceftriaxone.

	Cefotaxime (CTX)	Ceftriaxone (CRO)	p-Value[1]
Total patients	81	63	
No. patients with concomitant antibiotics (%)	64 (79.0)	47 (74.6)	0.6711[2]
Mean no. concomitant antibiotics/patients	1.4 ± 0.6	1.7 ± 1.2	0.0866
Concomitant antibiotic days/patient (Mean)			
Antianaerobe	243/46 (5.3)	175/33 (5.3)	0.4558
ß-lactam/Cephalosporin	70/18 (3.9)	32/12 (2.7)	0.6197
Aminoglycoside	32/8 (4.0)	20/2 (10.0)	0.1443
Macrolide	16/7 (2.3)	20/7 (2.9)	0.5795
Other antibiotics	18/5 (3.6)[3]	33/10 (3.3)[4]	0.8443

[1]Student's *t*-test unless otherwise stated

[2]Fisher's exact statistic

[3]Vancomycin (n=4); Doxycycline (n=1).

[4]Doxycycline (n=5); Vancomycin (n=4); Trimethoprim-Sulfamethoxazole (n=1).

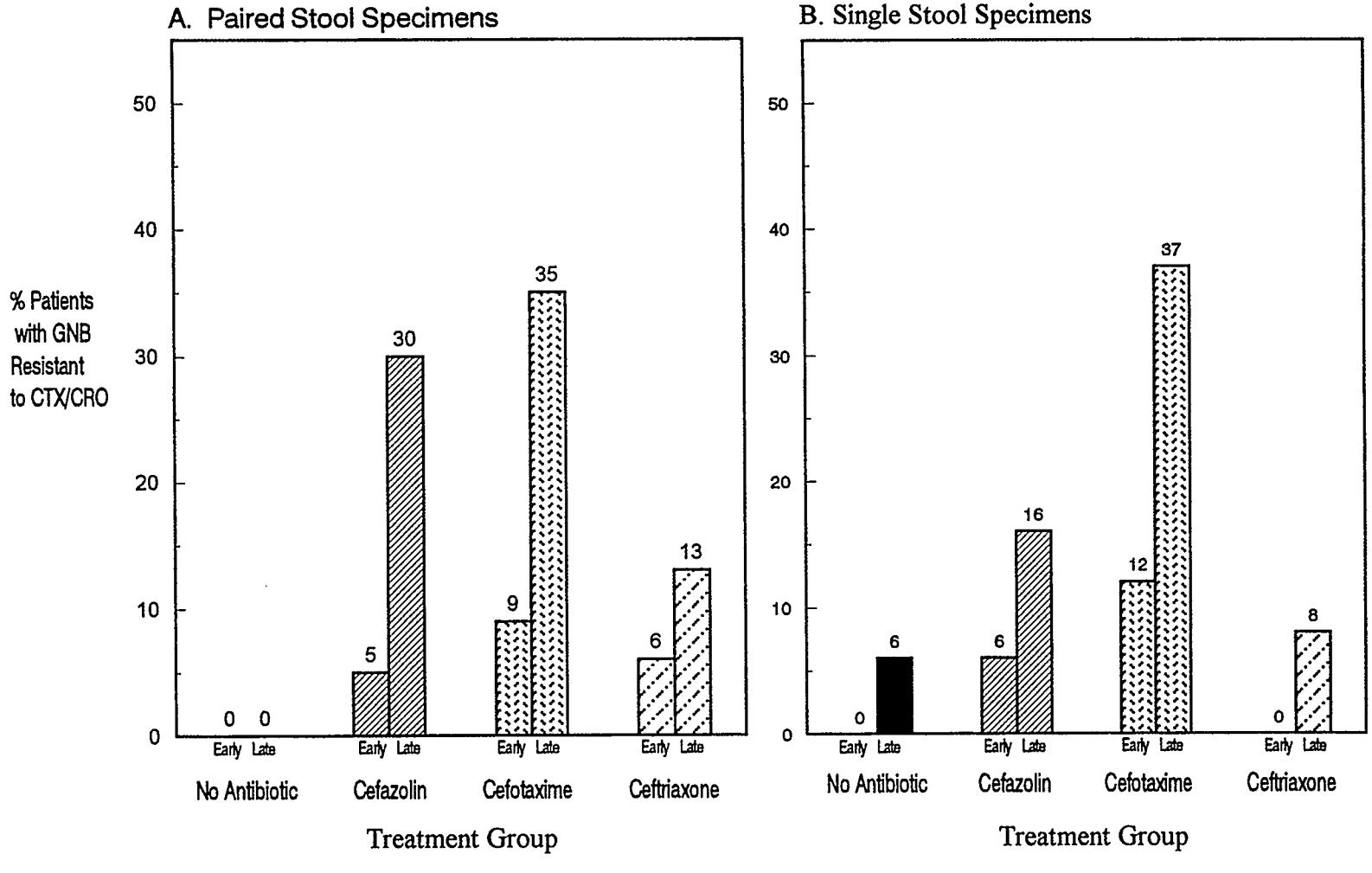
cephalosporin therapy. Metronidazole or clindamycin were administered to 46 patients (71.9%) treated with CTX and 33 patients (68.8%) treated with CRO. The number of concomitant antibiotic treatment-days did not differ between patients treated with CTX and patients treated with CRO ($p>0.05$; Student's *t*-test) for any of the antibiotic classes, which included antianaerobic, ß-lactam, aminoglycoside and macrolide agents, vancomycin, doxycycline and trimethoprim-sulfamethoxazole.

5.2. Proportion of Patients With Third-Generation Cephalosporin-Resistant Gram-Negative Bacilli

Ninety-six patients provided both initial and subsequent fecal specimens and was comprised of 21 untreated controls, 20 cefazolin treatment controls, 23 patients treated with CTX, and 32 patients treated with CRO. Two hundred and twenty-five patients provided a single specimen either earlier (day 1 to 4) or later during treatment (day 5+). This comprised, respectively, of 38 and 35 untreated controls, 33 and 29 cefazolin treatment controls, 29 and 26 patients treated with CTX, and 19 and 12 patients treated with CRO.

Third-generation cephalosporin resistance among fecal GNB was representative of growth on Mac_{16} CTX or Mac_{16} CRO. Similar trends of resistance were seen between patients from whom consecutive stools had been obtained (Figure 1A) and those from whom only one specimen was obtained

Figure 1. Comparison of the proportion of patients colonized with third-generation cephalosporin-resistant Gram-negative bacilli (GNB) during an early (Day 1-4) and a late (Day 5 to end of treatment) surveillance period. A: Patients from whom consecutive stool specimens were collected. B: Patients from whom only one stool specimen was collected. Stools were screened on MacConkey solid media containing 16 μg/ml cefotaxime (CTX) or ceftriaxone (CRO).


A. Paired Stool Specimens
B. Single Stool Specimens
% Patients with GNB Resistant to CTX/CRO
50
40
30
20
10
0
0 0
5
30
9
35
6
13
0
6
6
16
12
37
0
8
Early Late
No Antibiotic
Cefazolin
Cefotaxime
Ceftriaxone
Treatment Group

(Figure 1B). The similar proportion of resistant GNB recovered from paired and unpaired stool specimens suggested that these groups can be combined for analysis (Figure 2). Table 5 shows that the proportion of combined patients colonized with resistant GNB are significantly different among the untreated patients (2/93 [2.1%]) and patients receiving cefazolin (14/84 [16.7%]), CTX (20/81 [24.7%]), and CRO (6/63 [9.5%]) ($p<0.0001$; $\chi^2=22.69$).

In the first 48 hours after start of treatment, the proportion of resistant GNB in all treatment groups were similar to each other and to the baseline seen in patients not receiving any antibiotics ($p>0.05$, Fisher's exact test) (Figure 2; Table 5). By days 3 to 4, a marked increase in the proportion of patients colonized with cephalosporin-resistant GNB was seen among patients treated with CTX (6/19 [31.6%]) compared to untreated controls (0/21; $p=0.0071$). Resistant GNB occurred in 7.5 times more CTX-patients than cefazolin patients (1/24 [4.2%]; $p=0.0325$) and in 3.8 times more CTX-patients than CRO-patients - (1/12 [8.3%]; $p=0.2117$). The proportion of patients with resistant fecal GNB was not significantly different between patients treated with CRO and the other two groups, cefazolin ($p=1.0000$) or untreated patients ($p=0.3636$).

During the later part of the surveillance period, CTX patients had received a mean of 7.4 days of treatment prior to stool collection and CRO patients had received a mean of 7.6 days of therapy (Table 5). Neither CTX nor CRO patients received cephalosporin treatment for more than 17 days (Table 5). In this period, 17 of 52 patients (32.7%) receiving CTX harboured cephalosporin-

Figure 2. Proportion of the total number of patients colonized with third-generation cephalosporin- resistant Gram-negative bacilli (GNB) in patients receiving no antibiotics and in patients receiving cefazolin, cefotaxime (CTX) or ceftriaxone (CRO). Resistant GNB were selected from patient stool specimens on MacConkey solid media containing 16 μg/ml CTX or CRO.

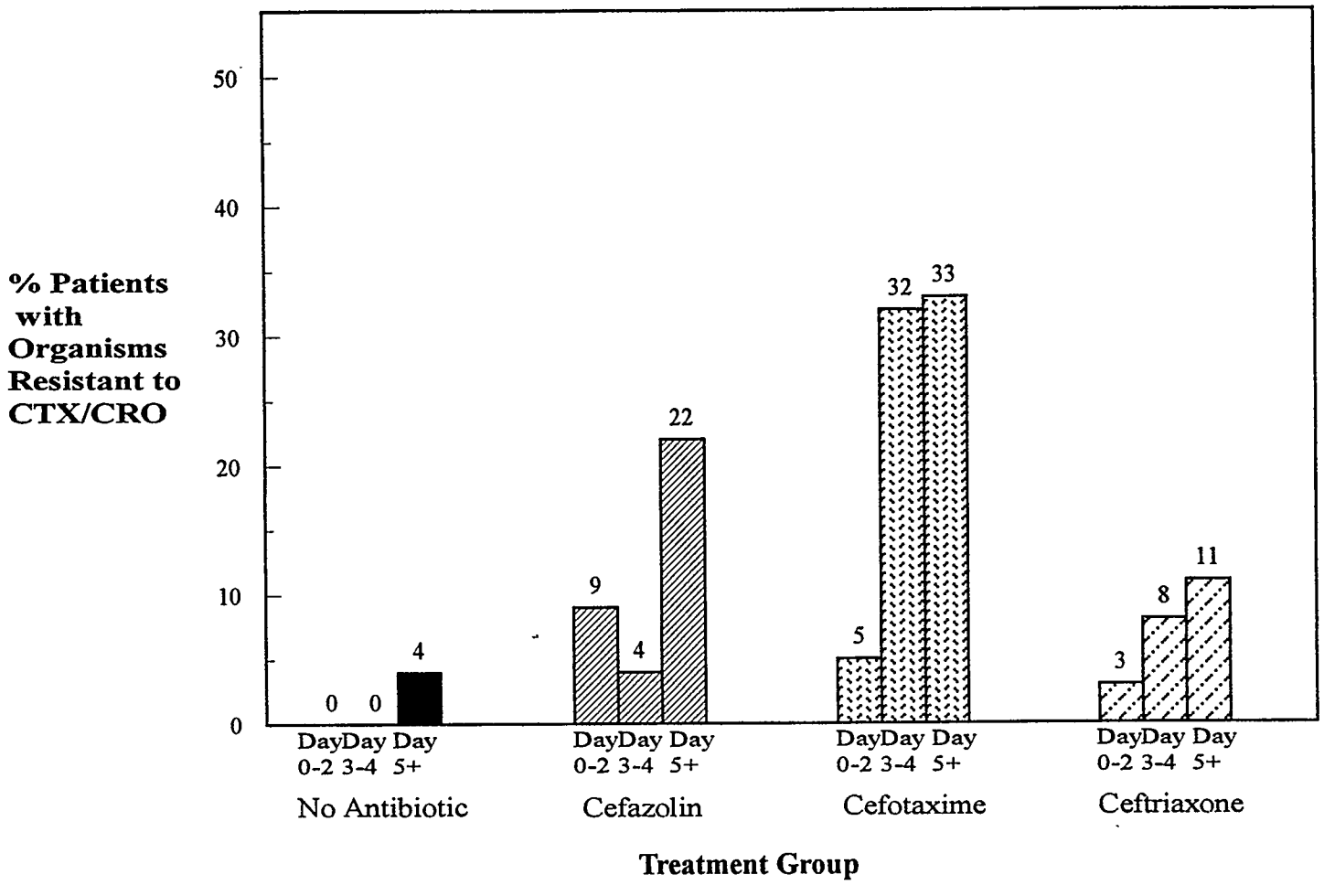

% Patients with Organisms Resistant to CTX/CRO
50
40
30
20
10
0
0
0
4
9
4
22
5
32
33
3
8
11
DayDay Day
0-2 3-4 5+
No Antibiotic
Cefazolin
Cefotaxime
Ceftriaxone
Treatment Group

Table 5. **Proportion of patients from which third-generation cephalosporin-resistant gram-negative bacilli were selected in stool specimens. Patient groups include untreated patients and patients receiving cefazolin, cefotaxime or ceftriaxone.**

	Treatment Groups			
	Untreated	Cefazolin	Cefotaxime (CTX)	Ceftriaxone (CRO)
A. Total No. Patients	93	84	81	63
No. Patients with resistant GNB (%)[1]	2 (2.1)	14 (16.7)	20 (24.7)	6 (9.5)
B. Resistant/Total Patients (%)				
Day 1 to 2	0/38 (0)	3/32 (9.4)	2/37 (5.4)	1/40 (2.5)
Day 3 to 4[2]	0/21 (0)	1/24 (4.2)	6/19 (31.6)	1/12 (8.3)
Day 5 to end of treatment[3]	2/56 (3.6)	11/51 (21.6)	17/52 (32.7)	5/44 (11.4)
C. Mean no. treatment days				
after 5 days	8.9 $\pm$4.2	8.9 $\pm$ 6.0	7.4 $\pm$ 2.6	7.6 $\pm$ 4.3
Range of treatment days	5 - 21	5 - 39	5 - 15	5 - 17

[1]$p<0.0001$; $\chi^2 = 22.69$

[2]Untreated vs. CTX p=0.0071; cefazolin vs. CTX p=0.0325 (Fisher's exact)

[3]Untreated vs. CTX p<0.0001; untreated vs. cefazolin p=0.0062; CTX vs. CRO p=0.0155

resistant GNB in stool specimens. Although this was not a significant increase from the resistance observed after 3 to 4 days of treatment with CTX, it was higher than what was observed among patients treated with CRO (p=0.0155). The relative risk for the selection of resistant GNB in gut microflora was 2.9 times higher in patients receiving CTX compared to CRO as cephalosporin-resistance was selected in only 5 of 44 CRO-treated patients (11.4%).

Untreated patients were hospitalized for a mean of 8.9 days up to a maximum of 21 days. This group of patients were at 9.2 times lower risk of colonization with resistant GNB than CTX-treated patients (2/56 [3.6%] vs. 17/52 [32.7%]; p<0.0001) whereas patients treated with cefazolin had similarly high resistance selection (11/51 [21.6%]; p=0.2689) and were only 1.5 times less likely to be colonized with resistant GNB as compared to patients treated with CTX. Cefazolin patients had treatment averaging 8.9 cefazolin-days including one patient who received cefazolin for 39 days. In the cefazolin group, selection of resistant GNB occurred 6.0 times more often than in the untreated group (p=0.0062) and 1.9 times more often than in patients treated with CRO although this was not significantly different (p=0.2721). Cephalosporin-resistance was found in 3.2 times more patients treated with CRO than with untreated patients (p=0.2353).

5.2.1 Proportion of Patients With "Intermediately-Resistant" Gram-Negative Bacilli

Fecal GNB isolated on Mac_2 CTX or Mac_2 CRO which did not also grow on Mac_{16} plates represent "intermediately-resistant" GNB. The proportion of

patients in each case and control group who were colonized with intermediately-resistant GNB did not differ significantly from each other during days 1 to 4 (χ^2=1.915; p=0.5902) or days 5+ (χ^2=5.1677; p=0.1599) of the surveillance period.

Organisms isolated from this lower selective screen were: *P. aeruginosa* (from n=15 patients), *E. cloacae* (n=4), *E. coli* (n=4), *A. calcoaceticus* (n=3), *Hafnia alvei* (n=2), and one each of *C. freundii, Enterobacter aerogenes* and *Klebsiella oxytoca.*

5.3 Types of Third-Generation Cephalosporin-Resistant Gram-Negative Bacilli Isolated From Stool Specimens

E. cloacae (n=16) and *C. freundii* (n=12) were the most frequently recovered resistant organisms from Mac_{16} screen media and were mostly selected from the stool flora of patients treated with CTX (Table 6). *Enterobacter* sp. included four *E. aerogenes* and one *Enterobacter sakazakii.* Five strains of *P. aeruginosa* and four *H. alvei,* were also recovered. Three *Morganella morganii* and one each of *Stenotrophomonas maltophilia, A. calcoaceticus,* and *Serratia liquefaciens* were each recovered from different patients.

The number of third-generation cephalosporin-resistant GNB recovered ranged from 1^+ to 4^+ growth on 16 μg/mL CTX/CRO media with 24/48 (50%) showing only 1^+ growth. Seven of forty-eight (14.6%) occurred at 2^+ growth, 10/48 (20.8%) occurred at 3^+ growth and 7/48 (14.6%) occurred at 4^+ growth.

Table 6. Number of third-generation cephalosporin-resistant Gram-negative bacilli recovered from the stool specimens of untreated patients and patients receiving treatment with cefazolin, cefotaxime (CTX) or ceftriaxone (CRO). Stool specimens were screened on MacConkey solid media containing 16 μg/ml CTX or CRO.

Organism	Untreated	Cefazolin	CTX	CRO	Total
Enterobacter cloacae	0	5	10	1	16
Citrobacter freundii	1	3	8	0	12
Enterobacter species[1]	0	2	2	1	5
Pseudomonas aeruginosa	0	0	4	1	5
Hafnia alvei	0	2	1	1	4
Morganella morganii	1	2	0	0	3
Stenotrophomonas maltophilia	0	0	0	1	1
Acinetobacter calcoaceticus	0	1	0	0	1
Serratia liquefaciens	0	0	0	1	1

[1]*E. aerogenes* (n=4); *E. sakazakii* (n=1)

5.4 In Vitro Antibiotic Susceptibilities of Gram-Negative Bacilli Selected on Screen Plates

5.4.1 Correlation of resistance screen with MICs

MICs correlated well with screening results - 45/48 (93.8%) of those selected on Mac_{16} had MICs $\geq$ 16 µg/mL for CTX and CRO. Of the 3 isolates that were susceptible to CTX and CRO, 1 was selected from fecal flora of patients treated with cefazolin (*E. cloacae*) and 2 were selected from fecal flora of patients treated with CTX (*E. aerogenes* and *E. cloacae*). The MICs for CTX and CRO of the 3 susceptible GNB were within one dilution of each other. In *E. cloacae* isolates, MICs ranged from $\leq$0.25 to 256 µg/mL where 2 of sixteen (13%) stool isolates were sensitive to CTX and CRO. All *C. freundii* isolates were resistant to CTX and CRO, having MICs $\geq$32 µg/mL. The MIC_{50} and MIC_{90} of other *Enterobacter* species, *P. aeruginosa, H. alvei,* and other GNB were $\geq$16 and $\geq$128 µg/mL, respectively, with MICs ranging from $\leq$0.25 to >512 µg/mL. One strain of *E. aerogenes* had an MIC of $\leq$0.25 for CTX and CRO.

Among all treatment groups, resistance rates were recalculated to exclude patients (n=3) colonized with organisms having low CTX and CRO MICs (<16 µg/mL). The recalculated rates reflected the same trend depicted by Figure 2 and Table 5.

5.4.2 In vitro susceptibilities of third-generation cephalosporin-resistant GNB to other ß-lactams, aminoglycosides, and fluoroquinolones

The MIC_{50} and MIC_{90} for other ß-lactams demonstrated broad cross-resistance to cefazolin, ceftazidime, piperacillin, piperacillin/tazobactam, ticarcillin and ticarcillin/clavulanate but most strains remained susceptible to imipenem (Table 7, Table 8). The exception was a strain of *S. maltophilia*, which had an MIC of >512 µg/mL for imipenem. All strains of *Enterobacter* species and *Citrobacter* tested had MIC_{90} of $\geq$64 µg/mL to cephalosporins and anti-pseudomonal penicillins. Cross-resistance was also observed in *P. aeruginosa*, *H. alvei*, and other GNB. The ß-lactamase inhibitors, tazobactam and clavulanate did not increase the activity of their respective ß-lactam counterparts as MICs remained the same or were decreased by only one dilution when these inhibitors were added to piperacillin or ticarcillin.

A. calcoaceticus and *S. maltophilia* were the only strains resistant to gentamicin. *S. maltophilia* was also resistant to tobramycin but *A. calcoaceticus* remained susceptible (MIC=8 µg/mL). Ciprofloxacin had limited activity against these two species (MIC 2 µg/mL) but ofloxacin and clinafloxacin remained active against them. *S. maltophilia* was susceptible to 0.125 µg/mL of clinafloxacin while *A. calcoaceticus* was susceptible to 0.25 µg/mL. Both were inhibited at an MIC of 1 µg/mL of ofloxacin.

Table 7. **Minimum inhibitory concentrations (μg/ml) of various antibiotics for *Enterobacter* sp. and *Citrobacter freundii* recovered from the stool specimens of untreated patients and patients treated with cefazolin, cefotaxime (CTX) or ceftriaxone (CRO). Stool specimens were screened on MacConkey solid media containing 16 μg/ml CTX or CRO.**

Drug	*E. cloacae* n=16			*C. freundii* n=12			*Enterobacter*[1] spp. n=5		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Cefotaxime	64	256	≤0.25-256	32	256	32-256	16	64	≤0.25-64
Ceftriaxone	64	256	≤0.25-256	64	256	16-512	16	64	≤0.25-64
Ceftazidime	32	64	≤0.25-128	64	256	1-256	16	64	≤0.25-64
Cefazolin	>512	>512	>512	>512	>512	>512	>512	>512	>512
Piperacillin	64	128	4-256	128	512	32-512	32	64	8-64
Pip/Tazo[2]	16	64	1-128	32	256	8-512	16	32	2-32
Ticarcillin	256	256	4->512	512	512	128->512	128	256	64-256
Ticar/Clav[3]	256	256	4-512	256	512	128-512	128	256	4-256
Imipenem	0.25	0.25	0.125-0.5	0.25	0.5	0.125-1	0.25	0.25	0.125-0.5
Clinafloxacin	≤0.015	≤0.015	≤0.015-0.06	≤0.015	0.03	≤0.015-0.125	≤0.015	0.06	≤0.015-0.06
Ofloxacin	0.125	0.25	0.06-0.25	0.125	0.5	0.06-2	0.125	0.25	0.06-0.25
Ciprofloxacin	0.03	0.06	≤0.015-.125	0.03	0.06	≤0.015-0.06	0.06	0.06	≤0.015-0.06
Tobramycin	0.25	0.5	≤0.06-0.5	0.25	1	≤0.06-2	0.5	1	0.25-1
Gentamicin	0.5	0.5	0.25-0.5	0.25	1	0.25-2	0.5	1	0.25-1

[1]*E. aerogenes* (n=4); *E. sakazakii* (n=1)
[2]Piperacillin/Tazobactam
[3]Ticarcillin/Clavulanate

Table 8. **Minimum inhibitory concentrations (μg/ml) of various antibiotics for *Pseudomonas aeruginosa*, *Hafnia alvei* and other Gram-negative bacilli[1] recovered from the stool specimens of untreated patients and patients treated with cefazolin, cefotaxime (CTX) or ceftriaxone (CRO). Stool specimens were screened on MacConkey solid media containing 16 μg/ml CTX or CRO.**

Drug	*P. aeruginosa* n=5			*H. alvei* n=4			Other GNB[1] n=6		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Cefotaxime	256	>512	8->512	32	512	16-512	16	64	16-64
Ceftriaxone	512	>512	8->512	128	512	32-512	16	128	1-128
Ceftazidime	8	32	1-32	256	512	256-512	16	64	8-128
Cefazolin	>512	>512	>512	>512	>512	>512	>512	>512	256->512
Piperacillin	32	128	4-128	64	512	64-512	16	128	8-128
Pip/Tazo[2]	16	128	4-128	64	512	64-256	16	128	≤0.25-128
Ticarcillin	256	256	4-256	64	512	64-512	32	256	4-512
Ticar/Clav[3]	256	256	4-256	64	512	64-512	32	256	2-512
Imipenem	2	4	2-4	0.25	2	0.125-2	0.5	2	0.25->512
Clinafloxacin	0.125	0.5	0.03-0.5	≤0.015	≤0.015	≤0.015-0.015	0.03	0.25	≤0.015-0.25
Ofloxacin	0.5	4	0.5-4	0.125	0.125	0.03-0.125	0.06	2	0.06-2
Ciprofloxacin	0.25	0.5	0.06-0.5	0.03	0.03	≤0.015-0.06	0.06	2	≤0.015-2
Tobramycin	0.5	0.5	0.5	0.25	0.5	≤0.06-0.25	0.5	8	0.125-32
Gentamicin	2	2	0.5	0.25	2	0.125-0.5	1	32	0.125-32

[1]*Morganella morganii* (n=3); One each *Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Serratia liquefaciens.*
[2]Piperacillin + Tazobactam
[3]Ticarcillin + Clavulanate

5.5 Presence of ß-lactamase Activity

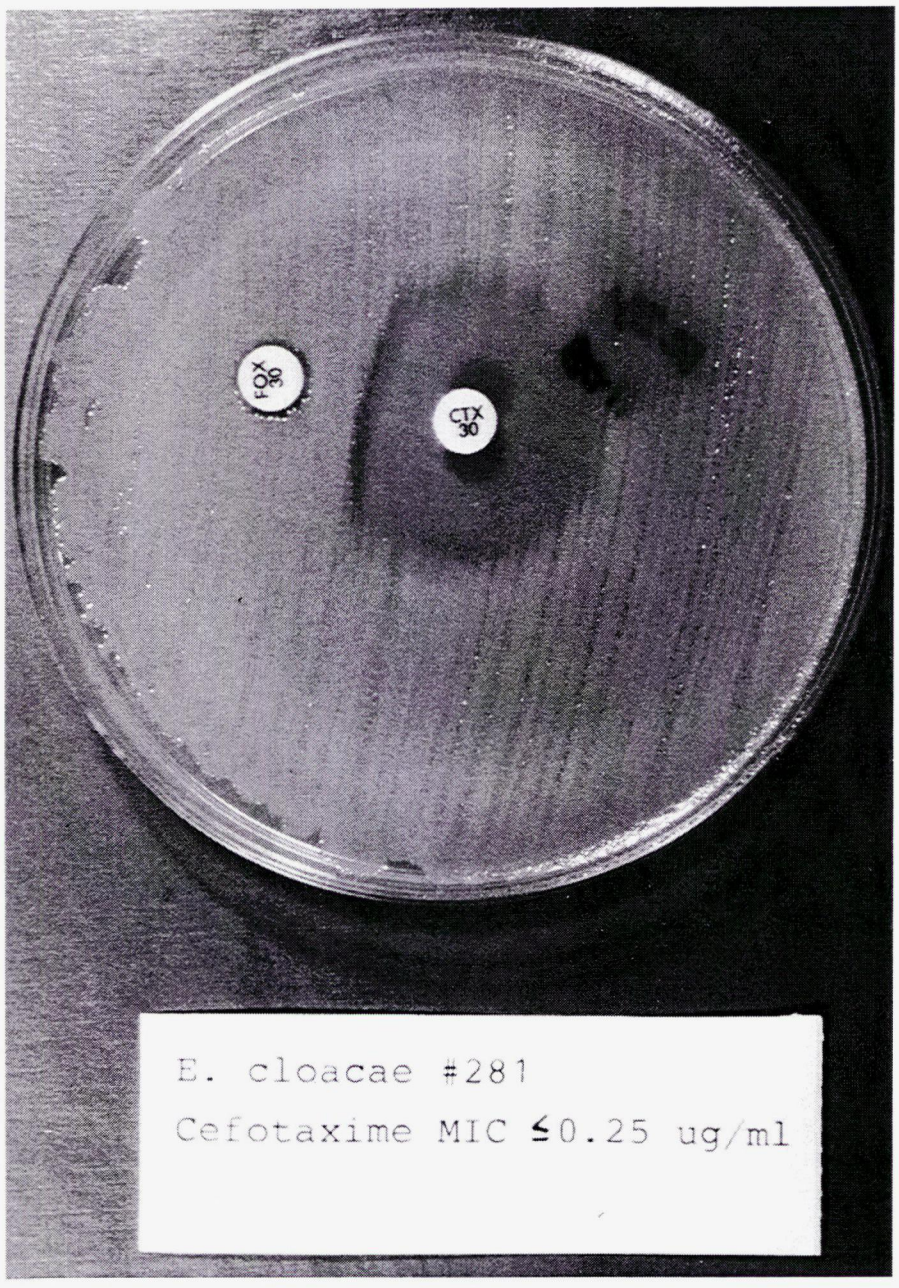
The nitrocefin assay revealed that 46/48 (95.8%) of the isolates were positive for ß-lactamase production. *S. aureus* ATCC 29213 tested positive for ß-lactamase, and *H. influenzae* ATCC 10211 tested negative. Although one *E. cloacae* isolate had an MIC of 0.5 µg/mL for CTX, ß-lactamase was detectable by this assay. CTX-sensitive *E. aerogenes* and an *E. cloacae* isolate, both with MICs of $\leq$0.25 µg/mL for CTX, did not express detectable ß-lactamase activity.

The 3 isolates with CTX MIC <16 µg/mL were further tested for inducible production of ß-lactamase by the disk approximation test. In the presence of cefoxitin, ß-lactamase production was induced and caused a truncated zone of growth around the test antibiotics CTX or CRO on Mueller-Hinton agar (Figure 3). *E. aerogenes* and the two *E. cloacae* isolates were positive for ß-lactamase production in the presence of cefoxitin.

5.6 Determination of Cefotaxime and Ceftriaxone Levels in Patient Stools

CRO and CTX levels in patients' stool specimens were determined using microbiological methods. The specimens analyzed were collected from patients who had received at least 5 days of therapy. The regression line relating zone sizes of growth inhibition to the $\log_{10}$ concentration of reference drugs were linear and corresponded to 0.25 to 32 µg/mL CTX and 0.125 to 32 µg/mL CRO. The mean concentration $\pm$ the standard deviation of CRO in the stool specimens of 7 CRO-treated patients was 316 $\pm$ 358 mg/kg stool and was significantly

Figure 3. The disk approximation test of an *E. cloacae* strain for the presence of inducible ß-lactamase. This isolate had a minimum inhibitory concentration of ≤0.25 μg/ml for cefotaxime (CTX). Cefoxitin (FOX) induces ß-lactamase production resulting in a truncated zone of inhibition around the CTX disk. Growth around the CTX disk appears only in closest proximity to the FOX disk.


CTX
30
E. cloacae #281
Cefotaxime MIC ≤0.25 ug/ml

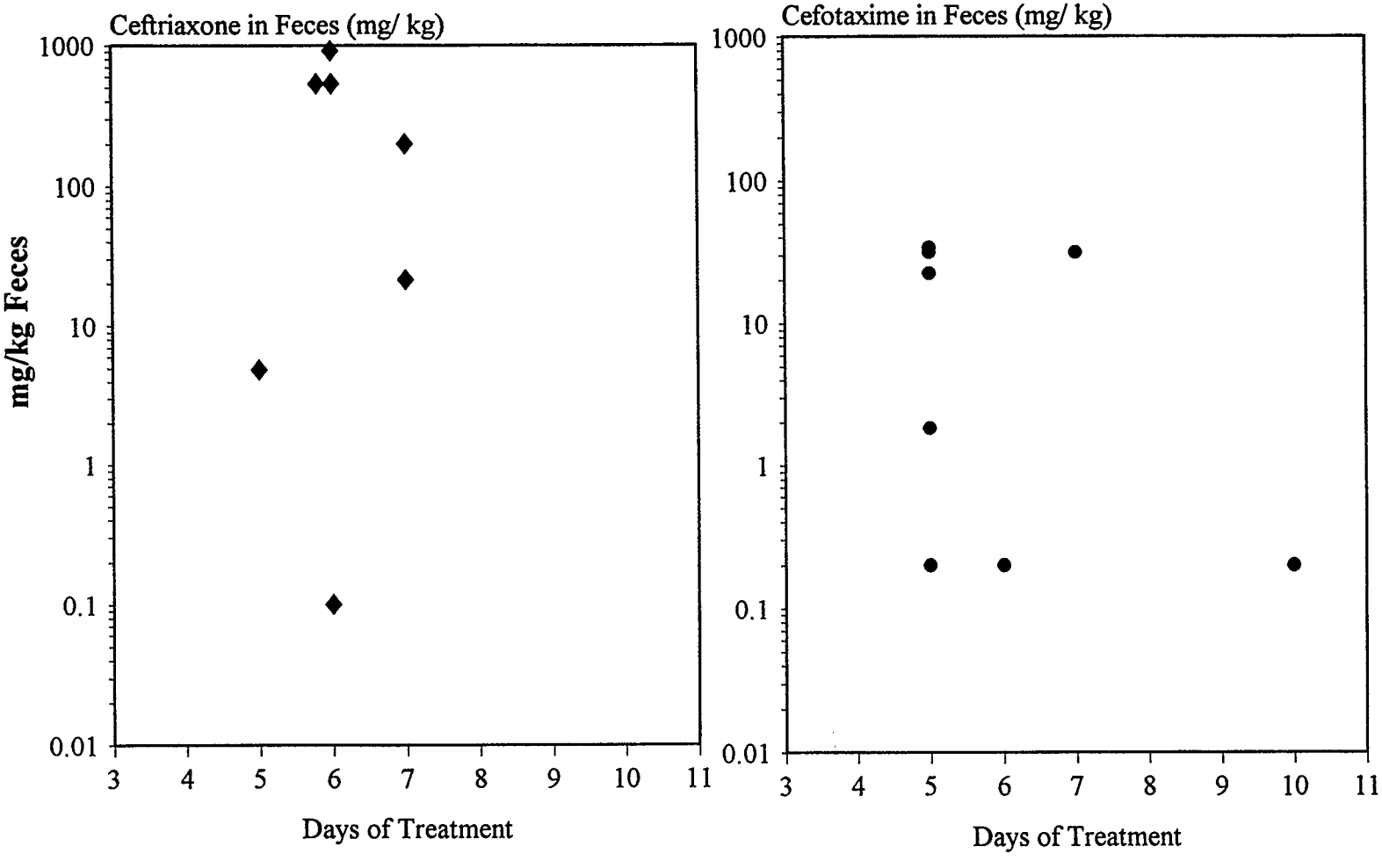
higher than the mean concentration of CTX (21 $\pm$ 24 mg/kg) in the stools of 8 CTX- treated patients (p=0.0328, Student's *t*-test).

CRO levels ranged from undetectable levels to >900 mg/kg of stool. In one patient no CRO was detectable (<0.125 mg/kg) from a stool specimen collected on day 6 of treatment. Another patient had 4 mg CRO/kg in a day 5 stool specimen and 23 mg CRO/kg was detected in a third patient with a day 7 stool specimen. In the fecal samples of four other patients, CRO concentrations were 214, 537, 537 and 919 mg/kg (Figure 4a). Cephalosporin-resistant fecal GNB were not isolated from any of these patients.

CTX levels in stools of patients receiving CTX ranged from undetectable levels to 64 mg/kg. In 3 patients, CTX was not detectable (<0.25 mg/kg) in stool specimens of patients who received treatment for as long as 10 days (Figure 4b). A fourth patient had 1.8 mg CTX/kg of a day 5 stool specimen. Three of four of these patients developed cephalosporin-resistant GNB. Concentrations of antibiotic in CTX patients were not attained to levels seen in the stool specimens of CRO patients. The highest fecal levels of CTX were seen in four patients who had concentrations of 27, 34, 39 and 64 mg CTX/kg of stool. In these latter four patients, cephalosporin-resistant GNB were not recovered.

The stool specimens of untreated controls (n=9) who had been hospitalized for at least 5 days did not produce zones of inhibition of growth of *C. perfringens*. Control distilled water containing no antibiotic also did not produce zones of inhibition.

Figure 4. Concentration of cephalosporin in stool specimens. Patients received at least 5 days of treatment with 1g ceftriaxone (CRO) I.V. q 24 hours or 1g cefotaxime (CTX) I.V. q 8 hours. A: CTX stool levels (mg/kg). B: CRO stool levels (mg/kg). In one patient, no CRO was detectable (<0.125 mg/kg) and in 3 patients, no CTX was detectable (<0.25 mg/kg).


Ceftriaxone in Feces (mg/ kg)
mg/kg Feces
1000
100
10
1
0.1
0.01
3
4
5
6
7
8
9
10
11
Days of Treatment
Cefotaxime in Feces (mg/ kg)
1000
100
10
1
0.1
0.01
3
4
5
6
7
8
9
10
11
Days of Treatment

5.7 Genotypic Analysis of Resistant Gram-Negative Bacilli

In 5.2% (5/96) of patients from whom more than one stool specimen was collected, a second GNB of the same species with a more resistant third-generation cephalosporin profile (two- to eight-fold increase in MIC) was subsequently isolated during therapy (Table 9; Table 10). Four (4.2%) other patients had resistant GNB showing the same subsequent MICs as therapy progressed (Table 11).

Thirty-two isolates were recovered from these nine patients. Two patients (Patient 1 and 2) had isolates that showed an increase in MIC for CTX with longer therapy (Table 9). Restriction fragment length polymorphisms were determined by pulsed-field gel electrophoresis (PFGE) and fragment patterns were distinguished by letter names (A to K). Comparison of the DNA restriction patterns showed that *P. aeruginosa* strains isolated from Patient 1 on days 4, 7 and 15 of CTX therapy were the same genotypically (DNA restriction pattern A).

The genotypic patterns of *E. cloacae* isolated from Patient 2 on day 1 and day 7 of CTX therapy were also identical (DNA restriction pattern B).

Fecal specimens obtained from Patient 3, 4 and 5 on day 2 and day 5 of cefazolin therapy yielded strains that were initially susceptible to CTX but were subsequently resistant (Table 10). In Patients 3 and 4, strains were genotypically identical (DNA restriction pattern C and D, respectively), while Patient 5 was colonized with two distinct clones, the first being susceptible to CTX (DNA restriction pattern E), the second, being resistant (DNA restriction pattern F). Only the second clone was selected from stool at day 5 of treatment.

Table 9. Genotypic pattern, as determined by pulsed-field gel electrophoresis (PFGE) of Gram-negative bacilli showing increasing resistance. Organisms were isolated from stool specimens of two patients receiving cefotaxime (CTX) and had minimum inhibitory concentrations (MIC) of >16 μg/ml for CTX.

	Treatment Group	CTX MIC (μg/mL)	Organism	DNA Restriction Pattern
Patient 1	Day 4 CTX	64	*P. aeruginosa*	A
	Day 7 CTX	64	*P. aeruginosa*	A
	Day 15 CTX	512	*P. aeruginosa*	A
		>512	*P. aeruginosa*	A
Patient 2	Day 1 CTX	32	*E. cloacae*	B
	Day 7 CTX	128	*E. cloacae*	B

Table 10. Genotypic pattern, as determined by pulsed-field gel electrophoresis (PFGE) of Gram-negative bacilli showing development of resistance. Organisms were isolated from stool specimens of three patients receiving cefazolin (CZ) and had minimum inhibitory concentrations (MIC) from ≤0.25 to 512 μg/ml for cefotaxime (CTX).

	Treatment Group	CTX MIC (μg/mL)	Organism	DNA Restriction Pattern
Patient 3	Day 2 CZ	≤0.25	*E. sakazakii*	C
	Day 5 CZ	64	*E. sakazakii*	C
		64	*E. sakazakii*	C
Patient 4	Day 2 CZ	≤0.25	*C. freundii*	D
	Day 5 CZ	32	*C. freundii*	D
		32	*C. freundii*	D
Patient 5	Day 2 CZ	≤0.25	*E. cloacae*	E
		256	*E. cloacae*	F
	Day 5 CZ	256	*E. cloacae*	F
		512	*E. cloacae*	F

Table 11. Genotypic pattern, as determined by pulsed-field gel electrophoresis (PFGE) of Gram-negative bacilli with persisting resistance to cefotaxime (CTX). Isolates were selected for in consecutive stool specimens of 3 patients receiving CTX and one patient receiving ceftriaxone (CRO).

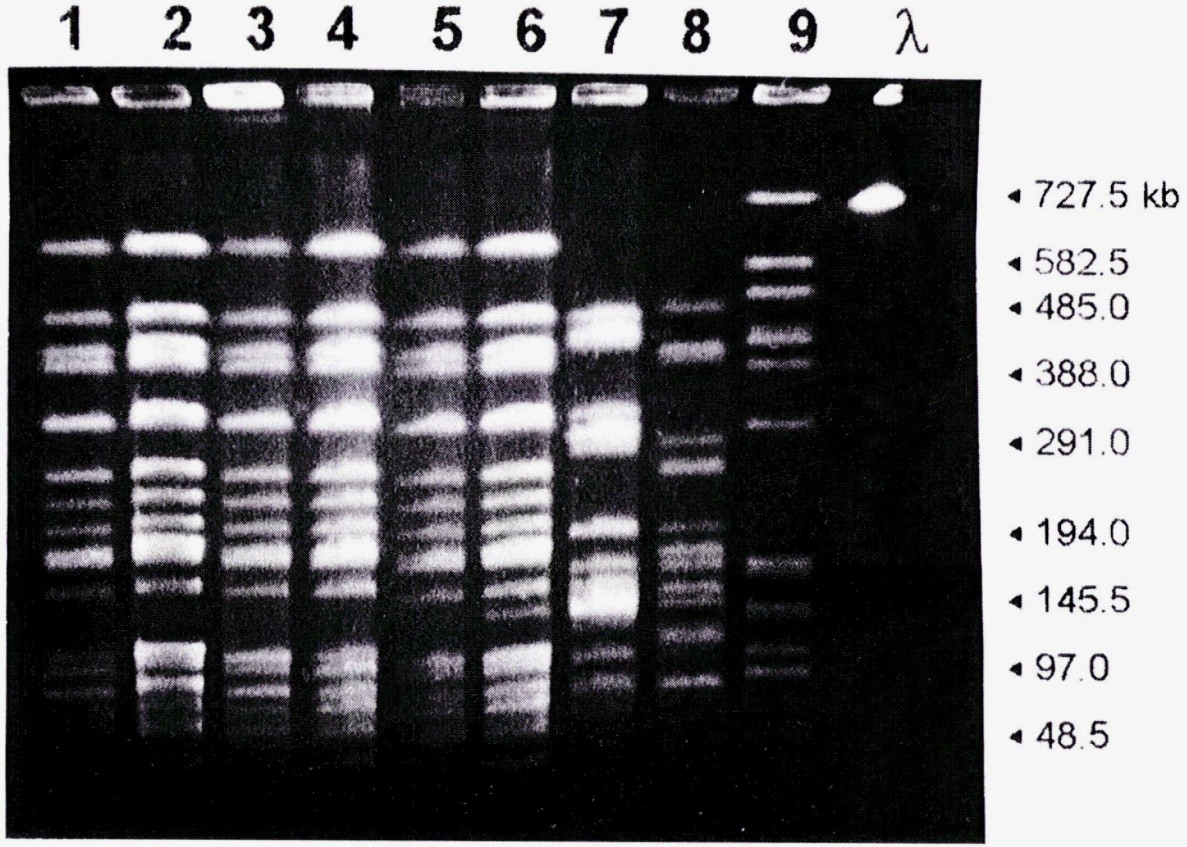
	Treatment Group	CTX MIC (μg/mL)	Organism	DNA Restriction Pattern
Patient 6	Day 1 CTX	64	*E. cloacae*	G
		64	*E. cloacae*	G
	Day 5 CTX	32	*E. cloacae*	G
		32	*E. cloacae*	G
		128	*E. cloacae*	G
		128	*E. cloacae*	G
Patient 7	Day 2 CRO	16	*P. aeruginosa*	H
	Day 6 CRO	16	*P. aeruginosa*	H
Patient 8	Day 3 CTX	64	*C. freundii*	I
	Day 5 CTX	64	*C. freundii*	I
Patient 9	Day 1 CTX	256	*E. cloacae*	J
		512	*E. cloacae*	J
	Day 9 CTX	128	*E. cloacae*	K

Patients 6, 7, 8 and 9 harboured strains that were initially resistant to CTX and showed the same MICs (within one dilution) in subsequent fecal specimens (Table 11). Figure 5 depicts the genotypic patterns of 3 patients as determined by PFGE. Lanes 1 to 6 represent DNA restriction patterns of isolates selected from fecal specimens of Patient 6 after 1 day of treatment with CTX (lanes 1 and 2) and after 5 days of treatment with CTX (lanes 3, 4, 5, 6). Like Patient 7 and Patient 8, the resistant isolates that were selected from the gut flora of Patient 6 after 5 days of treatment with a third-generation cephalosporin, were genotypically identical to isolates that were selected earlier during treatment (days 2, 3 or 1, respectively).

Patient 9 harboured two resistant clones, an *E. cloacae* strain from a day 1 fecal specimen (DNA restriction pattern J [Table 11]; lane 7 [Figure 5]) that was distinct from *E. cloacae* isolated from a day 9 fecal specimen (DNA restriction pattern K [Table 11]; lane 8 [Figure 5]). Although distinct from each other, genotypically, the resistant *E. cloacae* strains from Patient 9 were also genotypically distinct from the resistant *E. cloacae* strain from another patient who was hospitalized during the same time period (lane 9 [Figure 5]).

Sixty isolates of *E. cloacae* were recovered from 20 patients whose stool specimens were screened on Mac_{16} and Mac_{2} plates. Twenty-two different PFGE patterns were detected. Multiple isolates from the same patient all yielded identical DNA banding patterns except in two patients who had two distinct clones in their fecal flora.

Figure 5. Restriction fragment length polymorphism analysis of *Xba*I digested *Enterobacter cloacae* genomic DNA after pulsed field gel electrophoresis (PFGE) and ethidium bromide staining. Lanes 1-6 represent *E. cloacae* isolated from the stool specimen of Patient 6 during day 1 (Lanes 1 & 2) and day 5 (Lanes 3-6) of cefotaxime (CTX) treatment. Lanes 7 & 8 represent *E. cloacae* from Patient 9 during day 1 (Lane 7) and day 9 (Lane 8) of CTX treatment. Lane 9 represents *E. cloacae* isolated from a patient whose stool specimen also yielded *E. cloacae* with a minimum inhibitory concentration of $\geq$ 16 μg/ml for CTX. Lane 10 is a lambda (λ) PFGE marker. Results did not differ with *Not*I digested *E. cloacae* genomic DNA.


1
2
3
4
5
6
7
8
9
λ
727.5 kb
582.5
485.0
388.0
291.0
194.0
145.5
97.0
48.5

5.8 Analysis of Risk Factors Associated With Third-Generation Cephalosporin Resistance

Of 144 patients, 26 (18%) patients receiving CTX or CRO became colonized with third-generation cephalosporin-resistant GNB. In 118 patients (82%), resistant GNB were not selected within the fecal flora. A comparative analysis for risk factors associated with resistance showed there were no significant differences in the frequency of underlying disease between patients harbouring resistant GNB and patients with susceptible GNB ($p>0.05$, Figure 6). Malignancies (6/26, 23%) and respiratory disease (6/26) occurred the most in patients harbouring resistant GNB while cardiovascular (28/118, 24%) and respiratory disease (28/118) predominated among patients with susceptible GNB.

There was no association of previous courses of antibiotic therapy with resistance selection (Table 12) ($p=1.0000$, Fisher's exact test). Among resistant patients with prior antibiotic therapy (n=11), 10 (90.9%) patients had received ß-lactam agents which included cefazolin (n=5), ampicillin (n=4), CTX (n=3), CRO (n=1), cefuroxime (n=1) and cefoxitin (n=1). Six (54.5%) patients received metronidazole and 3 (27.3%) patients received gentamicin. The number of antibiotic-days did not differ between the three drug classes between susceptible and resistant patients ($p>0.05$, Student's *t*-test).

Figure 6. Underlying disease of patients with resistant Gram-negative bacilli (GNB) and susceptible GNB. A comparison between proportion of patients with third-generation cephalosporin-resistant GNB in gut microflora (n=26) and patients harbouring susceptible GNB (n=118). In resistant patients, other underlying diseases were: immunologic n=4, metabolic n=4, mixed connective tissue n=4. In susceptible patients, other underlying diseases were: head & neck n=5, immunologic n=3, metabolic n=2.

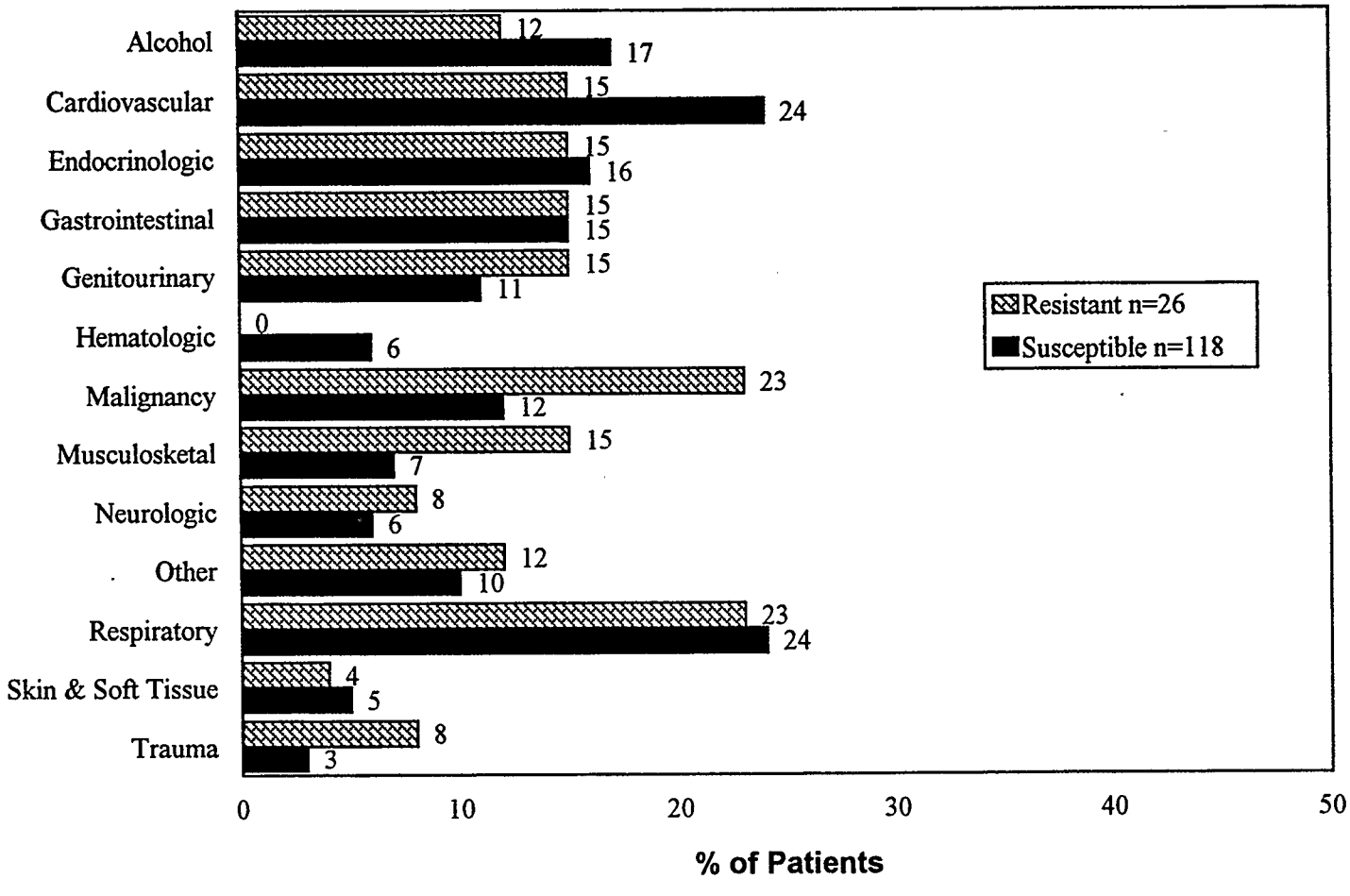

Alcohol
Cardiovascular
Endocrinologic
Gastrointestinal
Genitourinary
Hematologic
Malignancy
Musculosketal
Neurologic
Other
Respiratory
Skin & Soft Tissue
Trauma
12
17
15
24
15
16
15
15
15
11
0
6
23
12
15
7
8
6
12
10
23
24
4
5
8
3
Resistant n=26
Susceptible n=118
0
10
20
30
40
50
% of Patients

Table 12. Comparison of prior antibiotic use among CTX (CTX)- and ceftriaxone (CRO)-treated patients with resistant fecal Gram-negative bacilli (GNB) and CTX- and CRO-treated patients with susceptible GNB.

	Resistant	Susceptible	p -Value[1]
No. of patients	26	118	
No. of patients with prior antibiotics (%)	11 (42.3)	50 (42.4)	1.0000[2]
Prior antibiotic days/patient (Treatment Days)			
ß-lactam/Cephalosporin	31/10 (3.1)	121/39 (3.1)	0.9978
Antianaerobe	15/6 (2.5)	64/20 (3.2)	0.5733
Aminoglycoside	7/3 (2.3)	22/5 (4.4)	0.6100[3]
Macrolide/Azalide	0/0 (0)	28/8 (3.5)	
Trimethoprim-Sulfamethoxazole	0/0 (0)	13/4 (3.3)	

[1]Student's *t*-test unless otherwise stated

[2]Fisher's exact test

[3]Aminoglycoside, macrolide/azalide, trimethoprim-sulfamethoxazole were grouped for statistical analysis

Table 13. Comparison of concomitant antibiotic use among CTX (CTX)- and ceftriaxone (CRO)-treated patients with third-generation cephalosporin-resistant Gram-negative bacilli (GNB) and CTX- and CRO-treated patients with susceptible GNB.

	Resistant	Susceptible	p -Value[1]
No. of patients	26	118	
No. of patients with concomitant antibiotics (%)	24 (92.3)	87 (73.7)	0.0422[2]
Concomitant antibiotic days/patient (Mean)			
Antianaerobe	120/21 (5.7)	334/60 (5.6)	0.4208
ß-lactam/Cephalosporin	32/7 (4.6)	101/22 (4.6)	0.8227
Aminoglycoside	20/3 (6.7)	30/6 (5.0)	0.6804
Vancomycin	19/3 (6.3)	7/4 (1.8)	0.0797
Other	7/2 (3.5)[3]	49/20 (2.5)[4]	0.5333

[1]Student's *t*-test unless otherwise stated

[2]Fisher's exact test; p=0.0422 is significant

[3]Erythromycin n=1; Trimethoprim-Sulfamethoxazole n=1

[4]Doxycycline n=6; Erythromycin n=13; Trimethoprim-Sulfamethoxazole n=1

However, patients with resistant strains received concomitant antibiotics more frequently (24/26 [92.3%]) as compared to patients with susceptible (87/118 [73.7%]) (p=0.0422, Fisher's exact test). The number of treatment days, however, for each drug class, did not differ between patients with resistant strains and patients with susceptible strains (Table 13).

Patients colonized with resistant GNB also received antianaerobic agents (21/24 [87.5%]), in particular, metronidazole. Seven of twenty-four patients (29.2%) received another ß-lactam: ampicillin (n=6) or cloxacillin (n=1) and 3 others (12.5%) received an aminoglycoside: gentamicin (n=2) or amikacin (n=1). Three patients were also treated with vancomycin, one with trimethoprim-sulfamethoxazole and one with erythromycin.

6.0 DISCUSSION

The broad-spectrum of activity exhibited by third-generation cephalosporins has prompted increased use of CTX and CRO for empiric treatment of many Gram-negative nosocomial infections and Gram-positive infections of methicillin-sensitive *S. aureus*, *Streptococcus pneumoniae* and group A streptococci (Jones and Thornsberry, 1991; Klein and Cunha, 1995). Previous studies have shown emergence of resistant GNB in patients receiving third-generation cephalosporins (Chow *et al.*, 1991, Heusser *et al.*, 1990; Joffe *et al.*, 1995; Paull and Morgan, 1986), but few studies have examined the selective pressure of third-generation cephalosporins on the commensal flora in the human gut.

It is well-known that the administration of antimicrobial agents is the most common and significant cause of disturbances in the normal microflora (Nord *et al.*, 1984b), and the intestinal flora represents the largest reservoir from which resistant organisms can be selected. CTX and CRO were chosen for study of the their impact on the intestinal flora because these agents are therapeutically equivalent yet only 1-1.5% of CTX is eliminated via biliary excretion whereas 30-67% of CRO is so eliminated (Bergan, 1987). Both drugs possess similar lability to Type 1 cephalosporinase, and they are commonly prescribed for empiric treatment of serious infections (Klein and Cunha, 1995).

The study used a quasi-experimental design to compensate for the lack of full experimental control that can be encountered in an ecology study of hospitalized patients (Campbell and Stanley, 1963). Whereas most ecology

studies have been conducted during regulatory antimicrobial drug trials which dictate single drug evaluation, this study examined the ecological changes as they would occur in a normal hospital setting.

Treatment with CTX or CRO was allotted by random assignment after the initial decision to treat with a third-generation cephalosporin was made by staff physicians. At the CGH, CTX was used as the standard unrestricted third-generation cephalosporin because of ease of controlling dosage and lower costs, and because it was less likely to cause diarrhea and disturbances of gut microflora. Treatment with CRO required ID service review. Rapid discharge and the tendency to use the CRO once-daily regimen in outpatient therapy resulted in fewer CRO patients remaining in the study. Additionally, it was found that consecutive stool specimens were difficult to obtain because of early discontinuation of treatment, discharge of the patient from the hospital and incontinency of patients. Stool specimens were collected at various times during the surveillance period even if an initial specimen was not collected.

Among the strengths of the study is the examination for the selection of antibiotic resistance in a population of subjects where third-generation cephalosporins are used the most. The selection of resistance in the gut microflora of hospital patients has clinical implications for a hospital reservoir of resistant organisms transmissible to patients by the hands of medical personnel or by sharing of medical equipment outside an intensive care setting. At the CGH, the study sites included the general medical and surgical wards where the majority of patients were hospitalized, and not in the ICU setting where resistance acquisition may be even higher (Ledgerwood *et al.*, 1995).

The original intention was to compare the two third-generation cephalosporins only. However, realizing that one half of the patients in the hospital received antibiotics and 20-25% received multi-dose antimicrobial courses that included cefazolin, and given that the majority of cephalosporin use in the hospital was with a first- or second-generation cephalosporin (Point Prevalence Survey, CGH Antimicrobial Subcommittee, 1994), it was decided to add a treatment control. Furthermore, because of the bias that hospitalized patients routinely acquire hospital-associated antibiotic-resistant organisms (McGowan, 1983; Péchère, 1994; Tancrède *et al*, 1984), it was decided also to add a non-treatment control.

The study, therefore, allowed a comparison of the differential impact of CTX and CRO on gut microflora as well as a comparison for selection resistance in untreated hospitalized patients and patients treated with cefazolin. Cefazolin is the most extensively-used first-generation cephalosporin in many tertiary care centres and demonstrates the selection of resistance in patients treated with this cephalosporin (Flynn *et al.*, 1987). Its use, therefore, allowed for the validation of the methods employed for detecting resistance should resistance occur in patients receiving other cephalosporins. Untreated controls served as indicators for the development of third-generation cephalosporin-resistance that was due to other factors besides treatment with a cephalosporin agent.

The patient population under investigation was similar in age, underlying diseases, antimicrobial use prior to entering the study and concomitant antimicrobial use (Tables 1-4).

A comparison of these factors between patients treated with CTX and patients treated with CRO showed similar findings. Antibiotic use up to 6 months prior to current therapy was assessed for a more complete review of the patient's medical history. However, in individuals whose intestinal microflora was disrupted by the use of cephalosporins, normalization of the microflora occurred within 2 weeks (Arvidsson *et al.*, 1982; Nilsson-Ehle *et al.*, 1985). The amount of antibiotics used the previous 2 weeks and the amount of co-therapy was comparable between CTX and CRO groups. Slightly less than half of patients were already exposed to antimicrobials within 2 weeks before hospitalization, underscoring that patients are different from healthy volunteers that have been used in pharmacokinetic and drug effect studies (Arvidsson *et* al., 1982; de Vries-Hospers *et al.*, 1991).

In this study the sex of a patient was not matched between each group as gender has never been found to be a contributing factor to the development of resistance in patients. The significant difference found in sex among the four groups did not occur between the CTX and CRO groups as 68% of patients were male whether they received CTX or CRO.

Overall, the similarities found in host factors between CTX and CRO groups indicate that any differences in the selective pressure these antimicrobials exerted on the gut microflora could not be attributed to age, sex, underlying disease, prior or concomitant antimicrobial use.

Stool specimens obtained from 321 patients indicated a higher selective pressure for third-generation cephalosporin-resistant GNB among patients treated with CTX than among patients treated with CRO. In some instances,

patients consented to fecal sampling via rectal swab cultures because of stool incontinency. Nevertheless, resistance showed similar trends between patients with consecutive stool specimens (n=96) and patients with only single specimens (n=225) so that these results were combined for analysis. By having a larger sample size, the likelihood of correctly detecting a true difference in resistance between CTX- and CRO-treated patients was increased.

Parenteral administration of CTX in 81 patients had a substantial impact on the microbial flora of the gastrointestinal tract. Resistance occurred in 32.7% of patients and was manifested primarily in bacterial species known to possess chromosomal cephalosporinases (Bush, 1989; Sanders and Sanders, 1992). Cephalosporin-resistant *Enterobacter* species, *C. freundii*, and *P. aeruginosa* were isolated predominantly from CTX patients in counts up to 10^{10} CFU/g. The presence of ß-lactamase activity, as evidenced by the nitrocefin and double disk approximation test, resistance to other ß-lactam antibiotics, including the extended-spectrum penicillins and ceftazidime, and the lack of activity of clavulanate and tazobactam against these organisms implicates that resistance was mediated by the chromosomal Type 1 cephalosporinases (Bush, 1989).

Among 63 patients treated with CRO, the resistance observed was not higher than that observed among hospitalized patients not receiving any antibiotic even though the screening method allowed the detection of at least 10^2 CFU/g feces. Resistance was detected in up to 11.4% of patients with up to 17 days of treatment with CRO, a third of that detected in CTX-treated patients. In CTX patients, resistant GNB were detected as early as day 3 of treatment with CTX.

Findings from previous studies advocate less than 6 days of therapy with third-generation cephalosporins to avoid selection of resistant mutants (Dworzack *et al.*, 1987; Guggenbichler and Kofler, 1984) although the development of cephalosporin-resistance after only 3 days of treatment has been documented, albeit with the second-generation cephalosporin, cefamandole (Nord and Edlund, 1991).

Selection of third-generation cephalosporin-resistant GNB was favoured by CTX but was also seen with cefazolin. Cefazolin induces Type 1 enzymes strongly, but it is more likely that resistant mutants, which overproduce chromosomal ß-lactamases, arise under the selective pressure of second- or third-generation cephalosporins, (Livermore, 1991). In 84 patients, the administration of cefazolin selected for CTX- and CRO-resistant GNB in almost 1/4 of patients after an average of 9 treatment-days.

The fact that less antibacterial-efficient first-generation cephalosporins can select for Gram-negative species capable of inactivating third-generation compounds that have higher antibacterial activity, is of clinical importance. This likely constitutes the largest reservoir of these organisms in hospitalized patients due primarily, to the extensive use of cefazolin. By comparison, in the absence of antibiotic pressure, selection for cephalosporin resistance was infrequent (2.1%) in 93 patients not exposed to antibiotics.

Cephalosporin-resistant GNB were found to have in vitro resistance to the extended spectrum penicillins, ticarcillin and piperacillin, and other third-generation cephalosporins, including the anti-pseudomonad, ceftazidime. The addition of the ß-lactamase inhibitors, clavulanate and tazobactam to ticarcillin

and piperacillin did not increase the susceptibility of these isolates. Cross-resistance was restricted to most of the ß-lactam class of antibiotics with the majority remaining susceptible to imipenem, tobramycin, clinafloxacin and ciprofloxacin.

This finding is not surprising in view of the fact that any agent capable of selecting one of the mutant phenotypes of altered ß-lactamase expression will select for increasing resistance to all ß-lactam agents (except imipenem) (Garau, 1994; Liu *et al.*, 1992; Shah *et al.*, 1991). It also reflects that the mechanism of resistance to imipenem is different from that of other ß-lactam antibiotics, and has been described to be mainly due to altered OmpC proteins (Nikaido, 1988) or altered PBPs (Bellido *et al.*, 1990).

With the exception of *H. alvei* and *S. maltophilia,* the majority of isolates selected for in a high proportion of CTX- and to a lesser degree, CRO-treated patients are commonly known to possess Type 1 cephalosporinases. In particular, spontaneous mutants of *Enterobacter* species, that continuously manufacture the Type 1 enzymes at a high level, arise quite frequently (Livermore, 1991) and occurred the most among the patients of this study. Because members of this group of organisms are common causes of nosocomial infections (Chow *et al.*, 1994), selection of such resistant strains cause major problems within the hospital environment.

H. alvei is a Gram-negative enteric bacillus belonging to the *Enterobacteriaceae* family. In the past, *H. alvei* was considered a member of the genus *Enterobacter* because of biochemical similarities (Sakazaki, 1984). It is not surprising then, to have selected CTX-/CRO-resistant isolates displaying ß-

lactamase activity and multiple ß-lactam resistance that included the ß-lactamase inhibitors. Similar findings, in a respiratory isolate of *H. alvei* resistant to ceftazidime, led to characterization of the enzymes mediating the resistance (Thomson and Sanders, 1993). The results indicated that *H. alvei* possess Bush Type 1 cephalosporinases and should be included in the cluster of GNB capable of developing Type 1 resistance. This has not been recognized in previous epidemiological studies.

S. maltophilia are intrinsically resistant to all ß-lactam antibiotics, including cephalosporins and imipenem. This species possesses chromosomal cephalosporinases (L2 enzymes) and carbapenemases (L1 enzymes), the latter mediating resistance to imipenem or meropenem by participating directly in the hydrolysis of the carbapenem's ß-lactam ring (Saino *et al.*, 1982).

Changes in the outer membrane proteins causing resistance are particularly common in *P. aeruginosa* in which resistant mutants lack D2 outer membrane protein (Trias and Nikaido, 1990). The isolates of *P. aeruginosa* from this study remained susceptible to imipenem suggesting that the resistance was not mediated by outer membrane impermeability but rather, by the Type 1 cephalosporinases.

In vitro susceptibilities of the 48 isolates selected in patients' gut flora during treatment with CTX and CRO indicated that most (45/48 [93.8%]) were constitutive producers of cephalosporinase. This characteristic appeared stable since at least four serial passages were completed before susceptibility testing, and a reduction in MICs was not observed upon subsequent retesting. Inducible species require the presence of a ß-lactam to produce cephalosporinase

(Lindberg *et al.*, 1988) and in 3 susceptible strains (2 *E. cloacae*, 1 *E. aerogenes*), induction by cefoxitin in the disk approximation test resulted in resistance to CTX and CRO.

The presence of "intermediately-resistant" GNB in the gut flora of untreated patients and patients receiving treatment with cephalosporins underscores the potential vastness of the gut as a reservoir. Organisms that were isolated from the low selectivity screen (Mac + 2 μg/mL antibiotic) were mostly represented by members that elaborate inducible or constitutive amounts of cephalosporinase. Although this was not examined, it is expected that inducible species were present among the gut microflora of patients, including those who did not receive antibiotic therapy.

It is of interest to note the high frequency of *P. aeruginosa* (n=15) selected on the lower screen. This suggests that mutants expressing constitutive amounts of enzyme do not arise as frequently as observed in *E. cloacae* (Livermore, 1991). Additionally, 4 *E. coli* and 1 *K. oxytoca* strain were selected. These species are not known to produce inducible or constitutive ß-lactamases. Instead they produce basal amounts of enzyme, regardless of the presence of a ß-lactam drug, because the regulatory gene, *amp*R, is not present (Lindberg and Normark, 1986).

The results of the bioassay for detection of antibiotic levels in fecal specimens showed lower mean levels of CTX than CRO (21 mg/kg CTX versus 319 mg/kg CRO). In theory, the low levels of CTX in the gut would indicate less disturbance of the normal flora and preservation of colonization resistance. But resistant GNB were found less often in patients receiving CRO, an antibiotic that

was present in higher concentrations in the gut and so disrupted the normal flora to a greater extent than CTX.

The phenomenon of colonization resistance has been shown in animals (van der Waaij, 1971). It is difficult to demonstrate in humans because of factors involved with an intact mucosa, the production of gastric acid, salivation and swallowing (van der Waaij, 1983). Ethical concerns arise with protocols that require challenging humans with marker resistant strains and illness covariables make it difficult to interpret findings from these clinical studies.

Nevertheless, physicians are wary of administering broad-spectrum antibiotics which may disturb the normal fecal flora, despite the lack of data regarding colonization resistance in humans. The lower amount of resistance seen in patients treated with CRO indicates that high intraintestinal concentrations of CRO suppressed the normal flora but that inhibitory concentrations were also sufficiently high to avoid the selection of resistant mutants. CRO displayed low selective pressure during its administration but the ecological void that remained in the gut after treatment may have increased the risk of colonization with exogenous resistant pathogens. A very real threat is the occupation of this ecological void by vancomycin-resistant enterococci . Future studies will need to examine the risk of resistance development in the gut microflora of patients who have discontinued therapy with CRO.

Not all studies have reported high CRO concentrations in patients (Arvidsson *et* al., 1982; de Vries-Hospers *et* al., 1991). Low detectable concentrations of CRO have been attributable to the drug's inactivation with ß-lactamases of anaerobes, mainly *Bacteroides* species (de Vries *et al.*, 1991;

Léonard *et al.*, 1989). This may explain the high variance in stool concentration of antimicrobials in patient specimens. Nevertheless, the bioassay that was used was a good indicator of the biological activity of an antibiotic present in the gut.

The higher proportion of resistant GNB among CTX patients indicates that CTX has a more significant impact on the gastrointestinal flora whereas CRO has a lesser tendency to induce resistance development. That less than 2% of CTX is recovered in bile (Bergan, 1987) suggests that subinhibitory concentrations of CTX in the colon may select for stably-derepressed variants of enteric bacilli by inhibiting the susceptible population (Livermore, 1991). Conversely, if the active concentration of an antimicrobial agent in the bowel is effective against prevailing resistant mutants, selection of resistance will not occur (Vollaard and Clasener, 1994). Since CRO therapy results in high biliary concentrations, a recipient's colonic microflora is profoundly changed (Bodey *et al.*, 1983). It is concluded that the results of this study show that, while possibly being deleterious to the intestinal microflora, the high intestinal concentrations of CRO appears to suppress the rate of resistance development.

It has been suggested that indigenous bacteria are continuously translocating in low numbers from the GI tract, even in healthy immunocompetent hosts (Berg, 1995). Although there is little direct evidence of bacterial translocation in humans, indigenous intestinal bacteria comprise a large reservoir of potential and opportunistic pathogens. Reports of *Enterobacter* infections suggest that common reservoirs for the organism include the urinary, respiratory and GI tracts, in addition to surgical and burn wounds

(John *et al.*, 1982; Ristuccia and Cunha, 1985) although the gut is the most common portal of entry (Chow *et al.*, 1991).

The vast majority of *Enterobacter* infections in patients undergoing cardiac surgery developed in patients who already had *Enterobacter* species as part of their endogenous flora and horizontal transmission was responsible for only two of 12 *Enterobacter* infections (Flynn *et al.*, 1987).

In this study, analysis of chromosomal DNA restriction patterns by PFGE showed that 16 patients colonized with third-generation cephalosporin-resistant *E. cloacae* did not acquire resident *E. cloacae* hospital strains. The resistant *E. cloacae* were genotypically unique to each of the 16 patients suggesting the emergence of resistance during therapy with a cephalosporin agent.

Further evidence for the emergence of resistance was found in 4 of 9 patients with initial and subsequent stool specimens. In vitro susceptibilities showed that genotypic clones, distinct to each of 4 patients, were initially susceptible to CTX and CRO but became resistant or showed increasing resistance to CTX and CRO with prolonged therapy. That the source of third-generation cephalosporin-resistant GNB may be the patients themselves was also emphasized in 5 additional patients who remained colonized with a resistant clone that was unique to each patient.

Subsequent treatment with a different drug class showing in vitro activity may lead to eradication of the resistant clone or it may lead to further development of resistance. Discontinuing therapy, on the other hand, has led to the "normalization" of the intestinal microflora with the reappearance of aerobic bacteria to pretreatment CFU counts (Nilsson-Ehle *et al.*, 1985). In resistant

bacteria, no impact on susceptibility patterns were observed up to 1 week after cessation of CRO therapy (Guggenbichler and Kofler, 1984).

Limited data have been obtained in controlled studies to assess characteristics of patient populations that may be predisposed to the emergence of resistant pathogens. Several reports have associated certain underlying diseases and prior antibiotic use with the emergence of pathogens resistant to broad-spectrum cephalosporins (Chow *et al.*, 1991; Jacobson *et al.*, 1995; Sanders and Sanders, 1988).

In this study, underlying disease and prior antimicrobial use did not contribute to the selection of resistant GNB in patients receiving CTX or CRO. Patients with resistant strains had received concomitant antibiotics more frequently (24/26 [92.3%]) than those with susceptible strains (87/118 [73.7%]). Metronidazole was the most common antibiotic to be prescribed as co-therapy. This demonstrates the relative ease with which an inexpensive agent is used to provide a broader spectrum of activity regardless of whether it is indicated. The higher use of metronidazole amongst patients with resistant organisms supports the role the anaerobic flora may play in preventing the development of resistance.

In summary, the comparative selection, between CTX and CRO, of third-generation cephalosporin-resistance in fecal microflora was first reported in this study. Treatment with CTX selected for resistant GNB in a higher proportion of patients than CRO treatment. Almost all the GNB selected possessed a Type 1 cephalosporinase which conferred resistance to third-generation cephalosporins, extended-spectrum penicillins and ß-lactamase inhibitors.

CTX concentrations in stool specimens appeared lower than CRO concentrations. During therapy, resistance was due to the development of resistance in initially susceptible organisms, an increase in resistance in resistant organisms, or persistence of resistance. Patients treated with CTX are at higher risk of selecting third-generation cephalosporin-resistant strains from gut microflora. Factors such as age, sex and underlying illness, prior and concomitant antibiotic use did not contribute to the higher selective pressure of CTX compared to CRO. When treating with either CTX or CRO, however, cotherapy was a risk factor for the selection of third-generation cephalosporin-resistant GNB from gut microflora.

The most important finding was the rapidity and high frequency with which drug-resistant organisms emerged in the gut during cephalosporin therapy. The perception that antibiotic-treated patients are not an infection control hazard must be changed. The patients in this study are representative of the patient population on the medical and surgical wards of a typical Canadian acute care hospital. The higher proportion of patients who were colonized with third-generation cephalosporin-resistant GNB during treatment with CTX compared to CRO suggests that CRO is the better choice for reducing the potential of the gut as a reservoir for resistant GNB during treatment with a third-generation cephalosporin agent. In light of this, CTX should not be administered to patients at high risk of becoming infected with resistant organisms and should be avoided in neutropenic patients.

This information must be imparted to physicians in order to emphasize the need for judicious prescribing practices. Rotating treatment with a different class

of antibiotics may be a way to decrease the selective pressure of CTX before resistance becomes prevalent. When resistance does arise, strict handwashing practices must be stressed among health care workers to prevent the transfer of resistant organisms to debilitated patients. Future studies will be required to determine the role that transmission by hand plays in disseminating resistant organisms originating from a patient's gut flora. Perhaps, knowledge of the mechanisms for development of resistance and the role of hands in transmission will finally convince physicians, who have been shown consistently to be low compliers (Henderson *et al.*, 1995), to wash their hands.

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