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THE DEVELOPMENT AND APPLICATION OF A NOVEL FLUORESCENCE TEST TO DETERMINE THE SPECTRUM OF ß-LACTAMASES

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

by

Wendy Ferris

Wendy Ferris, Ottawa, Canada, 1995

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ABSTRACT

Development of a Novel Satellitism Fluorescence Test for the Detection of an Extended-Spectrum ß-Lactamase (ESBL) in Isolates of *Klebsiella pneumoniae*.

The work leading to the development of this fluorescence test was divided in to two parts. Initially, susceptibility of 94 *K. pneumoniae* isolates collected from three hospitals (Series I) to cefazolin (KZ), cefoperazone (CFP), ceftazidime (CAZ), and piperacillin (PRL) was determined by agar dilution and NCCLS disc diffusion methods. Detection of ß-lactamase was performed by the Masuda Test, the Double Disc Synergy Test (DDST), and a novel test developed during the course of this work for ESBL as follows: Serial dilutions of antibiotics against which enzyme activity is to be demonstrated are incorporated into Mueller-Hinton agar containing methylumbeliferyl-ß-D-glucuronide (MUG) 70 μg/mL. Growth of *Escherichia coli* on this medium gives fluorescence but growth of *K. pneumoniae* does not. These plates are seeded with an *E. coli* lawn and *K. pneumoniae* strains are spot inoculated by Steers Replicator onto the lawn. At antibiotic concentrations above the MIC of *E. coli*, fluorescence on the lawn is inhibited. On the same plates, satellite growth of *E. coli* occurs around enzyme-producing *K. pneumoniae* isolates and this is manifested as a ring of fluorescence. The incorporation into the medium of clavulanic acid indicates if the enzyme is neutralized. In the Series I the *K. pneumoniae* isolates positive by DDST were also positive by the satellite fluorescence test. None of the isolates were positive by DDST and negative by fluorescence testing.

SERIES I

	KZ	CFP	CAZ	PRL
Resistant	5	0	0	6
DDST +	23	19	2	39
Fluorescence +	41	34	2	73

Having established the validity of the fluorescence test with the Series I *K. pneumoniae* isolates, the actual incidence of resistance to ß-lactams in *K. pneumoniae* isolates was determined with this novel fluorescence test for ESBL in 127 consecutive Ottawa Civic Hospital *K. pneumoniae* isolates (Series II). The Series II *K. pneumoniae*'s had NCCLS disc diffusion and fluorescence tests done with CAZ, CFP, KZ, cefuroxime (CXM), cefotaxime (CTX), cefoxitin (FOX), ceftriaxone (CRO), and aztreonam (ATM).

SERIES II

	ATM	KZ	CFP	CAZ	CXM	CTX	CRO	FOX
Resistant	0	1	1	0	1	0	0	0
Fluorescence +	1	25	61	2	2	0	0	0

Conclusions are (1) Routine susceptibility tests do not detect ESBL in *K. pneumoniae* isolates which may be clinically important. (2) The satellite fluorescence test is a more sensitive test than the DDST. (3) This fluorescence test is a convenient, flexible, and sensitive test for screening ESBL production in *K. pneumoniae* isolates. (4) A small number of *K. pneumoniae* isolates in Ottawa show a decreased susceptibility to

third generation ß-lactams with evidence of enzyme degradation but are susceptible by zone diameter measurements.

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LIST OF ABBREVIATIONS

Amoxycillin / Clavulanic Acid	AMC
Ampicillin	AMP
Aztreonam	ATM
Clavulanic Acid	CA
Ceftazidime	CAZ
Cefoperazone	CFP
Colony Forming Units	CFU
Ceftriaxone	CRO
Cefotaxime	CTX
Cefuroxime	CXM
Double Disc Synergy Test	DDST
Extended-Spectrum ß-lactamase	ESBL
Cefoxitin	FOX
Cefazolin	KZ
Mueller-Hinton Agar	MHA
Minimal Inhibitory Concentration	MIC
Millimetres	mm
Methylumbeliferyl-ß-D-Glucuronide	MUG
National Committee For Clinical Laboratory Standards	NCCLS
Dicloxacillin	OX
Penicillin-binding-proteins	PBP
Phosphate Buffered Saline	PBS
Piperacillin	PRL
Ticarcillin	TIC
Tryptone Soya Broth	TSB

INTRODUCTION

1. PROBLEMS IN TREATMENT RESULTING FROM THE DEVELOPMENT OF RESISTANCE

Resistance to antimicrobial drugs is a worldwide problem that is becoming more extensive as more antimicrobial agents became available (1). Resistance was encountered soon after the introduction of penicillin for general use in the 1940's. By the mid-1940's there were reports of *Staphylococcus aureus* isolates that did not respond to treatment with penicillin. By the mid 1950's virtually all hospital acquired infections caused by *S. aureus* were resistant to treatment by penicillin (2,3). This problem has also occurred with gram negative bacteria. In 1974 isolates of *Haemophilus influenzae* were found to be resistant to ampicillin which at that time was the standard treatment for children suspected of having meningitis caused by *H. influenzae* (2). In 1976 the emergence of penicillinase-producing *Neisseria gonorrhoeae* was reported (2,4-6). In both these clinical situations treatment could no longer be initiated with ampicillin (AMP) or penicillin, respectively, which has had a profound effect on treatment. There are also problems in treating infections caused by *Pseudomonas aeruginosa* and *Burkholderia cepacia* in patients with cystic fibrosis where resistance to all the aminoglycosides and ß-lactams has emerged (7,8). *Mycobacterium tuberculosis*, often an infection seen in immunocompromised patients, has developed multi-drug resistance which hinders treatment (9).

Klebsiella pneumoniae is a gram negative bacillus that causes nosocomial infections in immunocompromised patients. These infections are a source of morbidity and mortality in intensive care wards, neonatal units and nursing homes (10-15). *K. pneumoniae* was always resistant to AMP but when reports of gram negative bacilli resistant to cefazolin

(KZ which is a first generation cephalosporin) (15a) and to the second generation cephalosporins surfaced, pharmaceutical companies developed third generation cephalosporins such as CFP, CAZ, CTX, and monobactams such as ATM to resist the action of the ß-lactamases known at that time(15a). Since 1983 there have been reports from Europe, South America, and the United States of *K. pneumoniae* isolates resistant to the third generation ß-lactam antibiotics by the action of novel ß-lactamases that were able to hydrolyse these antibiotics. These enzymes were called extended-spectrum ß-lactamases (ESBL's) (16). As *K. pneumoniae* developed resistance to the third generation cephalosporins, treatment of infections caused by this organism became increasingly more difficult.

2. REVIEW OF RESISTANCE MECHANISMS FOUND IN BACTERIA

Many antimicrobial agents have been found in nature or synthesized since the discovery of penicillin. Acquired resistance to antibiotics can occur by three major mechanisms: prevention of the antibiotic from reaching its target, production of altered targets, and destruction or modification of the antimicrobial agents (16). Bacteria can possess one or more of these mechanisms working either alone or simultaneously resulting in resistance to an antimicrobial agent. Factors that influence resistance are characteristics of the bacteria, the drugs, and the environment in which they are all brought together (7). The mechanisms of resistance found in bacteria are reviewed below with specific emphasis on the mechanism that is relevant in this study which is the production of ß-lactam inactivating enzymes (ß-lactamases) in *K. pneumoniae* isolates.

2.1 Intrinsic Resistance

Intrinsic resistance applies to the physical and chemical properties of a genus or species of bacteria. This resistance is common to all strains of a species and is associated with normal chromosomal determinants of a cell (7,16). This type of resistance is species specific and can be inferred from bacterial identification.

2.2 Alteration in Permeability

Antibiotics must enter the bacterial cell to act at its target site to exert a bactericidal or bacteriostatic effect on the bacterium. If an antimicrobial agent is unable to permeate the cell or to accumulate intracellularly, there is less chance that the drug will exert its effect (7,17,18,19). ß-lactams must enter the cell, gain access to the periplasmic space, and bind to the penicillin-binding proteins (PBP) on the cytoplasmic membrane. Gram negative organisms have a complex cell wall structure and the peptidoglycan layer is not in contact with the environment but is bound on the outer surface by the outer membrane. This outer membrane is the first obstacle that antibiotics encounter in finding their way to the target site in gram negative bacilli. Some antibiotics can pass through the lipid component of this membrane but ß-lactams cross by passing through aqueous transmembrane pores comprised of specialized proteins called porins (20-25). These porins act as a sieve to allow molecules of a particular size to enter and exit the cell. ß-lactams can readily penetrate the porins of gram negative bacteria (4,16,17,20). If there is a mutational event that alters the size of the porin, antibiotics may no longer gain access into that bacteria and resistance ensues due to an alteration in permeability (20,26).

2.3 Alteration of Target Site

An antibiotic must bind to a target structure within the bacterium on which it can exert its bactericidal or bacteriostatic effect. ß-lactam antibiotics (penicillins and cephalosporins) must bind to PBP's on the cytoplasmic membrane. ß-lactam antibiotics enter the gram negative bacterial cell by crossing the outer membrane via the porins and gain access into the periplasmic space. and bind to the PBP's (7, 17,18,26-29). The PBP's are required in the final stage of peptidoglycan synthesis (7,17,18,27,28,29). If there is a mutational change in the protein structure of the PBP, then the ß-lactam will not bind to the PBP and lyse the cell. Therefore, the bacteria will be resistant to that antimicrobial agent.

2.4 Production of Antibiotic Inactivating Enzymes

Resistance to ß-lactams, aminoglycosides, and chloramphenicol antibiotics can be mediated by antibiotic-inactivating enzymes. The ß-lactamases are the best known examples of hydrolytic enzymes which cleave the ß-lactam ring of penicillins and cephalosporins rendering the antibiotic ineffective and were first documented by Abraham and Chain in 1940 (30). They described the penicillin destroying activity in extracts of *E. coli* and showed that the process was enzymatic and classified the enzyme as a penicillinase. In 1942 Kirby reported that an extract of *S. aureus* could inactivate penicillin as described above (1). During the next decade penicillinase became the accepted term for this group of enzymes. In the 1960's there was a shift in the organisms causing nosocomial infections from gram positive to gram negative organisms, particularly *E. coli* which were originally susceptible to ampicillin (an aminopenicillin) (2). Soon

outbreaks of resistant organisms were reported (2,5,31).

The emergence of resistance in gram negative bacilli to the third generation cephalosporins by the production of novel ß-lactamases was not anticipated when these antimicrobial agents were developed. They were designed to resist the action of the currently recognized ß-lactamases that had limited the efficacy of older penicillins and cephalosporins (26,32,33). After ceftazidime (CAZ), cefotaxime (CTX), and aztreonam (ATM) became available for clinical use, the emergence of resistance to these drugs during therapy began to appear. This resulted in treatment failure or relapse of infection (26). As the resistance to the newer ß-lactams became more widespread, so did the number of ß-lactamases isolated which were classified as Extended-spectrum ß-lactamases (ESBL's). (This is discussed below in more detail.) (34-52).

3. DESCRIPTION OF GENERATIONS OF CEPHALOSPORINS

Cephalosporins are a group of antimicrobial compounds whose structure consists of a ß-lactam dihydrothiazine ring system. Substitutions of different side-chains have given rise to semi-synthetic antibiotics. The modifications at position 1 (which is usually a sulphur), addition or substitutes at positions 3 of the dihydrothiazine ring or position 7 of the ß-lactam have resulted in the various generations of cephalosporins. The substitutions at position 3 changes metabolic and pharmacokinetic activity while modifications at position 7 alter antimicrobial activity (15a).

These cephalosporins have been grouped into generations based on antimicrobial spectrum. The first generation cephalosporins (which include cephalothin and cefazolin) are active against gram positive cocci with moderate activity against *E. coli, Proteus*

mirabilis, Moraxella catarrhalis, and *K. pneumoniae*. The activity of first generation cephalosporins against other *Enterobacteriaceae* is unpredictable and cannot be assumed without susceptibility testing (15a,52a).

The second generation cephalosporins (which inlcude cefuroxime) have enhanced activity against gram negative bacteria. This enhanced activity is accomplished by the replacement of the hydrogen at position 7 with a methoxy group. This substitution results in increased stability against ß-lactamases. Cefuroxime has a furyl group at position 7 which retains its activity against *Staphylococci* as well as ß-lactamase stability (15a,52a).

The third generation cephalosporins (which include ceftazidime and cefotaxime) are considered the most effective against the facultative gram negative bacteria as well as against *Staphylococci* and *Streptococci*. These cephalosporins have further modifications to the side chains at position 7 which enhances their penetration into gram negatives (15a,52a),

4. HISTORY AND REVIEW OF ß-LACTAMASE CLASSIFICATIONS

In 1965 The Enzyme Commission described the enzyme that inactivated penicillin as "penicillin-amido-ß-lactam hydrolase " (3). In the mid-1960's introduction of broad-spectrum penicillins and cephalosporins provided additional substrates for enzyme characterization. Enzymes that hydrolysed the newly introduced cephalosporins were referred to as cephalosporinases. In 1972 The Enzyme Commission designated penicillinase as ß-lactamase I and cephalosporinase as ß-lactamase II. These enzymes which can be plasmid or chromosomally mediated have been found in virtually all bacteria (3,5,6). As enzymes were isolated, it was evident that this nomenclature would not reflect

the variety of enzymes found within these two classes. There have been several classification schemes proposed as newer enzymes with different properties were isolated. These classification schemes have been based on:

1. Substrate Profile which indicated the antibiotics inactivated by that enzyme.
2. Sensitivity to Inhibitors which indicated the compounds that could inactivate the ß-lactamase by binding with it. These inhibitors include oxacillin, clavulanic acid, sulbactam, and tazobactam. The latter three compounds were specifically designed to inhibit plasmid-mediated enzymes .
3. Isoelectric Points (53,54)
4. Molecular Weight
5. Location of the gene that encodes for the ß-lactamase indicating whether the enzyme is chromosomally or plasmid-mediated (1,2,3,5,6,55,56,57,58).

4.1 Sawai

In 1968 Sawai et al divided ß-lactamases into three groups based on substrate profile as follows (4,7,58):

Group 1. Inducible Cephalosporinases

Group 2. Broad Spectrum Enzymes which were cephalosporinases that had the properties of penicillinases

Group 3. Penicillinases

4.2 Pitton

In 1972 Pitton subdivided the TEM-type enzymes which are described under "Plasmid-Mediated ß-Lactamases" into two groups on the basis of isoelectric points and substrate profile. The Type I TEM enzymes, which at that time were TEM-1 and TEM-2, were those commonly found in *E. coli*. The enzymes that comprised Type II were plasmid-mediated enzymes differentiated from the TEM enzymes by isoelectric focusing (3,53,54,55,56).

4.3 Jack and Richmond

The Jack and Richmond Classification (3) of ß-lactamases was based on substrate profile like the Sawai et al classification but had four groups which were:

Group 1. Broad Spectrum Enzymes

Group 2. Penicillinases

Group 3. Cephalosporinases with little or no activity against penicillins

Group 4. Cephalosporinases with some activity against penicillins (3) .

4.4 Richmond and Sykes

These initial classifications schemes were replaced by a more definitive scheme in 1973 by Richmond and Sykes (2,3). This scheme contained five groups based on substrate profile and inhibition studies. Each group contained a number of enzyme types as follows: (2,3,31,56,59,60)

Class 1. Chromosomally mediated cephalosporinases that were constitutive

or inducible.

Class 2. Chromosomally mediated penicillinases

Class 3. Plasmid-mediated broad-spectrum enzymes with equal activity against penicillins and cephalosporins. The TEM-type enzymes fall in this category.

Class 4. Broad-spectrum enzymes as in Class 3 but these are resistant to inhibition by cloxacillin. These constitutive enzymes were found in Klebsiella.

Class 5. Penicillinases that were capable of hydrolysing oxacillin. The OXA and PSE-type enzymes fall into this class.

4.5 Matthew

Matthew described and classified eleven types of plasmid-mediated ß-lactamases in 1976 and put them into three groups. These groups of enzymes all were constitutive and have unique isoelectric points. The three groups were broad-spectrum enzymes, oxacillinases, and carbenicillinases (31,56).

4.6 Ambler

In the intervening years many of these ß-lactamases were isolated and their amino acid structure was determined. In 1980 Ambler et al devised a ß-lactamase classification scheme based on amino acid sequences (1,2,3,31,56,61). There were three classes in this scheme which were:

Class A. This penicillinase has a molecular weight of 29,000 daltons and has

a serine residue at the active site. There is considerable homology between these enzymes which include the gram positive and the TEM-type enzymes of the gram negatives.

Class B. These cephalosporinases require the metal co-factor Zn^{++} for activity. These enzymes have a molecular weight of 23,000 daltons.

Class C. These are chromosomally mediated cephalosporinases with a structural Amp C gene in gram negative bacteria. These enzymes have a molecular weight of 39,000 daltons. Like the Class A enzymes, these cephalosporinases have a serine residue at the active site but there is no amino acid homology between these two classes.

By the late 1980's thirty plasmid-mediated ß-lactamases had been identified on the basis of substrate profile, reaction with inhibitors, and isoelectric points. TEM-1 is the most commonly found plasmid-mediated enzyme while among the *Klebsiella species* the SHV-1 enzyme predominates. Mutations in these two originally isolated enzymes resulting in amino acid changes at particular sites of the active enzyme led to extended spectrum enzymatic activity. These new enzymes were able to hydrolyse the third generation cephalosporins and were called ESBL's (5,6,47,62-67).

4.7 Bush

The most inclusive classification scheme developed that attempts to combine all the previous schemes was that proposed by Bush in 1989 (59). This scheme did not use the

location of the gene that encodes for the ß-lactamase as the primary classification factor (1,56,59). Bush has four groups in her classification scheme with subgroups in Class 2. This classification encompasses all the ß-lactamases including the ESBL's that have been described and isolated since 1983.

Class 1. Chromosomally-mediated cephalosporinases that are inhibited by aztreonam and dicloxacillin but not by clavulanic acid or sulbactam. This group includes the inducible cephalosporinases of *Enterobacter* and *Pseudomonas*.

Class 2. ß-lactamases with a diversity of substrate profiles but all are inhibited by clavulanic acid, sulbactam, and tazobactam. This group includes the most prevalent plasmid-mediated ß-lactamases found in *E. coli* (TEM-1) and *K. pneumoniae* (SHV-1) as well as derivatives of these enzymes responsible for resistance to newer ß-lactam antibiotics.

Class 2a. Penicillinases from Gram positive organisms and not inhibited by oxacillin.

Class 2b. Broad-spectrum ß-lactamases that hydrolyse broad-spectrum ß-lactams (TEM-1 and SHV-1).

Class $2b^1$ Extended-spectrum ß-lactamases (TEM-3, SHV-2, SHV-3) which hydrolyse the third generation cephalosporins, CAZ, CTX, and the monobactam, ATM.

Class 2c. Carbenicillinases (PSE-1,2,3).

Class 2d. Oxacillinases.

Class 2e. Cephalosporinases

Class 3. ß-lactamases that require a metal ion (Zn++) for enzymatic activity. Not all these enzymes are inhibited by clavulanic acid.

Class 4. Penicillinases not inhibited by clavulanic acid. These enzymes are rarely encountered.

5. CHROMOSOMAL AND PLASMID MEDIATED ß-LACTAMASES

5.1 Chromosomally-Mediated ß-Lactamases

Chromosomally-mediated cephalosporinases are large proteins with a molecular weight of 30 - 42 kd. These enzymes are inhibited by dicloxacillin but not by clavulanic acid or sulbactam. Enhanced expression of chromosomally-mediated cephalosporinase can occur via two mechanisms: exposure to an inducer or mutation to a derepressed state (5,68,69,70). Although chromosomally-mediated ß-lactamases are not within the scope of this research, it is appropriate to include them in this historical review and to include them in the classification schemes developed since 1973 (2,3,31,58). The expression of chromosomally mediated ß-lactamases is regulated by the structural Amp C gene with a molecular weight of 39,600. Chromosomal ß-lactamases are found in many gram negative bacteria and hydrolyse KZ at a higher rate than the commonly found and first isolated plasmid-mediated ß-lactamase, TEM-1. There is DNA sequence homology between the chromosomally mediated ß-lactamases in *E. coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Shigella flexneri*, and *Proteus vulgaris* (58). In gram

negative bacteria, ß-lactam resistance can be caused by genetic events leading to an increased activity of chromosomal ß-lactamase (3,31,58,60).

ß-lactamase induction can occur when there is an increased rate of synthesis of ß-lactamase by a bacterium in response to the presence of an inducer in the growth medium (3,58,69,70,71). Two potent inducers are the antimicrobial agents cefoxitin and imipenem (72). While the inducer is present within the media, whether it be in laboratory growth media or present within a patient due to antimicrobial treatment, the rate of induction of chromosomally mediated enzyme can be variable depending on the culture media, the length of induction, the concentration of inducer present, and the stability of the inducer to the induced enzyme (6). With induction, the enzyme stable ß-lactam ring will encounter one of the numerous molecules of ß-lactamase in the periplasmic space and will be bound. Once bound the ß-lactam may or may not be hydrolysed. This non-hydrolytic trapping of the antibiotic by the enzyme leads to the genesis of a long-lived biologically inactive (60,73,74) complex.

Gram negative organisms that possess chromosomally mediated ß-lactamases can mutate to an increased rate of enzyme synthesis, known as derepression. The increase in the number of ß-lactamase molecules per bacterium is achieved by an increased rate of enzyme synthesis (71,75,76,77). Mutants with an increased rate of ß-lactamase synthesis have either an increase of Amp C gene copies produced by amplification of the gene or an increased rate of transcription (58,60).

5.2 Plasmid-Mediated ß-Lactamases

The existence of ß-lactamases that render gram negative organisms harbouring them resistant to the bactericidal effect of ß-lactams has been known since the 1960's (2,31,55). In 1965 an *E. coli* resistant to ampicillin was isolated from a patient named Temoniara in Athens, Greece. This resistance was due to the presence of a plasmid-mediated ß-lactamase. In honour of the patient from whom it was isolated, the enzyme was called TEM-1 (5,6,31). Although there have been a plethora of plasmid-mediated ß-lactamases isolated and characterized since then, TEM-1 is still the most commonly encountered plasmid-mediated enzyme in gram negative bacteria (2,31,55,56). In 1970 a plasmid-mediated enzyme in *Klebsiella* that conferred resistance to ampicillin and first generation cephalosporins but differed biochemically from TEM-1 was isolated and classified as SHV-1 (2,11,31,55).

Since 1980 there has been an outbreak in Europe and later in South and North America of novel plasmid-mediated enzymes that re-emphasized the role that ß-lactamases play in resistance and that was the result of natural selection and the use of newly introduced antibiotics (34). In 1983 in Germany strains of *Klebsiella* and *Serratia* resistant to cefotaxime and ceftazidime were reported (41). This resistance was found to be due to a novel plasmid-mediated ß-lactamase. By 1987 there were a number of reports from various Western European countries of plasmid-mediated resistance to extended-spectrum antibiotics. This resistance mirrored the clinical therapy with ß-lactam antibiotics as resistance was seen to those extended-spectrum ß-lactams that were used most often as treatment for gram negative infections. By 1991 there were 19 novel enzymes that had

been identified as derivatives from TEM and eight derivatives from SHV enzymes based on DNA hybridization techniques (1,34,79). These extended spectrum ß-lactamases (ESBL's) are believed to be the result of mutations of the plasmid-carrying genes encoding for TEM-1 and TEM-2 which are widely spread among gram negative bacilli and SHV-1 prevalent in *Klebsiella* (80,81). These ESBL's confer resistance to oxyamino-ß-lactams (cefotaxime and ceftazidime) and monobactams (aztreonam) and show broader substrate profiles caused by mutations which lead to minor alterations in the amino acid configuration of the active site of the original enzyme (45,46,47,48,49,52,64,66,67,82).

As organisms that harbour plasmids encoding for the ESBL's become more prevalent, treatment becomes more difficult. These resistance plasmids have also been shown to be easily transferable to other susceptible gram negative bacilli which can become an infection control problem within a hospital or nursing home if the recipient organisms are the cause of nosocomial infections in these institutions (10,11,13,41,64,65). Although these ESBL's have been documented around the world their incidence is low. However, these enzymes constitute a serious threat to the effectiveness of current ß-lactam therapy (83).

When it became evident that gram negative bacteria could genetically alter the sequence of the original plasmid-mediated enzymes to extend the substrate profile, the pharmaceutical companies then developed ß-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) which when combined with a ß-lactam antibiotic would be able to avoid hydrolysis by ß-lactamase (50,84,85,86,87,88). The inhibitor would bind with the ß-lactamase enzyme and allow the ß-lactam to exert its bactericidal effect on the cell

(50). Clavulanic acid resistant TEM type ß-lactamases were first described in 1991 and given the notation of TRI-1 and TRI-2 (TEM resistant to inhibitors), BIL-1 (82) and TRC-1 (TEM Resistant to Clavulanic Acid) (89).

6. ß-LACTAMASE DETECTION

There are a number of methods by which ß-lactamase activity can be detected. ß-lactamases confer resistance to ß-lactams by their ability to hydrolyze the antibiotic within the periplasmic space before the antibiotic reaches its targets which are the PBP's on the cell membrane. The methods for the detection of ß-lactamases are based on the changes that take place following hydrlysis of the amid bond of the ß-lactam ring. This process can result in

1. the loss of antimicrobial activity
2. the spectral shift in UV radiation
3. hydrolytic products that can be detected by
    a. acidimetric methods
    b. iodometric methods
    c. chromogenic methods (52a,89a,89b)

Cephalosporins usually possess adequate levels of absorption in the UV spectrum to allow the measurment of the loss of absorption when the ß-lactam ring is hydrolyzed (89c). The definitive method of ß-lactamase detection is the spectrophotometric method in which the rate of hydrolysis of the ß-lactam ring is measured by the rate of decrease in optical density of the ß-lactam solution at the wavelength of maximum absorption associated with the ß-lactam ring (89a). This method, however, because of the necessity to extract and

purify the enzyme, is not suitable as a screening test for ß-lactamase in a routine, diagnostic laboratory.

The acidimetric method of ß-lactamase detection utilizes pH indicators to detect a decrease in pH resulting from the cleavage of the ß-lactam ring. A suspension of ß-lactam, bacteria, and phenol red as the pH indicator is incubated. If the isolate possesses a ß-lactamase the solution will turn from purple to yellow as the pH decreases (52a).

The iodometric method of ß-lactamase detection is based on the decolorization of a starch-iodine mixture as a result of the ability of acid to reduce iodine. Iodine is reduced by the action of the acid produced when a isolate suspension is incubated with ß-lactam and a starch-iodine solution. Reduced iodine is lost from the starch-iodine complex which in turn causes a reduction of the blue colour of the complex to colourless (52a).

The chromogenic detection of ß-lactamase depends on the colour change resulting from the hydrolysis of the ß-lactam ring of a chromogenic cephalosporin, nitrocefin. This test is simple, can be applied directly to the colonies, and can be used as a rapid screening test (89a,89b). This rapid screening of clinical isolates for ß-lactamase was particularly important in the 1970's with penicillinase-producing *N.gonorrhoeae* and *H. influenzae* to indicate to the physician whether the isolate possessed a ß-lactamase even if the isolate was susceptible on routine testing (2). Although these screening tests are still used, they only indicate if an isolate has a ß-lactamase but do not indicate the full antibiotic spectrum inactivated by the enzyme.

7. SUSCEPTIBILITY TESTING METHODS

7.1 DILUTION TECHNIQUES

The determination of bacterial susceptibility to antimicrobial agents by dilution techniques is a quantitative methodology. Dilution tests are used to determine the minimum concentration of an antimicrobial agent required to inhibit or to kill an organism and is referred to as the minimum inhibitory concentration or MIC. Serial dilutions of the antimicrobial agent are inoculated with the organism and are incubated at the appropriate temperature and conditions. The MIC is the lowest concentration of the antibiotic with no visible growth. MIC's can be done on agar or in broth (in test tube or in microtitre wells)(52a).

7.2 DISC DIFFUSION

Disc diffusion tests are the most widely used procedures for susceptibility testing. This is a qualitative test that indicates whether an isolate is susceptible, moderately susceptible, or resistant to a particular antimicrobial agent. This test is flexible in the number of antibiotics that can be tested and is technically easy but rigorously controlled. Disc diffusion is not suitable for testing organisms that are slow-growing as the result is based on the diffusion of the antibiotic into the agar and logarithmic growth of the organism (52a).

7.3 E TEST

The E Test is a diffusion technique that gives a quantitative MIC result. This test is based on diffusion of a continuous gradient of antibiotic from a plastic strip into agar medium. The strip is physically inert with a predefined concentration of dried stabilized

drug on one side of the strip and a continuous MIC interpretive scale of antibiotic concentration on the upper side. After the agar plate has been inoculated with the test organism the E Test strips are placed drug side down and incubated. After incubation, an ellipse of inhibition is formed around the strip. The MIC is read at the point where the ellipse intersects the strip edge (89d).

OBJECTIVES

Clinically the physician depends on antimicrobial susceptibility reports from the microbiology laboratory to choose the antibiotic for therapy. With most routine susceptibility tests, many of the *K. pneumoniae* strains do not appear to be resistant to ß-lactams by standard disc diffusion tests or by automated micro-dilution techniques. Small increments of decreased susceptibility which may indicate resistance to ß-lactams by enzymatic activity would not be detected. This failure to detect the presence of an ESBL in an organism could lead to subsequent treatment failures (80,83,90,91). In order to monitor slight decreases in susceptibility to extended-spectrum ß-lactams which may have clinical significance, new tests need to be developed specifically for the detection of ESBL's.

In view of the reports of *K. pneumoniae* resistance to third generation cephalosporins, the incidence of resistance of *K. pneumoniae* to these antimicrobial agents in Ottawa was studied. Thompson and Saunders (83,91) had reported that some *K. pneumoniae* isolates that were susceptible to extended-spectrum ß-lactams on routine laboratory susceptibility also possessed a plasmid-mediated ß-lactamase that was able to inactivate third generation cephalosporins. Saunders has also reported that *K. pneumoniae* may show slight increases in MIC's due to ß-lactamase (83). With these parameters, the incidence of resistance of *K. pneumoniae* isolates to extended-spectrum ß-lactams by comparing disc diffusion susceptibility patterns with agar dilution MIC determinations were studied to see if there were slight decreases in susceptibility. If the resistance was due to ß-lactamase, could a flexible, reliable, sensitive screening test for the detection of plasmid-mediated ß-lactamase and to indicate the spectrum of ß-lactams

inactivated by the enzyme be developed.

In 1990 Vatopolous (80) developed a disc diffusion test that indicated the presence within a *K. pneumoniae* isolate of a ß-lactamase that could be inactivated by clavulanic acid (CA). This was an important step in ß-lactamase detection as all the plasmid-mediated ß-lactamases isolated at that time were inactivated by clavulanic acid. This test is known as the double disc synergy test (DDST). There were several problems in utilizing this test as a screening test for ß-lactamase in *K. pneumoniae* isolates in the routine laboratory namely: disc placement (82), the length of time to obtain a result, and the fact that there have since that time been plasmid-mediated ß-lactamases that are not inhibited by CA (15,43,50,65,92,93,94).

Previously, Masuda had described a method by which ß-lactamase (94a) could be detected in crude enzyme extracts from gram negative organisms. This test was a more time consuming procedure than the DDST which negated its value as a screening test. The screening test that is required is one in which a number of *K. pneumoniae* isolates could be tested against a variety of ß-lactam antibiotics within as short a time as possible in order to inform the physician of the presence of a ß-lactamase in a *K. pneumoniae* isolate that could result in treatment failure if utilized and to evaluate the overall susceptibility patterns of a large number of organisms.

The objectives of this research were :

1. To determine the incidence of resistance to extended-spectrum ß-lactams in *Klebsiella pneumoniae* isolates in Ottawa .

2. To develop a sensitive and reliable screening test to detect ß-lactamase.
3. To detect a decrease in zone diameter and an increase in MIC's to various extended-spectrum ß-lactams occur before observing resistance according to NCCLS?
4. To study representative collection of *K. pneumoniae* to determine the incidence of resistance in *K. pneumoniae* isolates and screen these isolates for ESBL's with the fluorescence test.

MATERIALS AND METHODS

1. BACTERIAL STRAINS

Series I *Klebsiella pneumoniae* isolates were chosen to compare susceptibility tests and to develop a screening test for ß-lactamase. These *K. pneumoniae* isolates were chosen to include a high incidence of resistance. A series of 94 of these isolates were received from the following hospitals: The Children's Hospital of Eastern Ontario, The Ottawa Civic Hospital, and The Oshawa General Hospital. These isolates were collected between the period of June 1991 and August 1992. They were used to develop the fluorescence test for ß-lactamase and were designated as KP- #.

Series II *K. pneumoniae* were chosen to examine the true prevalence of resistance in a collection of consecutive *K. pneumoniae* isolates from The Ottawa Civic Hospital. These 127 isolates had susceptibility testing done by zone diameter measurements and were screened for ß-lactamase by the fluorescence test developed in this study. These *K. pneumoniae* isolates were designated as OCH-#.

The control organisms were *Escherichia coli* ATCC 25922 used in the determination of agar dilution minimal inhibitory concentrations (MIC's) and as the indicator organism in the fluorescence test, *Micrococcus lutea* ATCC 9431 as the indicator for the Masuda Technique, and a *Klebsiella pneumoniae* strain which contains a TEM-10 plasmid (received from Dr. Sharon Walmsley, Toronto Hospital) for DDST and MUG fluorescence tests. See Appendix 1 for a list of the isolates used in this study.

2. ANTIBIOTICS

Antibiotic powders utilized in agar dilution minimal inhibitory concentration (MIC)

by NCCLS agar dilution determinations (96a) and fluorescence testing were as follows: ceftazidime and cefuroxime (Glaxo Canada Inc. Toronto, Ontario), cefoperazone (Pfizer Canada Inc. Kirkland, Quebec), cefotaxime (Roussel Canada Inc. Montreal, Quebec), piperacillin (Cyanamid Canada Inc. Markham, Ontario), ticarcillin and clavulanic acid (CA) (Beecham Laboratories Inc. Pointe Claire, Quebec), cefazolin (Eli Lily Canada Inc., Scarborough, Ontario), oxacillin (OX) (Sigma, St. Louis, Missouri, U.S.A.) ceftriaxone (Hoffmann - LaRoche Ltd. Mississauga, Ontario), Aztreonam (Bristol - Meyers Squibb Canada Inc., Montreal, Quebec), cefoxitin (Merck Frosst Canada Inc. Dorval, Quebec), and ciprofloxacin (Miles Laboratories, Etobicoke, Ontario).

The antibiotic discs and their content in micrograms (μg) which were used for disc diffusion testing according to NCCLS (96b) and for double disc synergy tests (DDST) were as follows: ampicillin 10 (AMP), ceftazidime 30 (CAZ), cefuroxime 30 (CXM), cefoperazone 30 (CFP), cefotaxime 30 (CTX), cefazolin 30 (KZ), ciprofloxacin 5 piperacillin 100 (PRL), ticarcillin 75 (TIC), ceftriaxone 30 (CRO), aztreonam 30 (ATM), and amoxycillin/clavulanic acid 2:1 30 (AMC). All these discs were obtained from Oxoid Laboratories, Unipath Ltd. Basingstoke, England.

3. MEDIA

The media utilized in broths, agar plates, and fluorescence tests were Tryptone Soya Broth (TSB) from Oxoid, Unipath Ltd., Basingstoke, England, Mueller-Hinton Agar (MHA) from Becton Dickenson Microbiology Systems, Mississauga, Ontario and methylumbeliferyl-ß-D-Glucronide (MUG) from Sigma (St. Louis, Missouri, U.S.A.).

4. SUSCEPTIBILITY TESTING

Susceptibility testing was carried out by two methods: NCCLS Agar dilution MIC determinations (94b) for Series I and NCCLS disc diffusion (94c) for both Series I and Series II isolates. See Table 1 for the NCCLS criteria for agar dilution and zone diameter interpretive criteria.

4.1 Agar Dilution Minimal Inhibitory Concentration (MIC) Determinations

Agar dilution MIC's were determined for all Series I *Klebsiella pneumoniae* isolates to CAZ, CFP, CXM, CTX, KZ, PRL, and TIC by NCCLS. Ten doubling dilutions of each antibiotic ranging from susceptible to resistance values were incorporated into MHA. See Table 1a for antibiotic ranges tested. The isolates and control organisms (*E. coli* ATCC 25922 and *K. pneumoniae* with a TEM-10 plasmid) were grown overnight in TSB at 37°C. The next day the broths were diluted with sterile normal saline to a 0.5 MacFarland Standard and further diluted by adding 10 μl of broth into 1.0 ml. of sterile saline. Each isolate was pipetted into one of the 37 wells of a Steer's Replicator template (95). Then each MHA plate containing antibiotic was inoculated with these isolates by the replicator prongs. Each of the 37 dots contained a *K. pneumoniae* isolate at a concentration of 10^4 cfu/spot. The plates were incubated overnight at 37°C. The next day the MIC of each isolate to each antibiotic was determined as the lowest antibiotic concentration with no visible growth on the MHA plate. The MIC's were done in triplicate for each isolate.

4.2 NCCLS Zone Diameter Determinations

Each *K. pneumoniae* isolate of Series I was grown in tryptone soya broth (TSB) overnight. The next day the broths were diluted with sterile normal saline to a 0.5 MacFarland Standard and then spread on to two petri plates of MHA. Six antibiotic discs were placed one plate and five on the other so that eleven antibiotics (AMP, CAZ, CXM, CFP, CTX, KZ, TIC, PRL, CRO, ATM, and AMC) were tested for each *K. pneumoniae* isolate. The plates were incubated overnight at 37°C. The next day zone diameters for each antibiotic were measured by calipers to the nearest mm. and recorded according to NCCLS specifications (94c). Zone diameter measurements for KZ, CXM, CAZ, CTX, CRO, CFP, ATM, and FOX were done on the Series II isolates. The disc diffusion test were carried out in duplicate for Series I and once for Series II isolates.

TABLE 1: NCCLS SUSCEPTIBILITY CRITERIA OF ANTIBIOTICS STUDIED

	ZONE DIAMETER (mm)			MIC (μg/mL)		
	Resistant	Moderately Susceptible	Susceptible	Resistant	Moderately Susceptible	Susceptible
Ampicillin	≤ 13	14 - 16	≥ 17	≥ 32	16	≤ 8
Ticarcillin	≤ 15	15 -19	≥ 20	≥ 128	32 - 64	≤ 16
Piperacillin	≤ 17	18 -20	≥ 21	≥ 128	32 -64	≤ 16
Cefazolin	≤ 14	15 - 17	≥ 18	≥ 32	16	≤ 8
Cefoperazone	≤ 15	16 - 20	≥ 21	≥ 64	32	≤ 16
Cefuroxime	≤ 14	15 - 22	≥ 23	≥ 32	16	≤ 8
Ceftazidime	≤ 14	15 - 17	≥ 18	≥ 32	16	≤ 8
Cefotaxime	≤ 14	15 - 22	≥ 23	≥ 64	16 - 32	≤ 8
Ceftriaxone	≤ 13	14 - 20	≥ 21	≥ 64	16 - 32	≤ 8
Aztreonam	≤ 15	16 - 21	≥ 22	≥ 32	16	≤ 8
Cefoxitin	≤ 14	15 - 17	≥ 1 8	≥ 32	16	≤ 8

Table 1 lists the NCCLS susceptibility criteria for MIC and zone diameter determinations for all the antibiotics utilized in this study (94b, 94c)

TABLE 1A ANTIBIOTIC CONCENTRATION RANGES TESTED IN AGAR DILUTION MIC TESTS

ANTIBIOTIC	LOWEST CONCENTRATION (μg/mL)	HIGHEST CONCENTRATION (μg/mL)
	0.5	256
Piperacillin	0.5	256
Ticarcillin	0.25	128
Cefazolin	0.25	128
Cefuroxime	0.06	32
Cefoperazone	0.03	16
Ceftazidime	0.03	16
Cefotaxime		

5. DOUBLE DISC SYNERGY TEST (DDST)

The DDST which tests for ß-lactamase inhibition by CA was done on all the *K. pneumoniae* strains in Series I with CAZ, CFP, PRL, and KZ. All the isolates were grown at 37°C overnight in TSB and diluted with sterile normal saline the next day to a 0.5 MacFarland Standard in TSB in logarithmic growth phase and spread onto a MHA plate. An antibiotic disc containing AMC was placed in the centre of the petri plate. The CAZ, CFP, PRL, and KZ discs were placed at a calculated distance from the AMC disc. The calculated distance for each isolate was $0.5\ (d_1 + d_2) + 5$ mm. where d was the zone size diameter to the ß-lactam and d_2 was the zone size diameter to AMC. The derivation of this equation is described in the Results. (The d_1 and d_2 were previously measured by the NCCLS Disc Diffusion tests). After overnight incubation at 37°C a positive DDST was observed as zone size enhancement of the ß-lactam zone towards the AMC disc. This is a presumptive test for the presence in an isolate of a clavulanic acid-sensitive ß-lactamase with activity against the antibiotic being tested (6,80,83) (see Figures 1 and 2).

Figure 1: This figure illustrates the result of the DDST with the ß-lactam and AMC discs 30 mm apart. This is the test procedure as described in the literature by Vatopoulos (80). The enhancement of the zone of inhibition around the CFP disc was a positive result indicating the presence of a CFP-inactivating enzyme in this *K. pneumoniae* isolate. The zones of inhibition around both the KZ and PRL discs were small and were not enhanced towards the AMC disc. This would be classified as a negative result.

Figure 1

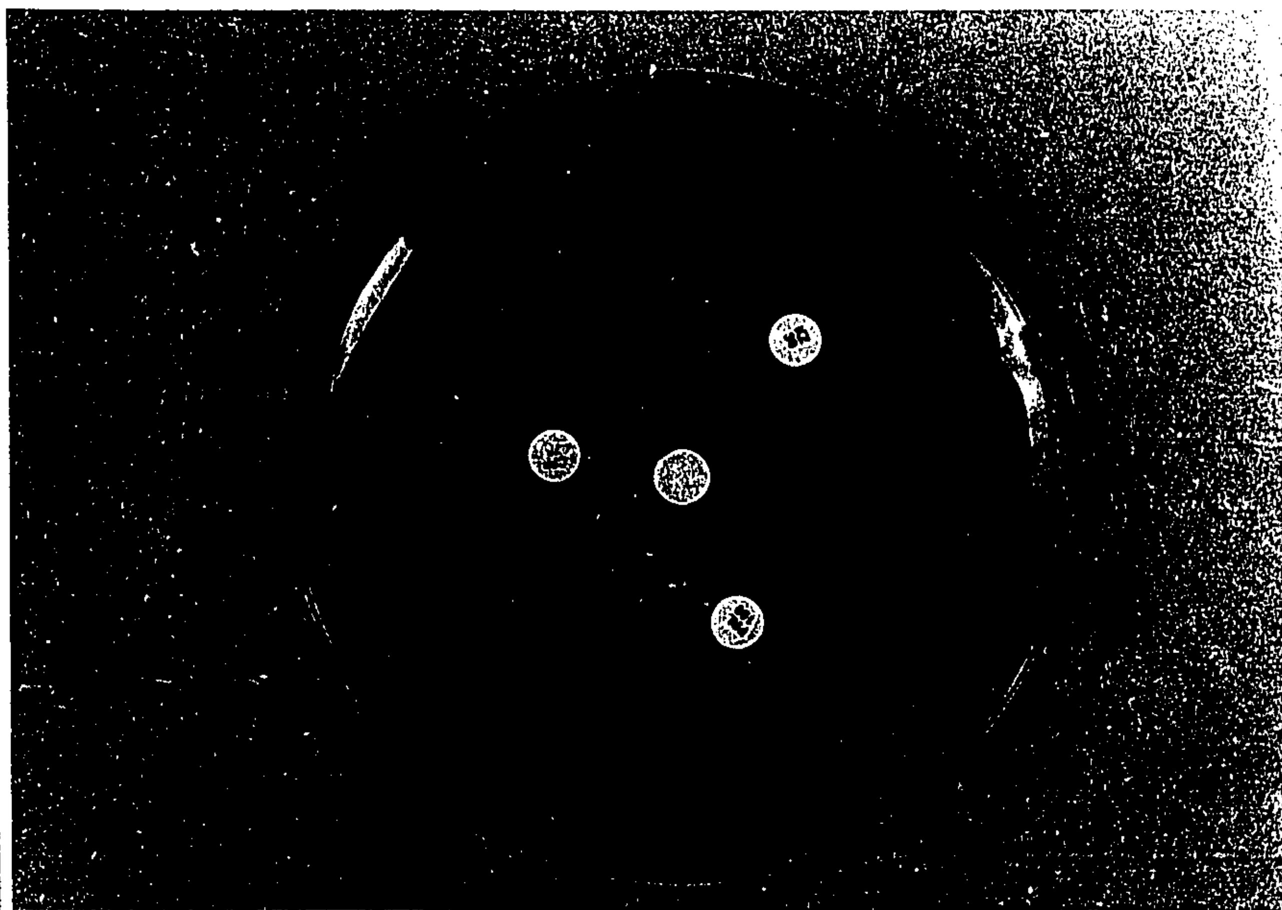
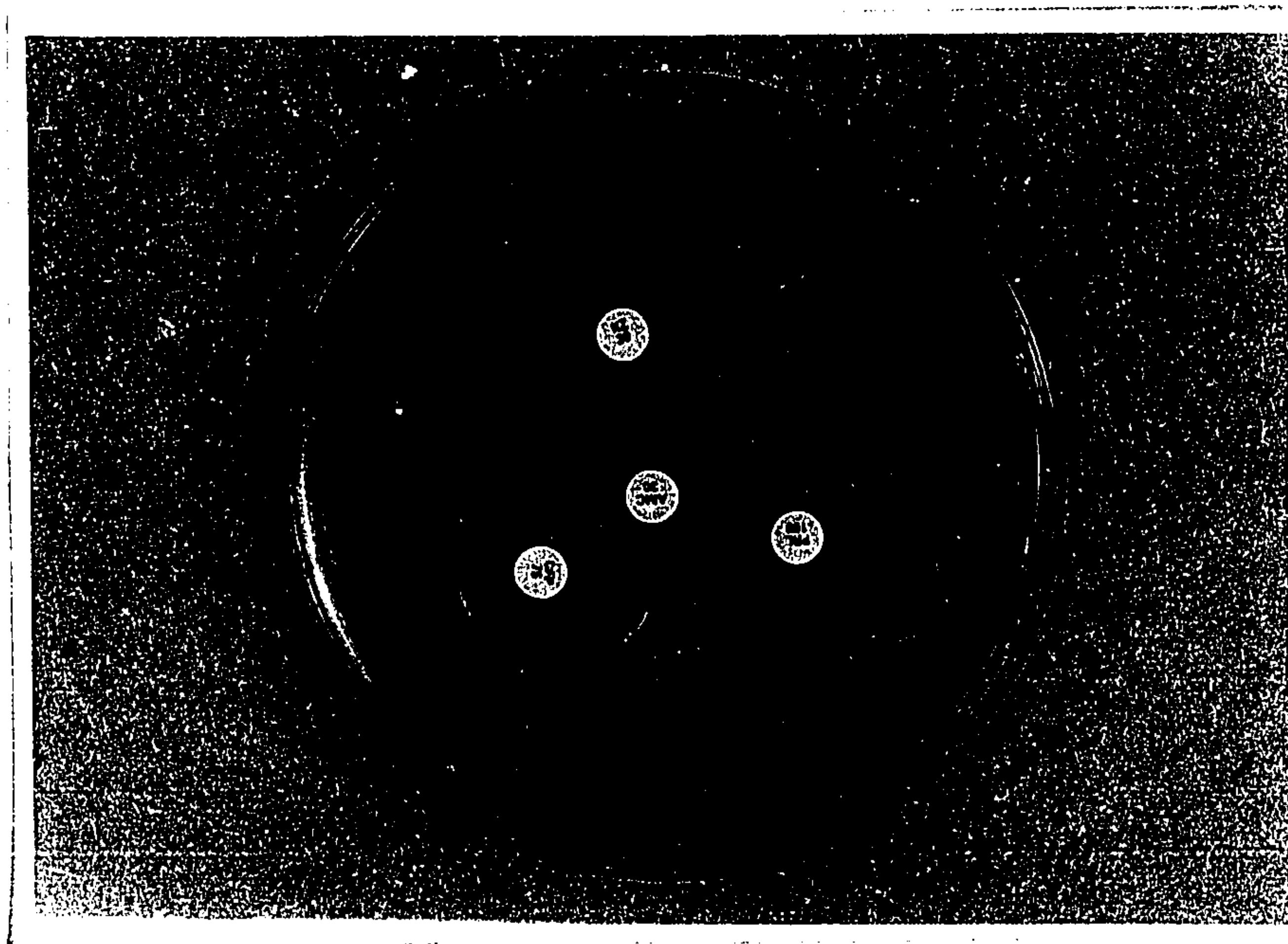


Figure 2: This figure illustrates the result of the DDST with the same *K. pneumoniae* isolate as in Figure 1. This DDST procedure utilized the calculation for disc distance determined by the zone diameters to the ß-lactam and AMC on initial susceptibility tests. With the placement of the CFP, KZ, and PRL discs at the calculated distance from the AMC disc, there was zone diameter enhancement towards the AMC disc for all three ß-lactams. The DDST results were positive for all three ß-lactams.

Figure 2

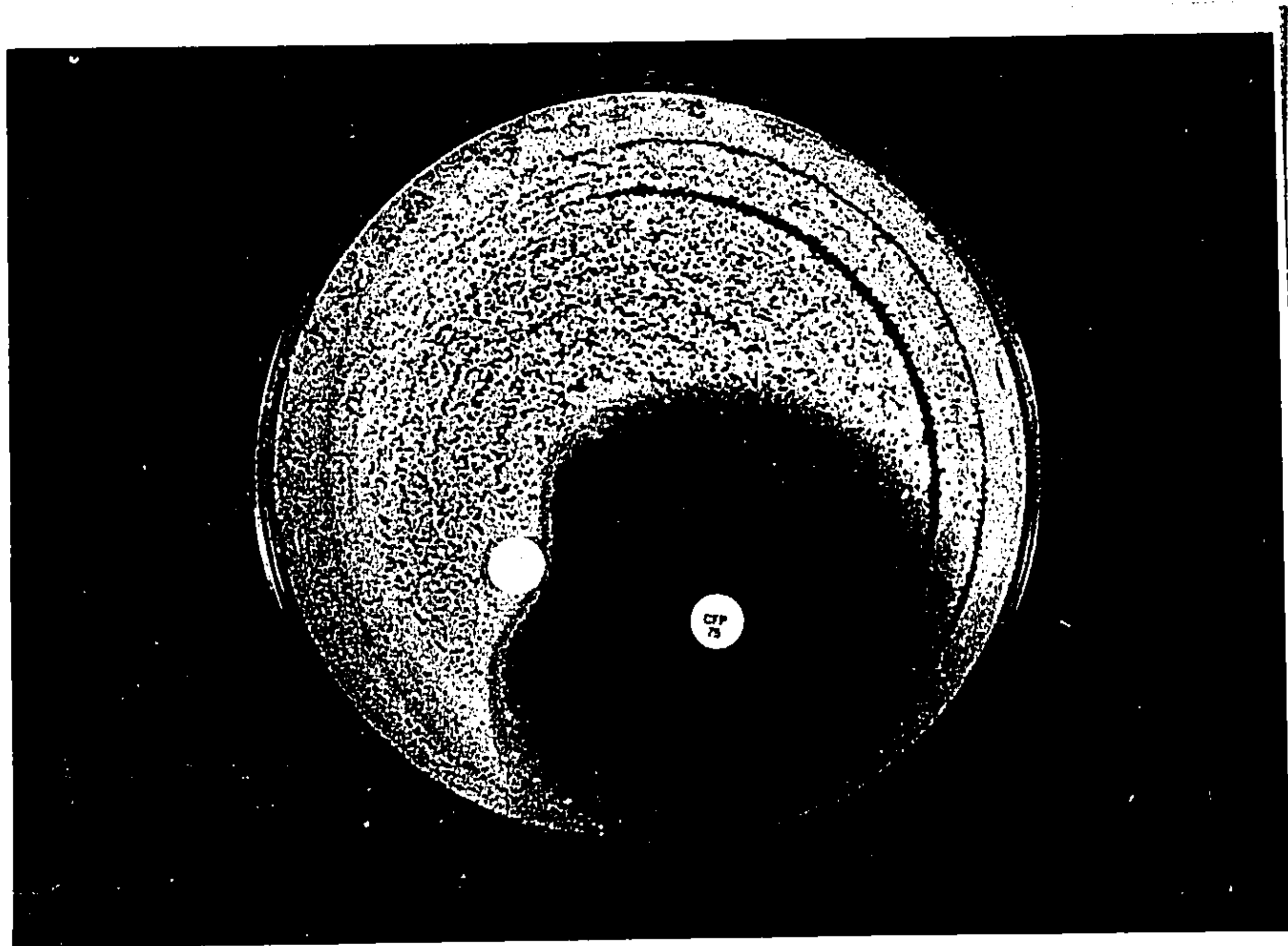


6. MASUDA TECHNIQUE

The Masuda Technique was performed to determine ß-lactam inactivation by crude ß-lactamase extract as described by Masuda(94a). Three isolates (KP-1212, KP-1, KP-68) that showed a positive DDST to ß-lactam antibiotics CAZ and CFP were repeatedly frozen to -70°C and thawed seven times to release the ß-lactamase from the periplasmic space. The isolates were washed with phosphate buffered saline (PBS) and then centrifuged at 2500 rpm for 15 minutes. The supernatant containing the enzyme was saved and the cellular debris discarded. A MHA plate was spread with a lawn of *Micrococcus lutea* ATCC 9431 (indicator strain). Blank antibiotic discs impregnated with 15 μl of enzyme extract at a concentration of 1:1, 1:2, 1:4, and 1:8 of the crude enzyme extract diluted with PBS were placed at a distance from the CAZ and CFP discs that would be within the periphery of the zone diameter to that particular antibiotic. (These zones had been previously measured by the NCCLS Disc diffusion tests). These plates were incubated overnight at 37°C. Inactivation of the ß-lactam was noted by growth of the indicator strain within the expected zone of inhibition (94a). (See Figure 3).

Figure 3: The Masuda Technique described the inactivation of ß-lactamase by crude enzyme extract. A MHA plate was spread with *Micrococcus lutea* ATCC 9431. Blank discs were impregnated with crude ß-lactamase extract from *K. pneumoniae* isolates. This disc was placed within the zone of inhibition to CFP by *Micrococcus lutea*. The growth of the indicator *Micrococcus* around the disc into the normal zone of inhibition is a positive.

Figure 3



7. FLUORESCENCE TEST

7.1 Principle of the Test

Methylumbeliferyl-ß-D-Glucuronide (MUG) is a fluorogenic compound used routinely in microbiology to detect the presence of *E. coli* in urine and water samples (96). *E. coli* contains glucuronidase which hydrolyzes non-fluorescence MUG to free glucuronide and a fluorescent product, methylumbeliferone. This enzyme is not present in *K. pneumoniae* so that growth of *E. coli* on MUG gives fluorescence whereas growth of *K. pneumoniae* does not. Using this fluorogenic compound and the principle satellitism, a novel test for ESBL was developed during the course of this work as follows: Serial dilutions of antibiotics against which enzyme activity was to be demonstrated were incorporated into Mueller-Hinton agar containing methylumbeliferyl-ß-D-glucuronide (MUG) 70 μg/mL (see Figures 4 and 5). These plates were seeded with an *E. coli* lawn and spots of the *K. pneumoniae* strains were then inoculated on the lawn. At antibiotic concentrations above the MIC for *E. coli*, fluorescence of the lawn was inhibited. If a *K. pneumoniae* spot on the *E. coli* lawn synthesized a ß-lactamase that hydrolysed the ß-lactam in the MHA, satellite growth of *E. coli* would occur due to degradation of the antibiotic at that spot. Growth of *E. coli* results in the splitting of MUG and appears as a ring of fluorescence under ultraviolet light. The incorporation into the medium of CAindicated if the enzyme was neutralized.

7.2 Development of the Fluorescence Test

On initial qualitative fluorescence testing there was good correlation with the DDST. The range of antibiotic concentrations required were those that spanned the MIC of *E. coli* to whatever ß-lactam was being tested 1/2 MIC, MIC, 2 x MIC and 4 x MIC for *E. coli* were utilized. A positive fluorescence at 4 x MIC was graded as 3+, at 2 x MIC as 2+, and at the MIC as 1+. At the ß-lactam concentration of 1/2 MIC for *E. coli* there were no discrete rings of fluorescence due to the confluent growth of the *E. coli* lawn with visible spots of *K. pneumoniae* (see Figures 6,7,8,9).

Figure 4: Illustrates the fluorescence of *E. coli* when grown in the presence of MUG.

This is the positive control for the fluorescence test.

Figure 4

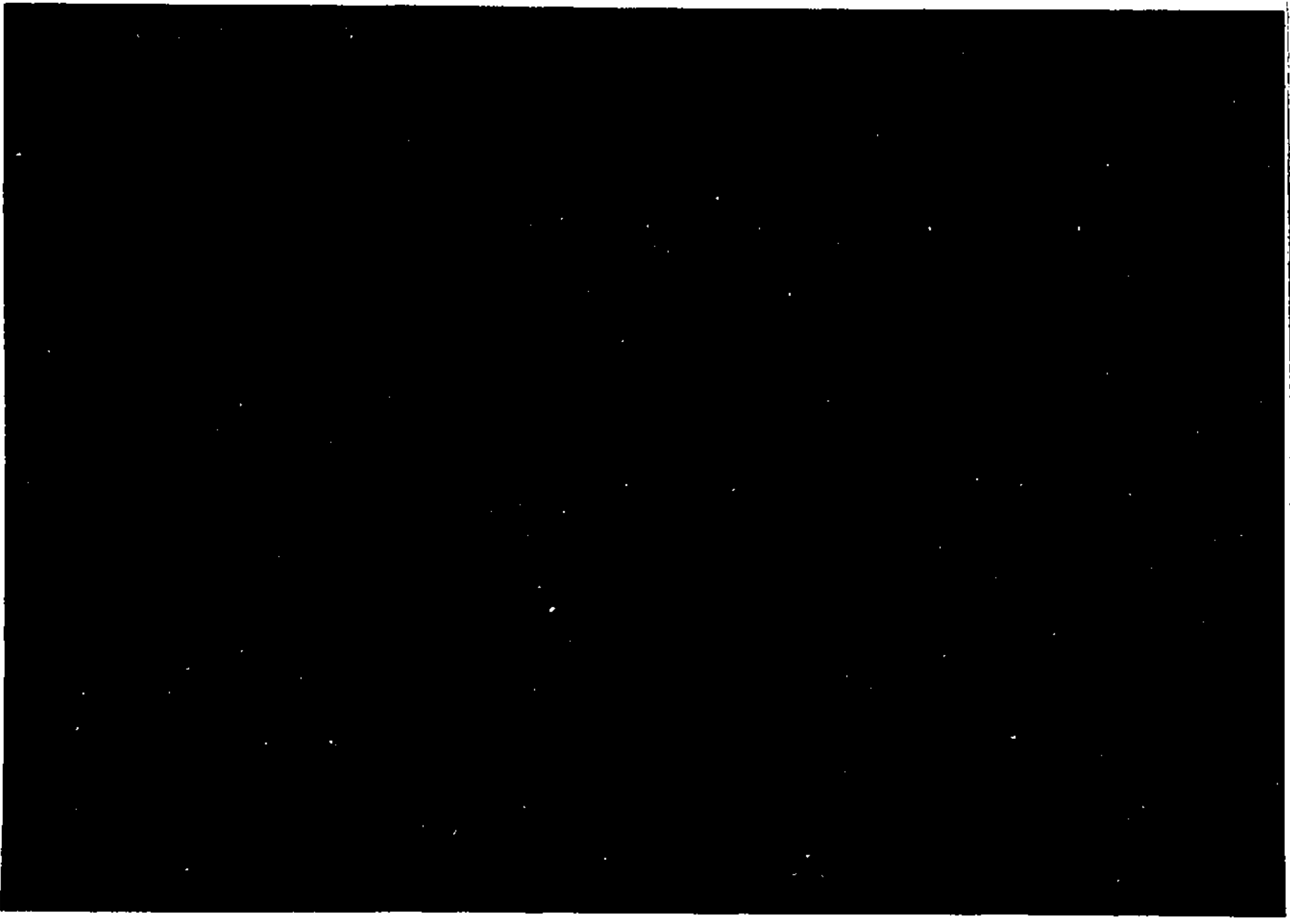


Figure 5: Illustrates the negative fluorescence of *K. pneumoniae* when grown in the presence of MUG. *K. pneumoniae* does not possess the enzyme glucuronidase. This plate represents the negative control for the fluorescence test.

Figure 5

Figure 6: Illustrates a 3+ positive fluorescence with the top two and bottom four *K. pneumoniae* dots on this MHA-MUG plate. The two centre *K. pneumoniae* dots are negative. This plate contains ß-lactam 4x MIC of *E. coli*. The satellite colonies of *E. coli* around the *K. pneumoniae* dots are visible particularly in the upper left and the bottom dots.

Figure 6

Figure 7: Illustrates a 2+ fluorescence for the two top and bottom four *K. pneumoniae* dots. The middle two *K. pneumoniae* dots are negative. The concentration of ß-lactam in this MHA-MUG plate is 2 x MIC for *E. coli*. The satellite colonies of *E. coli* growing around the *K. pneumoniae* dots are visible particularly around the upper two dots and bottom dots.

Figure 7

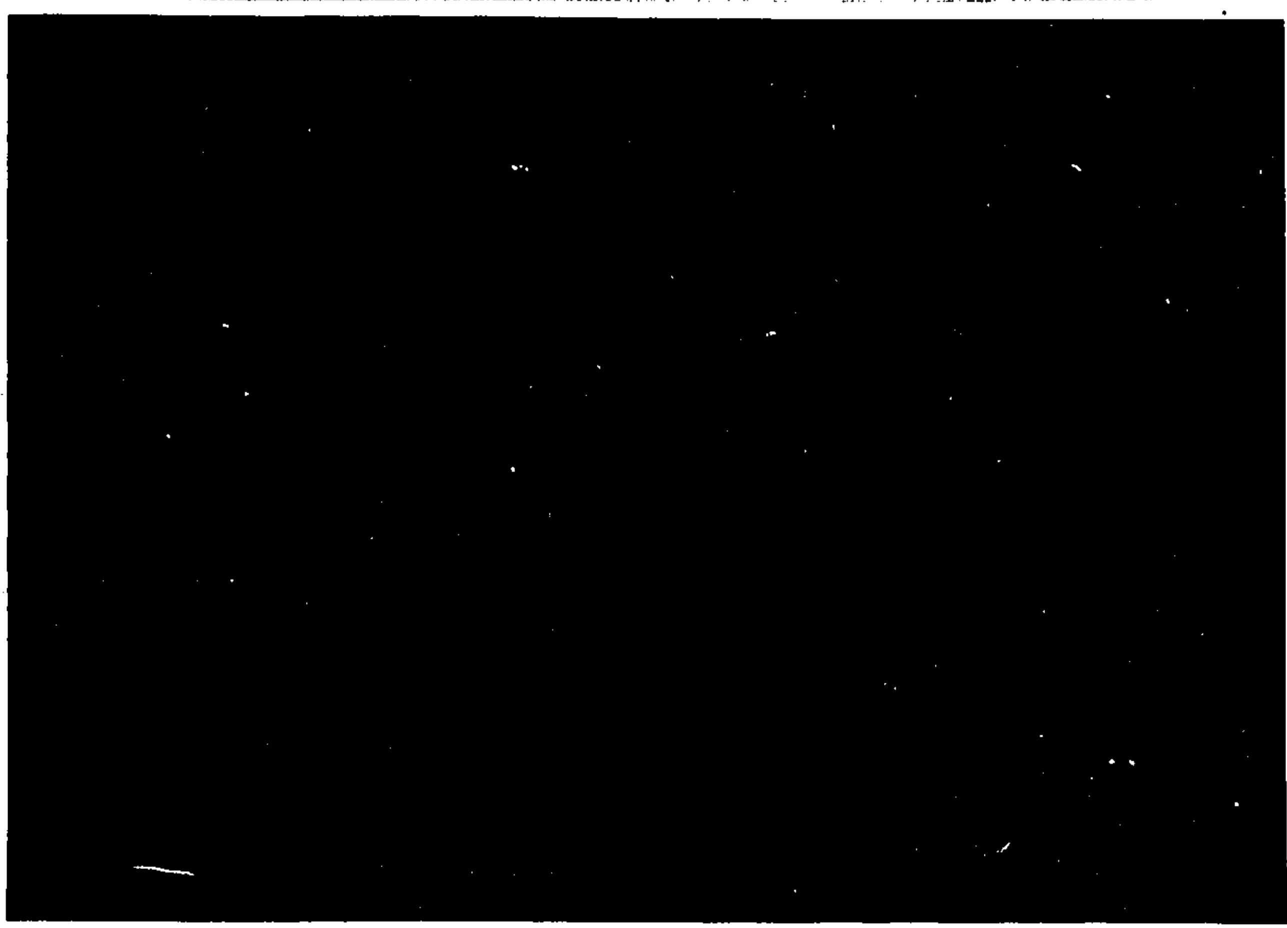


Figure 8: Illustrates a 1+ fluorescence in the middle two *K. pneumoniae* dots which were negative in Figures 6 and 7. The concentration of ß-lactam in this MHA-MUG plate was the MIC for *E. coli*.

Figure 8

Figure 9: This MHA-MUG plate contained ß-lactam at 1/2 MIC for *E. coli*. At this concentration the growth of *E. coli* is not inhibited which is seen by diffuse growth and subsequent fluorescence on the entire plate. If a *K. pneumoniae* dot exhibits fluorescence around it only at this concentration, the result is classified as negative as the *E. coli* grows readily at this concentration of ß-lactam without enzyme degradation.

Figure 9

7.3 Fluorescence Test Procedure

All Series I isolates were screened with the fluorescence test. Four serial dilutions of each ß-lactam antibiotic was incorporated into MHA containing MUG at 70 μg/mL . *E. coli* ATCC 25922, grown in TSB for two hours at 37°C and diluted with sterile normal saline to a 0.5 MacFarland Standard in stationary phase (obtained by placing the broth at 4°C for one hour), was seeded on to the MHA-MUG plates. On this seeded MHA-MUG plate were placed 37 dots of *K. pneumoniae* isolates by a Steers Replicator (95). The concentration of *K. pneumoniae* used for the dots was 0.5 MacFarland Standard in logarithmic phase obtained by growing the *K. pneumoniae* isolates in TSB for three hours at 37°C and diluting the broth with sterile normal saline to a 0.5 MacFarland Standard. The MHA-MUG plates were incubated at 37°C overnight. The next day all the plates were examined under a uv light source for fluorescence.

For all *K. pneumoniae* isolates that had a 1+, 2+, or 3+ fluorescence, the test was repeated with the ß-lactam as well as a series of MHA-MUG plates with ß-lactam and CA at 1.0 μg/mL and MHA-MUG with OX at 30 μg/mL to determine enzyme type. The specificity of this fluorescence test to detect resistance by enzymatic activity was determined by testing the *K. pneumoniae* isolates with ciprofloxacin. (See Discussion). Table 2 lists the ß-lactams and ciprofloxacin tested and the concentration of antibiotic in each plate.

TABLE 2: ANTIBIOTICS AND CONCENTRATIONS FOR FLUORESCENCE TEST

ANTIBIOTIC	ANTIBIOTIC CONCENTRATION (μg/mL)			
	4 x MIC	2 x MIC	MIC	1/2 MIC
Cefazolin	4	2	1.0	0.5
Cefoperazone	2	1.0	0.5	0.25
Cefuroxime	16	8	4	2
Ceftazidime	0.5	0.25	0.12	0.06
Cefotaxime	0.5	0.25	0.12	0.06
Ceftriaxone	0.5	0.25	0.12	0.06
Cefoxitin	16	8	4	2
Aztreonam	0.5	0.25	0.12	0.06
Ciprofloxacin	0.5	0.25	0.12	0.06

Table 2 lists all the antibiotics and the concentrations tested have a *K.pneumoniae* for fluorescence. Four concentrations of each antibiotic spanning 4x the MIC to 1/2 the MIC for *E. coli* was tested for each isolate.

RESULTS

1. SUSCEPTIBILITY TESTS

1.1 MIC Determinations

Agar dilution MIC's were determined for Series I isolates using NCCLS criteria with PRL, TIC, KZ, CXM, CFP, CAZ, and CTX (94b). There were 5/94 (5%) isolates resistant to PRL and 68/94 (72%) isolates were resistant to TIC among the Series I isolates. Cefazolin resistance (MIC ≥32 μg/mL) was seen in 6/94 (6%) of the Series I isolates. There were 5/94 (5%) isolates resistant to CXM. All Series I *K. pneumoniae*'s were susceptible to CAZ, CTX, and CFP. Five isolates, KP-1212, KP-1730, KP-1409, KP-7538, and KP-68 had an MIC > 2 μg/mL to CAZ and one (KP-68) had an increased MIC > 1 μg/mL to CTX. These MIC's were still within the susceptible range (see Tables 2 and 3). Agar dilution MIC's were done in triplicate. None were greater than one dilution variation. The result was taken as the MIC in two of the three results.

1.2 NCCLS Zone Diameter determinations

Zone diameters were done in duplicate for Series I isolates and variations were less than 3 mm. Series II isolates were done once. All this procedures were carried out according to NCCLS guidelines (94c).

The zone diameters for TIC, PRL, and AMP were measured only on the Series I *K. pneumoniae* isolates. There were 50/94 (53%) isolates resistant to TIC and 20/94 (23%) isolates resistant to PRL. AMP resistance was seen in 91/94 (97%) of the isolates (see Table 3).

Zone diameters to KZ were measured for both Series I and II *K. pneumoniae*

isolates. In Series I there were 7/94 (7%) isolates moderately susceptible or resistant to KZ with a zone diameter of ≤17 mm of which one isolate was resistant with a zone of ≤ 14 mm. In Series II, 2/127 isolates (OCH-59 with a zone of 16 mm and OCH-118 with a zone of 15 mm) were moderately susceptible and one (OCH-71 with a zone of 12 mm) was resistant to KZ (see Table 3).

The second generation ß-lactam tested on Series I and II was CXM which is susceptible to hydrolysis by derivatives of the TEM-1 and SHV-1 enzymes such as TEM-3, TEM-9, TEM-10 (33,38,42,46). Five isolates in Series I (KP-1409, KP-1730, KP-33, KP-68 and KP-73) were resistant to CXM. In Series II one isolate, OCH-45, with a zone diameter <14 mm, was resistant to CXM while all the other isolates were susceptible (see Table 3).

Although CFP is a third generation cephalosporin, it is very susceptible to ß-lactamase hydrolysis. It is no longer used clinically for treating patients but we utilized it in this study because it was a suitable antibiotic for the development of the fluorescence test. One isolate of Series I, KP-83, and one isolate in Series II, OCH-81, were resistant to CFP (see Table 3).

In Series I (94 isolates) and Series II (127 isolates) *K. pneumoniae* isolates, the zone diameters indicated that all of these organisms were susceptible to CAZ, CTX, CRO, and ATM. Zone diameters for FOX were measured for Series II isolates and all 127 *K. pneumoniae* isolates were susceptible (see Table 3).

TABLE 3: SUSCEPTIBILITY OF *K. pneumoniae*ISOLATES (SERIES I AND II) TO 8 ß-LACTAMS

	NCCLS DISC TEST		MIC	
ANTIBIOTIC	# KP R	% R	# KP R	% R
SERIES I (n=94)				
Ampicillin	91	97	ND	ND
Ticarcillin	50	53	68	72
Piperacillin	22	23	5	5
Cefazolin	1	1	6	6
Cefuroxime	5	5	5	5
Cefoperazone	1	1	0	0
Ceftazidime	0	0	0	0
Cefotaxime	0	0	0	0
SERIES II (n=127)				
Cefazolin	1	0.8		
Cefuroxime	1	0.8		
Cefoperazone	1	0.8		
Ceftazidime	0	0		
Cefotaxime	0	0		
Ceftriaxone	0	0		
Aztreonam	0	0		
Cefoxitin	0	0		

The susceptibility tests results for the NCCLS disc diffusion and the NCCLS agar dilution MIC results on Series I isolates and the NCCLS disc diffusion test on the Series II are listed in this table. The number and percentage of resistant isolates are inidicated for each antibiotic tested.

2. DOUBLE DISC SYNERGY TEST (DDST)

Although all the 94 Series I *K. pneumoniae* isolates were susceptible to third generation cephalosporin by both the zone diameter and MIC determinations, those organisms that showed a minor decrease in susceptibility by zone diameter testing and/or that had an increased MIC to the ß-lactam were a concern. Was this decreased susceptibility due to ß-lactamase activity? The DDST was done on all 94 *K. pneumoniae* isolates with CAZ, CFP, KZ, and PRL. When this test was done according to the procedure reported in the literature (80) all of the results were negative. The procedure reported in the literature was to place the AMC disc and the ß-lactam disc 30 mm apart. If the organism had a zone diameter less than 20 mm to one of those antibiotics, then the 30 mm distance was too great to give a positive result by which there was zone diameter enhancement towards the AMC disc. (Figures 1 and 2). Therefore, a calculation was devised to take into account the individual zone sizes of each isolate: distance apart = 0.5 (d_1 + d_2) mm + 5 mm where d_1 is the zone diameter of that isolate to the ß-lactam and d_2 is the zone diameter to AMC for that isolate. Once this calculation was applied for each antibiotic and each isolate, it was evident that there were *K. pneumoniae* isolates that did possess ß-lactamase enzyme inhibited by clavulanic acid. The isolates in Series I which had a positive DDST were more numerous than those *K. pneumoniae*'s resistant to the ß-lactams in the initial susceptibility testing. The DDST results for each antibiotic tested are listed in Table 4.

With PRL 39/94 (41%) of the isolates had a positive DDST with 22/94 (23%) of the isolates resistant by zone diameter and 5/94 (5%) resistant by MIC. This is likely due to

the prominence of TEM-1 and SHV-1. In this series 23/94 (24%) had a positive DDST to KZ. Only one of these 23 isolates was resistant by zone diameter while 6/94 were resistant to KZ by MIC. Two of the Series I isolates (KP-1212 and KP-40) had a positive DDST to CAZ. Both of these isolates had zone diameters and MIC's indicating susceptibility, although KP-1212 had a MIC of 4 μg/mL. which is probably a significant decrease in susceptibility (83).

The DDST results indicated that although the majority of *K. pneumoniae* were susceptible by zone diameter and MIC determinations, some of the isolates did harbour a ß-lactamase enzyme that was inhibited by CA which could indicate a plasmid-mediated ß-lactamase (see Table 4).

3. MASUDA TECHNIQUE

The Masuda Technique (94a) was applied to three isolates (KP-1212, KP-1, KP-68) to determine if this test was more sensitive and thus a better screening test than the DDST in the detection of ß-lactamases. All these three organisms had positive DDST to CFP. KP-1212 was also DDST positive for CAZ. In the results for Masuda Test, the only positive for CFP was KP-1212 (see Table 5). At this point, it was decided that this test was not as sensitive as the DDST. Therefore, this test was not done on any other of the 94 isolates in Series I and the test was not included further in this study (see Table 5 and Figure 3).

TABLE 4: DDST RESULTS

ANTIBIOTIC	# POSITIVE / TOTAL ISOLATES	% POSITIVE
SERIES I (n=94)		
Piperacillin	39/94	41
Cefazolin	23/94	24
Cefoperazone	19/94	20
Ceftazidime	2/94	2

Table 4 lists the DDST results for the four antibiotics tested with the Series I isolates. Both the number and percentage of positives by DDST are listed for each antibiotic.

TABLE 5: COMPARISON OF SENSITIVITY IN SCREENING TESTS FOR THE DETECTION OF ß-LACTAMASE

STRAIN	DDST		MASUDA		FLUORESCENCE	
	CAZ	CFP	CAZ	CFP	CAZ	CFP
KP-1212	+	+	-	+	3+	3+
KP-1	-	+	-	-	1+	2+
KP-68	-	+	-	-	3+	1+

Table 5 compares the results of the DDST, Masuda and Fluorescence tests on three *K. pneumoniae* isolates. This table indicates that the fluorescence test is more sensitive than the DDST and Masuda for the detection of ESBL.

4. FLUORESCENCE TESTING

The fluorescence test was developed utilizing methylumbeliferyl-ß-D-Glucuronide (MUG) in order to have a reliable and sensitive screening test to determine whether a *K. pneumoniae* isolate did possess an extended-spectrum ß-lactamase (ESBL) that could hydrolyse extended-spectrum (third generation) ß-lactam antibiotics. The principle of the test is described in Materials and Methods under Fluorescence Test. All Series I isolates were tested for fluorescence with CAZ, CFP, KZ, PRL (see Table 6).

Many experiments were done exploring the impact of varying the concentrations of both the indicator (*E. coli*) and the test (*K. pneumoniae*) organisms and incubation time during the development process. Both the *E. coli* and *K. pneumoniae* isolates were grown in TSB for four hours and diluted to a 0.5 MacFarland Standard. The *K. pneumoniae* isolates were diluted 1/10 and 1/100 with PBS before being placed into the Steer's Replicator (95) and spotted onto the MHA-MUG plates with ß-lactam. After overnight incubation there was no fluorescence around the spots of *K. pneumoniae* that had been diluted 1/100.

It was found that using *K. pneumoniae* at a 0.5 MacFarland Standard in logarithmic growth phase was critical for the best results. This was obtained by incubating the *K. pneumoniae* in TSB at 37°C for three hours and diluting with sterile normal saline to a 0.5 MacFarland Standard. The concentration of *E. coli* was not as critical and it was not necessary to have the *E. coli* in logarithmic growth phase. The *E. coli* was grown in TSB at 37°C for two hours, diluted to a 0.5 MacFarland Standard and then refrigerated at 4°C

until the MHA-MUG plates were seeded with the *E. coli*. These conditions resulted in the best fluorescence of the satellite colonies around the spots of *K. pneumoniae*. The explanation for this is probably that the enzyme production is maximal during logarithmic growth phase. The delay in growth resulting from the use of stationary phase culture of *E. coli* allowed maximum time for the enzyme to inactivate the antibiotic. An incubation period longer than 18 hours caused a diffusion of the fluorescence outward in the MHA-MUG plates at which point discrete rings of fluorescence were not discernible.

The fluorescence test was done using PRL with Series I isolates and 73/94 (78%) of these *K. pneumoniae* isolates had enzymatic activity. There were a larger number of isolates that showed enzymatic activity against KZ (a first generation cephalosporin) than to the third and second generation cephalosporins. In Series I there were 41/94 (44%) with activity against KZ indicated by the positive fluorescence test. Two *K. pneumoniae* isolates showed fluorescence against CAZ (KP- 1212 and KP-40) indicating that these *K. pneumoniae*'s possessed an enzyme that had activity against this third generation ß-lactam. In Series I 34/94 (36%) were fluorescence positive with CFP (see Table 6).

After developing this fluorescence test with the Series I *K. pneumoniae* isolates we then looked at the consecutive *K. pneumoniae* isolates in Series II to determine the actual incidence of ESBL's in *K. pneumoniae*'s isolated in the Ottawa area. With the fluorescence test there were fewer isolates in Series II with 25/127 (20%) that were fluorescence positive than in Series I 41/94 (44%) for KZ. The number of strains that had a positive fluorescence test in Series II for CXM was 2/127 (1.5%), for CFP was 61/127

(48%), for CAZ was 2/127 (1.5%), and for ATM was 1/127 (0.8%). All the Series II *K. pneumoniae* isolates were negative for fluorescence with CRO, CTX, and FOX (see Table 6).

The specificity of the Fluorescence Test was determined by examining the neutralization of ß-lactamase activity by CA (see Figures 10 and 11). In the Series I *K. pneumoniae* isolates 32/34 that had enzymatic activity against CFP, 38/41 that had activity against KZ and 69/73 that had activity against PRL were neutralized by CA. All four isolates, KP-1212, KP-40, OCH-45, and OCH-81, that had enzymatic activity against CAZ were all neutralized by CA. The enzymatic activity against ATM seen with OCH-45 was neutralized by CA (see Table 7). Specificity was also determined by testing a *K. pneumoniae* with known resistance to Ciprofloxacin but negative by fluorescence. The results with both CA and Ciprofloxacin establish specificity of this fluorescence test (see Discussion).

4.1 Correlation between Fluorescence and DDST

Although the DDST is the gold standard test for the detection of CA-susceptible ß-lactamases, the fluorescence test developed was more sensitive. Both the DDST and Fluorescence test were done on Series I *K. pneumoniae* isolates to establish the sensitivity of the fluorescence test. With all the antibiotics tested there was a higher incidence of positives with the fluorescence compared with the DDST. All the isolates that were DDST positive were also positive for fluorescence. Table 8 compares the positives for both the DDST and Fluorescence Test in the Series I isolates. The sensitivity of the fluorescence

test is established in that none of the isolates were DDST positive and Fluorescence negative.

Figure 10: Illustrates a 3+ fluorescence with the top two and bottom four *K. pneumoniae* dots. This MHA-MUG plate contains ß-lactam at 4 x MIC for *E. coli*.

Figure 10

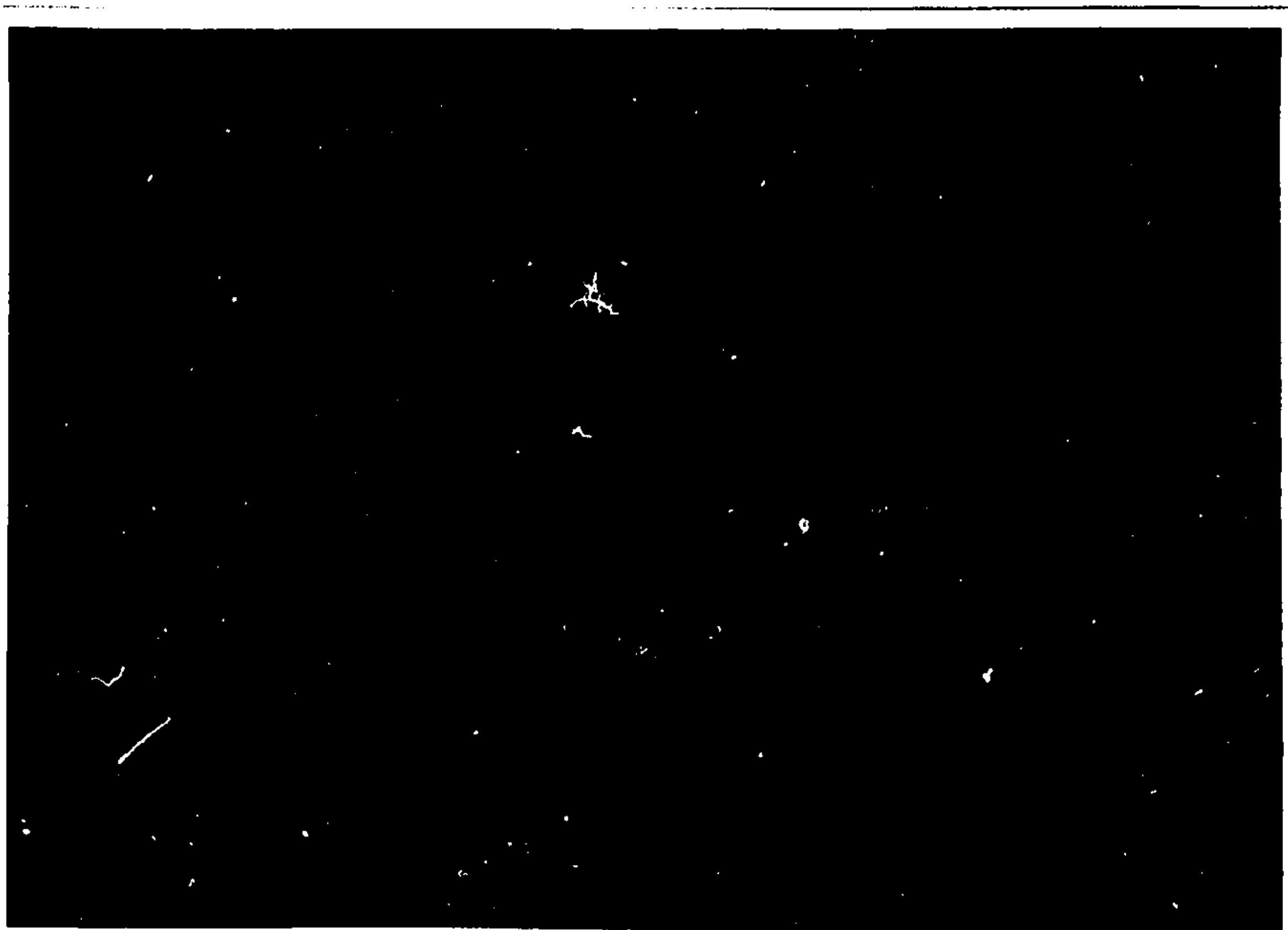


Figure 11: Illustrates the neutralization of the enzyme seen in Figure 10. This plate was in the same series as the plate in Figure 10 but contains both ß-lactam at 4 x MIC for *E. coli* and CA at 1.0 μg/mL. In comparison to the plate in Figure 10 all but one *K. pneumoniae* dot has been neutralized. The growth of one of the upper dots and all of the four lower dots has been inhibited. The CA acid neutralized the enzyme in those dots, the ß-lactam was not hydrolysed, and there was no satellite growth of *E. coli*.

Figure 11

TABLE 6: POSITIVE FLUORESCENCE TEST

ANTIBIOTIC	# KP FLUORESCENCE +	% +
SERIES I (n=94)		
Piperacillin	73	78
Cefazolin	41	44
Cefoperazone	34	36
Ceftazidime	2	2
SERIES II (n=127)		
Cefazolin	25	20
Cefoperazone	61	48
Cefuroxime	2	1.5
Ceftazidime	2	1.5
Aztreonam	1	0.8
Ceftriaxone	0	0
Cefotaxime	0	0
Cefoxitin	0	0

Table 6 lists the number and percentage of isolates positive by fluorescence in both the Series I and II isolates with the antibiotics tested.

TABLE 7: NEUTRALIZATION OF FLUORESCENCE BY CLAVULANIC ACID

ANTIBIOTIC	# ISOLATES MUG +	# ISOLATES NEUTRALIZED	%
SERIES I (n=94)			
Piperacillin	73	69	95
Cefazolin	41	38	93
Cefoperazone	34	32	94
Ceftazidime	2	2	100
SERIES II (n= 127)			
Cefazolin	25	24	96
Cefoperazone	61	54	89
Cefuroxime	2	2	100
Ceftazidime	2	2	100
Aztreonam	1	1	100

Table 7 shows the results of the neutralization of fluorescence by clavulanic acid in both Series I and II isolates that were fluorescence positive to the antibiotics listed here. The number of fluorescence positive, the number of those positives neutralized by clavulanic acid and the percentage of those neutralized are listed.

TABLE 8: COMPARISON BETWEEN DDST AND FLUORESCENCE TESTS

	DDST + MUG+	DDST+ MUG-	DDST- MUG+	DDST- MUG-
SERIES I (n=94)				
Ceftazidime	2	0	0	92
Cefoperazone	19	0	15	60
Cefazolin	24	0	17	53
Piperacillin	39	0	21	21

Table 8 shows the comparison of the fluorescence and DDST results on the Series I isolates with CAZ, CFP, KZ and PRL.

4.2 Correlation between Fluorescence and DDST with MIC's in Series I

In Figure 12 the comparison of Fluorescence and MIC's with PRL indicated that all the *K. pneumoniae* isolates that were moderately susceptible or resistant (MIC ≥32 μg/mL) were fluorescence positive. This is what we would expect with resistant organisms. There were a significant number of *K. pneumoniae* isolates that were susceptible but were able to hydrolyse PRL indicated by positive fluorescence. There were only 39/94 isolates that were positive with DDST but as indicated in Figure 12, there was a higher incidence of positive fluorescence with 73/94 exhibiting fluorescence. In the Series I isolates 6/94 were resistant to KZ by MIC seen in Figure 13. Forty-one isolates were fluorescence positive of which only 23 were DDST positive. There were isolates that were positive by fluorescence at the lowest MIC 0.06 μg/mL for CFP. The majority of isolates with a MIC to CFP ≥2 μg/mL were positive by fluorescence seen in Figure 14. In Series I all the isolates were susceptible to CAZ by MIC as seen in Figures 15. Two isolates (KP-1212 and KP-40) exhibited both positive fluorescence and a positive DDST. KP-1212 had MIC of 4 μg/mL while KP-40 had a low susceptible MIC of 0.06 μg/mL.

4.3 Correlation between Fluorescence and DDST with Zone Diameters in Series I

The comparison between zone diameter and fluorescence test with PRL is documented in Figure 16. In the isolates with the larger zones, indicating susceptibility, there were more *K. pneumoniae*'s isolates positive on fluorescence testing than those that were negative. Figure 17 indicates that the incidence of positive fluorescence was seen

throughout the entire zone diameter range for KZ but only one isolate was resistant with a zone of ≤14 mm. All the *K. pneumoniae* isolates with a zone diameter ≥22 mm. would be reported as susceptible even though there was evidence of enzymatic activity. There were 34 Series I isolates positive with the fluorescence test for CFP of which only 19 were also positive with DDST. See Figure 18. All the *K. pneumoniae* isolates were susceptible to CAZ by zone diameter but two had enzymatic activity. One isolate had a zone of 25 mm. and the other 29 mm as seen in Figure 19.

4.4 Correlation between Fluorescence and Zone Diameter in Series II

With the Series II *K. pneumoniae* isolates the true incidence of resistance was determined by screening for the presence of ß-lactamase comparing the zone diameters and the fluorescence test with the following antibiotics: KZ, CXM, CFP, CAZ, CTX, CRO, ATM, and FOX. The one *K. pneumoniae*, OCH-71, that was resistant to KZ with a zone diameter of 12 mm was fluorescence positive (these results are shown in Figure 20). There were 25/127 Series II isolates that were fluorescence positive with KZ with the majority of the *K. pneumoniae* isolates susceptible. One isolate of the Series II *K. pneumoniae*'s was resistant to CXM. This isolate with a zone of < 14 mm and the other with a zone of 21 mm were fluorescence positive (see Figure 21). The later would have been reported as susceptible yet possessed a ß-lactamase capable of hydrolysing CXM. Series II had 61 isolates with positive fluorescence test for CFP seen in Figure 22. Only OCH-81 was resistant on susceptibility testing with a zone diameter of 13 mm and all the other 126 isolates were susceptible. There were ten isolates (OCH-31, OCH-33, OCH-51, OCH-53,

OCH-61, OCH-79, OCH-87, OCH-95, OCH-96, and OCH-97) that were moderately susceptible to CFP by zone diameter and positive on fluorescence. Although the majority of the isolates were susceptible to CFP, 61/127 (48%) had ß-lactamase activity as indicated by the positive fluorescence test. There were two *K. pneumoniae* isolates in Series II that were fluorescence positive with CAZ. OCH-45 had a susceptible zone diameter to CAZ of 21 mm (see Figure 23). This isolate was also fluorescence positive with ATM and was susceptible with a zone of 27 mm (see Figure 24). OCH-81 was fluorescence positive with a susceptible zone diameter of 23 mm to CAZ. The remaining Series II *K. pneumoniae* isolates were fluorescence negative to all the third generation cephalosporins tested. The incidence of ESBL's in *K. pneumoniae* isolates in Ottawa is low but monitoring their presence is important in avoiding an outbreak of these resistant *K. pneumoniae*'s within an institution or a community.

Figure 12: This figure compares the susceptibility to PRL by MIC with fluorescence test in the Series *K. pneumoniae* isolates. Susceptibility to PRL is indicated by MIC 16 μg/mL. Resistance is indicated by MIC $\geq$128 μg/mL. Isolates with MIC's 32 - 64 μg/mL are considered moderately susceptible to PRL. The incidence of enzymatic activity against PRL in the Series I *K. pneumoniae* isolates spans the range of MIC's. At the susceptible MIC's from 1 to 16 μg/mL there are a majority of *K. pneumoniae*'s with enzymatic activity as indicated by the clear bars. Moderately susceptible isolates are all fluorescence positive. At MIC $\geq$256 μg/mL there was one isolate which was fluorescence negative.

Figure 12

PIPERACILLIN SERIES I

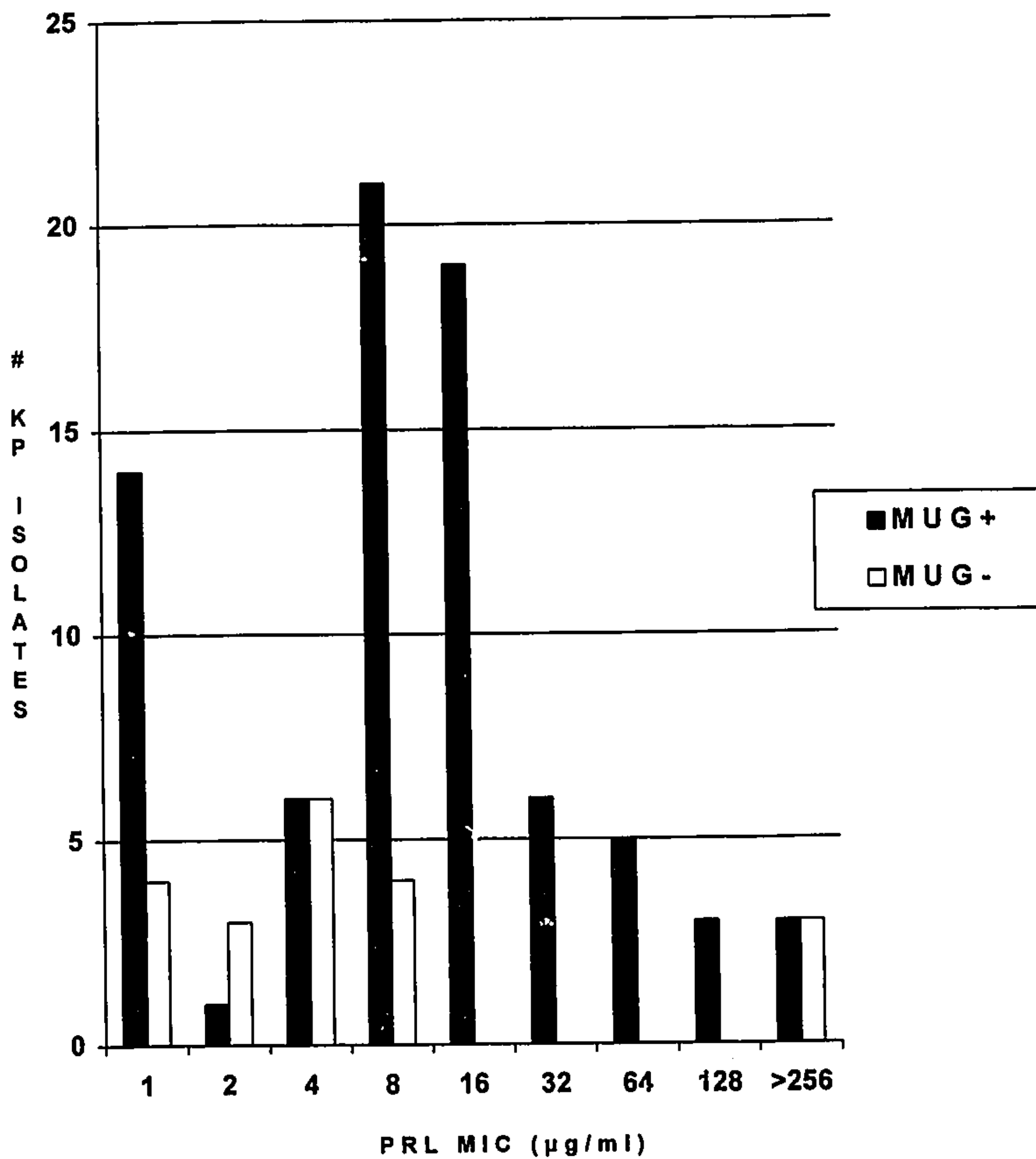


Figure 13: This graph compares the susceptibility by MIC with the fluorescence test to KZ with Series I isolates. All isolates with MIC ≤8 μg/mL are susceptible to KZ. There were susceptible *K. pneumoniae*'s with positive fluorescence. All the resistant isolates (MIC ≥32 μg/mL) were fluorescence positive.

Figure 13
CEFAZOLIN SERIES I

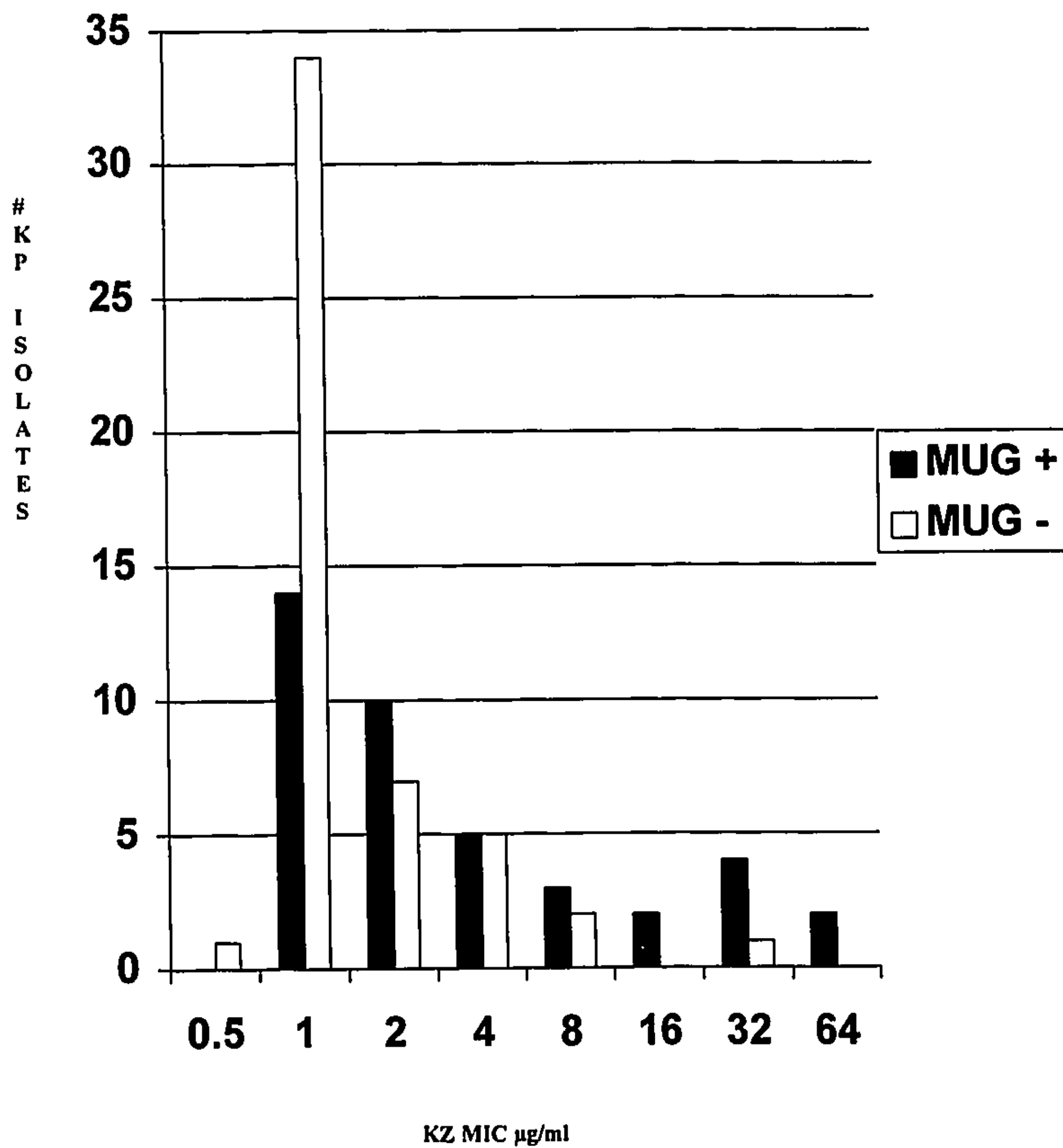


Figure 14: This graph compares the susceptibility by MIC for CFP to the fluorescence test in Series I. There was evidence of enzymatic activity against CFP at susceptible MIC's (≤16 μg/mL). As the MIC to CFP increased more isolates possessed enzymatic activity which can be noted at MIC ≥1.0 μg/mL the number of isolates with a positive fluorescence were greater than those with negative fluorescence. All these *K. pneumoniae* isolates are susceptible to CFP.

Figure 14

CEFOPERAZONE SERIES I

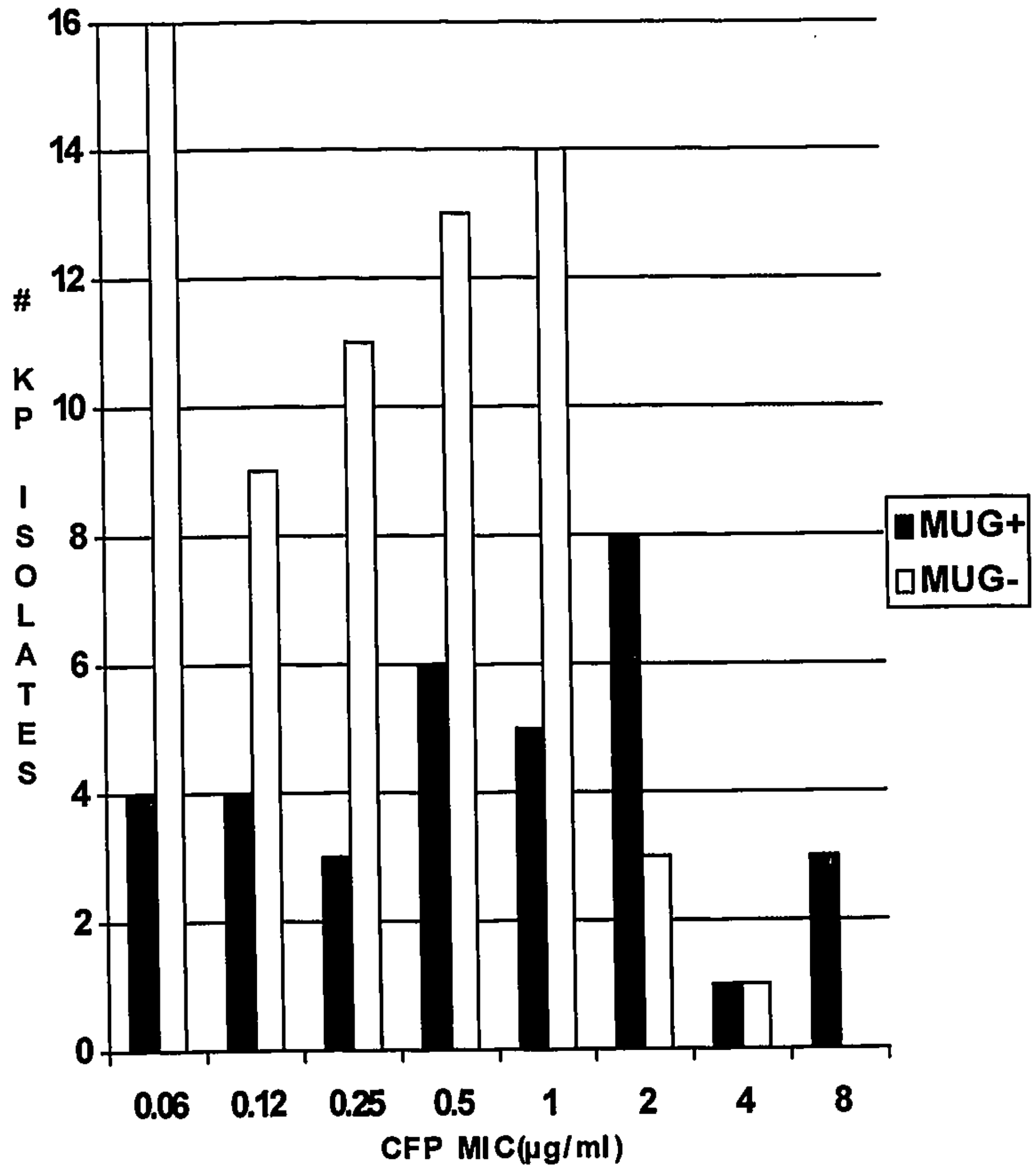


Figure 15: Susceptibility by MIC to the third generation cephalosporin CAZ compared to fluorescence test with the Series I *K. pneumoniae* isolates. All the isolates were susceptible with a MIC ≤8 μg/mL. Two isolates, one with MIC of 0.06 μg/mL and the other with MIC of 4 μg/mL were fluorescence positive.

Figure 15

CEFTAZIDIME SERIES I

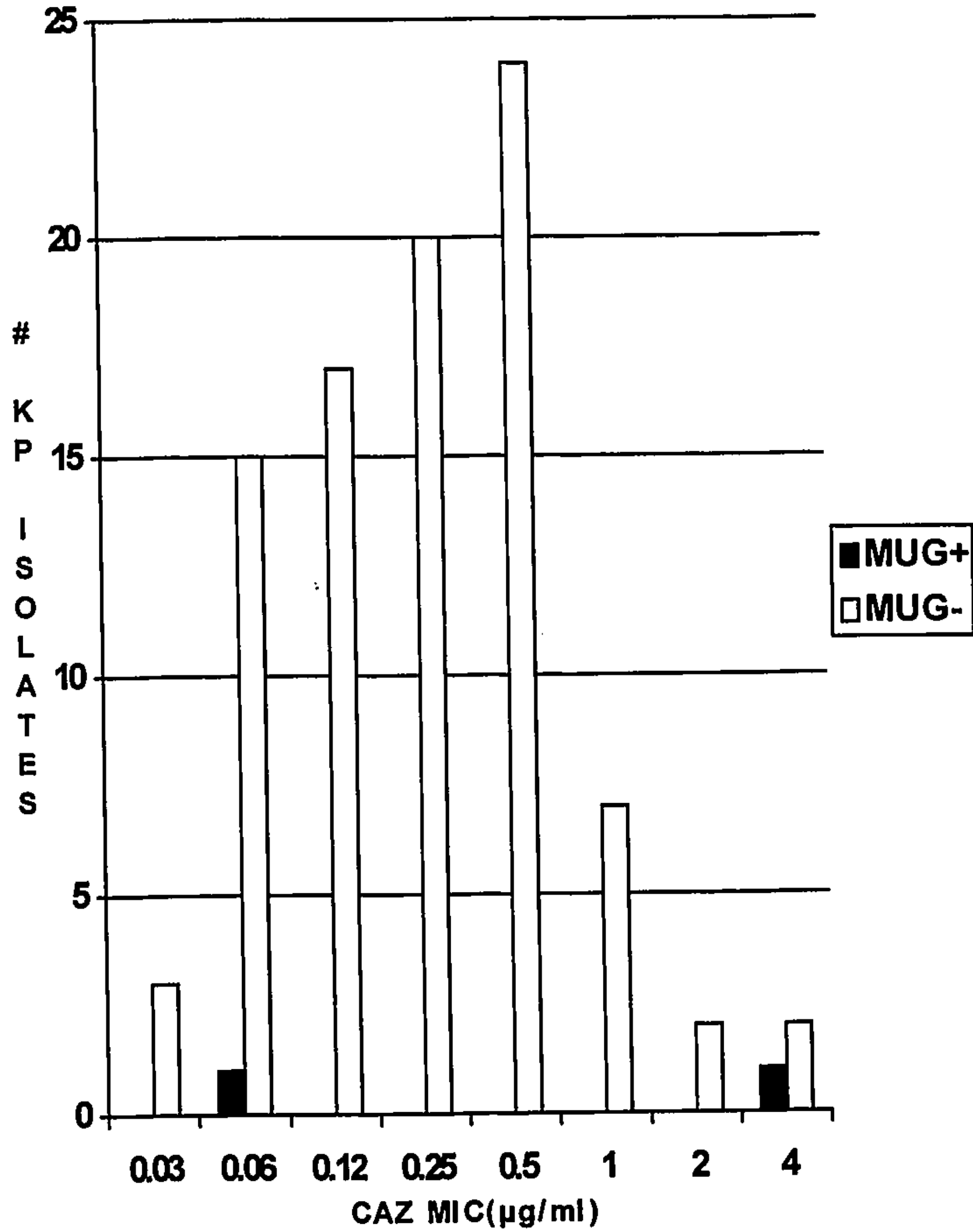


Figure 16: This graph compares the susceptibility to PRL by zone diameter with fluorescence in the Series I isolates. All the isolates with resistant zone diameters (≤17 mm) were fluorescence positive. This is expected with resistant organisms. The frequency of enzymatic activity was higher in moderately susceptible isolates with zone diameters of 18 - 20 mm than those isolates with a negative fluorescence. At the susceptible zone diameter (≥21 mm) there were more isolates with positive fluorescence than with negative fluorescence.

Figure 16

PIPERACILLIN SERIES I

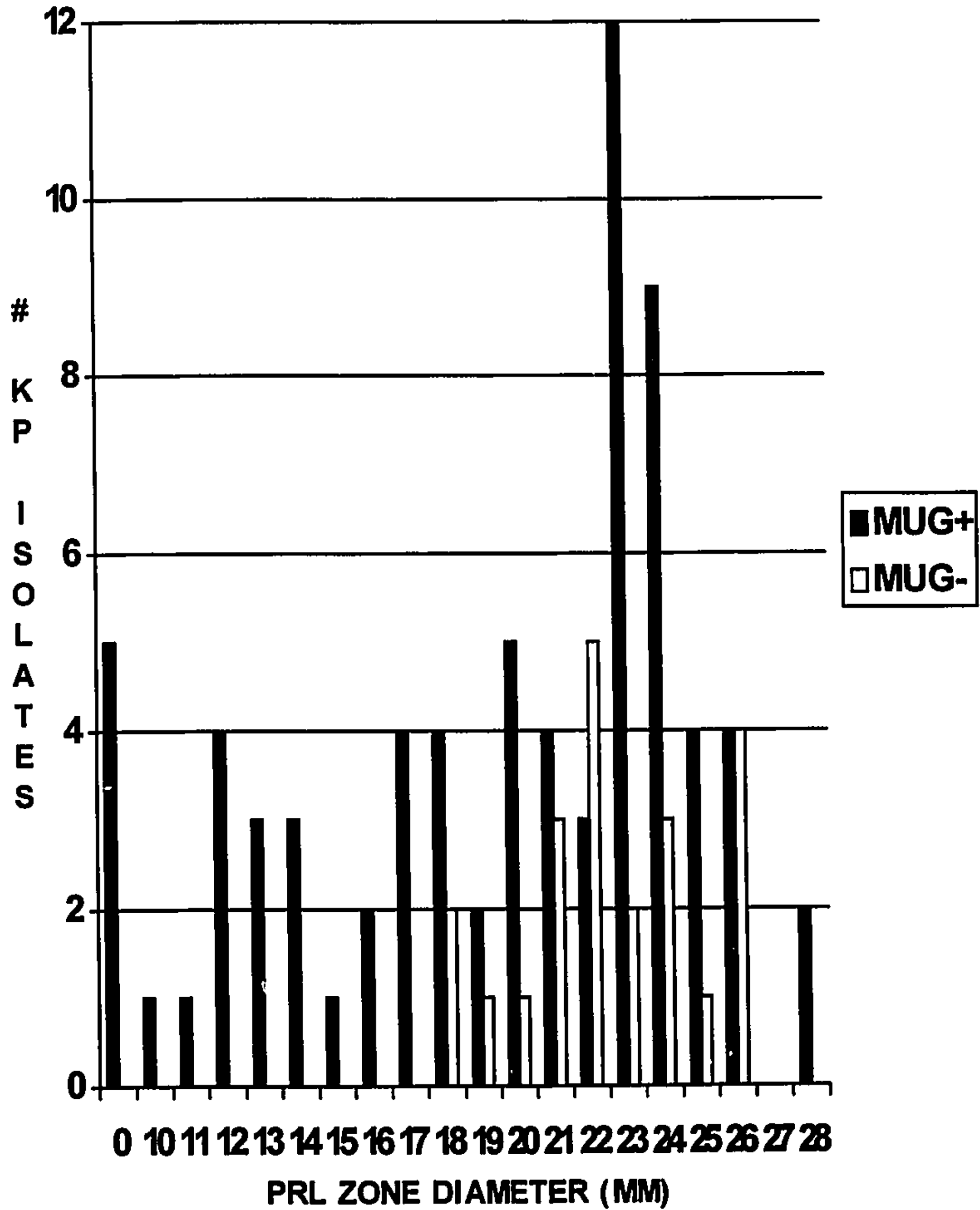


Figure 17: This graph illustrates the comparison of zone diameter to KZ with fluorescence test. There was evidence of enzymatic activity indicated by positive fluorescence throughout the zone diameter range. One isolate with a resistant zone (≤14 mm) was positive by fluorescence. There were equal numbers of positive and negative fluorescence isolates at moderately susceptible zones of 15 - 17 mm. At susceptible zone diameters (≥18 mm) there was evidence of enzymatic activity with isolates having positive fluorescence.

Figure 17

CEFAZOLIN SERIES I

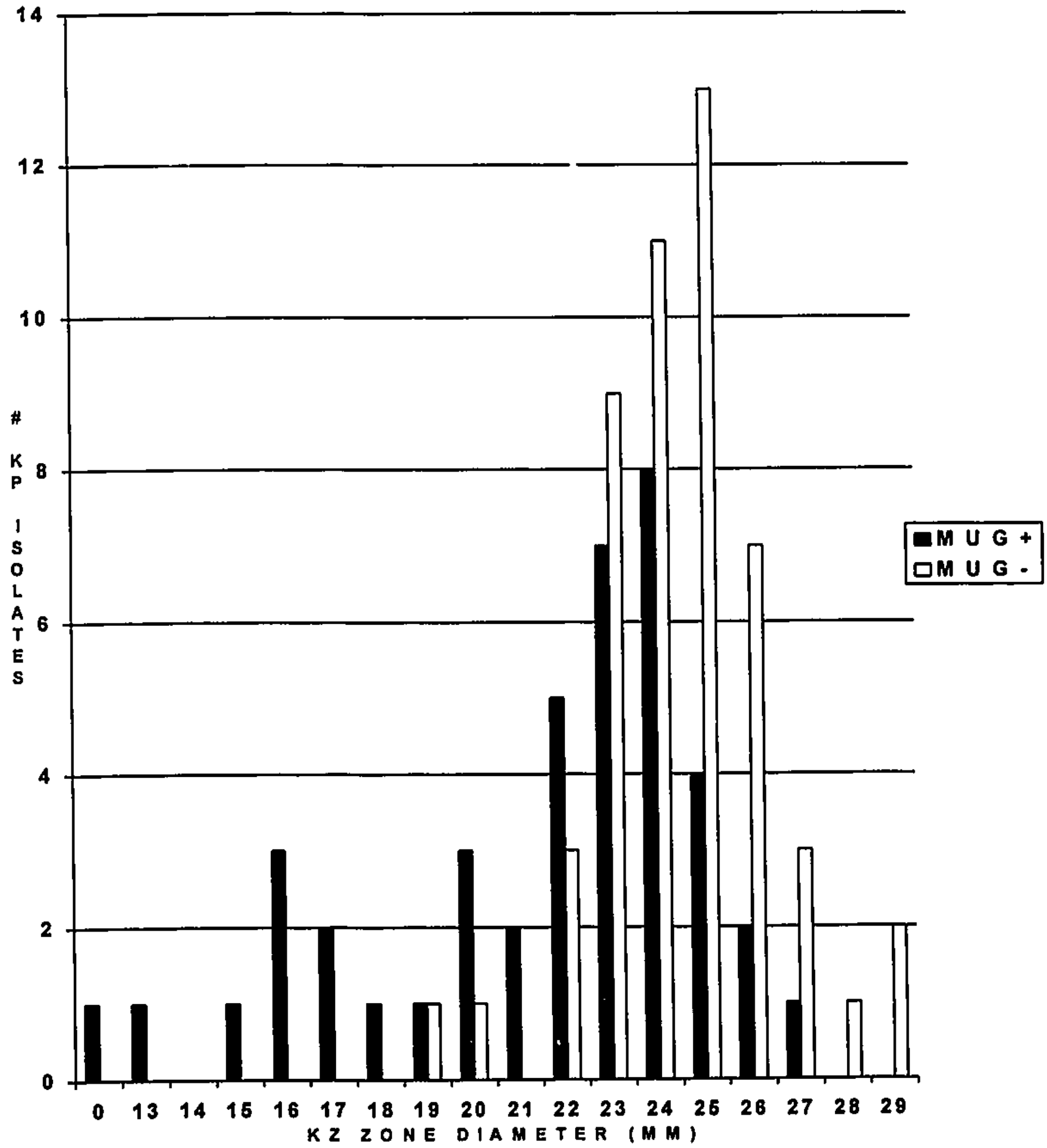


Figure 18: This graph compares zone diameters to CFP with fluorescence Series I *K. pneumoniae* isolates. One isolate with a resistant zone (≤15 mm) was positive by fluorescence. All the isolates with moderate susceptibility to CFP with zone diameters 16 - 20 mm were fluorescence positive. Isolates with zone diameters ≥21 mm are susceptible but in this study there was evidence of enzymatic activity in these susceptible isolates with positive fluorescence.

Figure 18

CEFOPERAZONE SERIES I

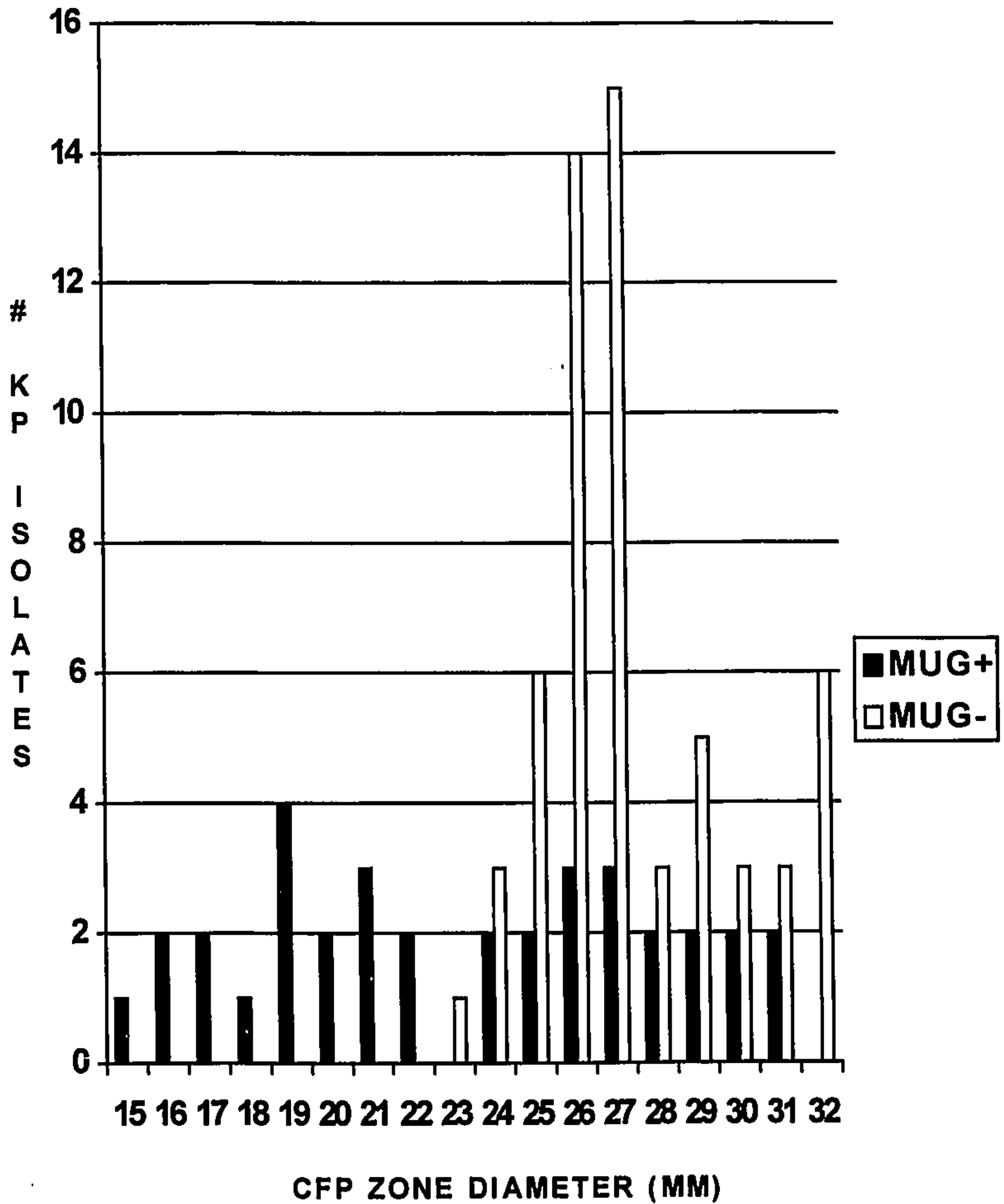


Figure 19: The incidence of enzymatic activity to CAZ was low in the Series I isolates as seen in this graph. All the *K. pneumoniae*'s were susceptible to CAZ with zone diameters ≥17mm. There were 2/94 isolates with a positive fluorescence, one with a zone diameter of 25 mm and the other of 29 mm.

Figure 19

CEFTAZIDIME SERIES I

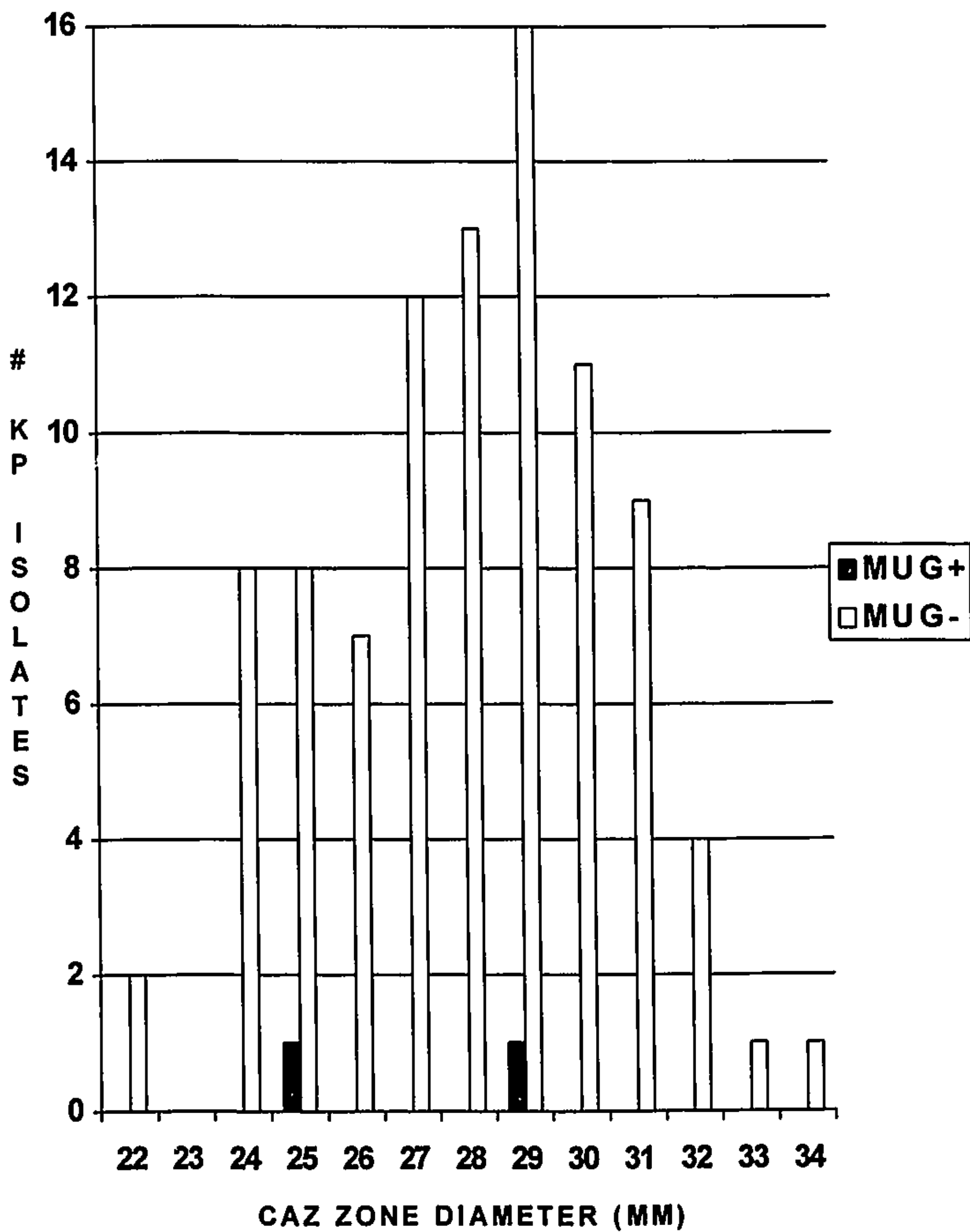


Figure 20: This figure compares the zone diameter to KZ with fluorescence test in the Series II *K. pneumoniae* isolates. The one *K. pneumoniae* with a resistant zone diameter 14 mm was also positive on fluorescence. All the isolates moderately susceptible to KZ with zone diameters 15 - 17 mm were also positive on fluorescence tests. There was evidence of enzymatic activity against KZ in susceptible isolate with zone diameters ≥18 mm.

Figure 20

CEFAZOLIN SERIES II

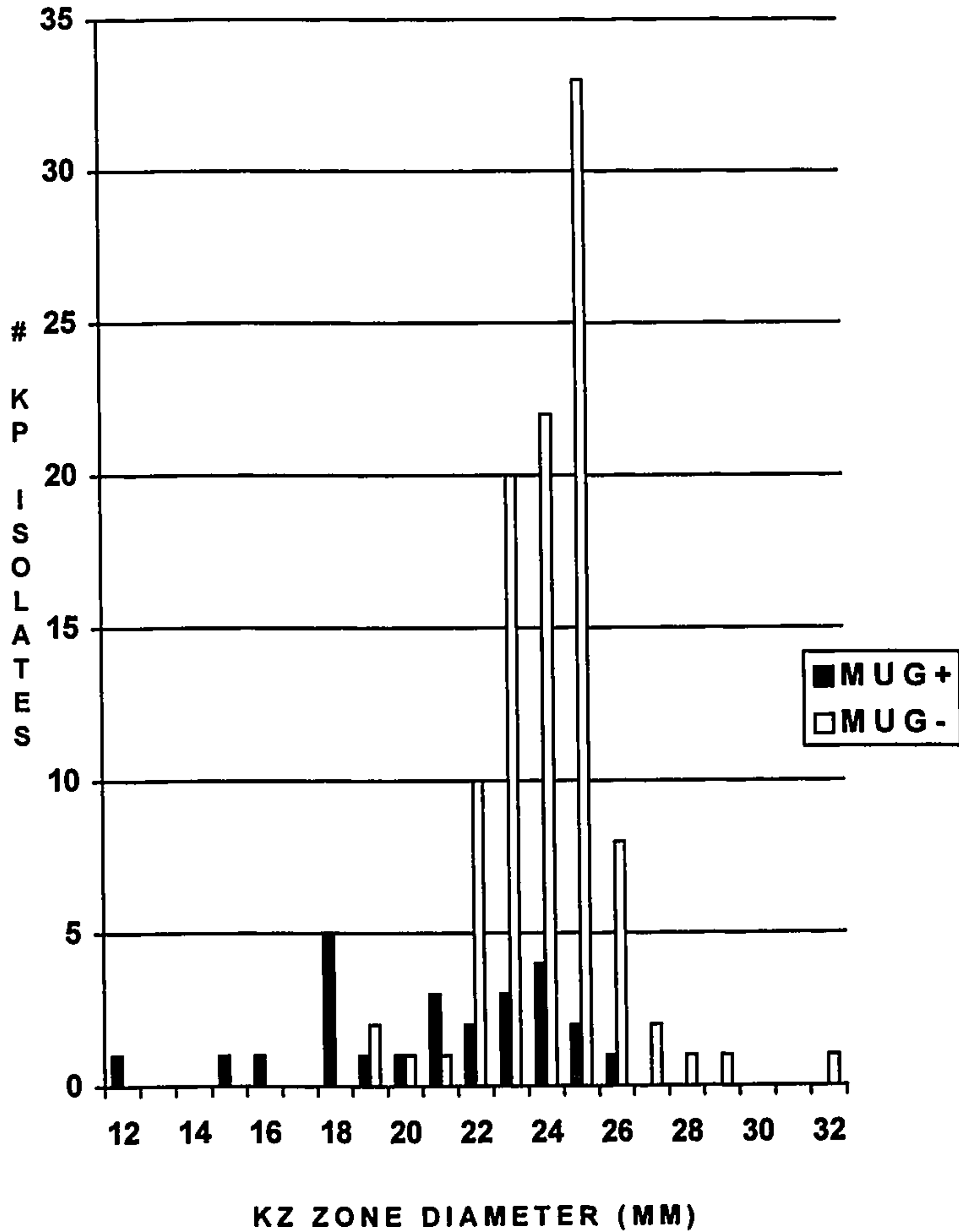


Figure 21: In Series II isolates two isolates were positive on fluorescence with CXM. One isolate was resistant with a zone diameter ≤14 mm and the other was moderately susceptible with a zone diameter between 15 - 22 mm. All the other isolates were susceptible with zone diameters ≥23 mm and negative on fluorescence tests.

Figure 21

CEFUROXIME SERIES II

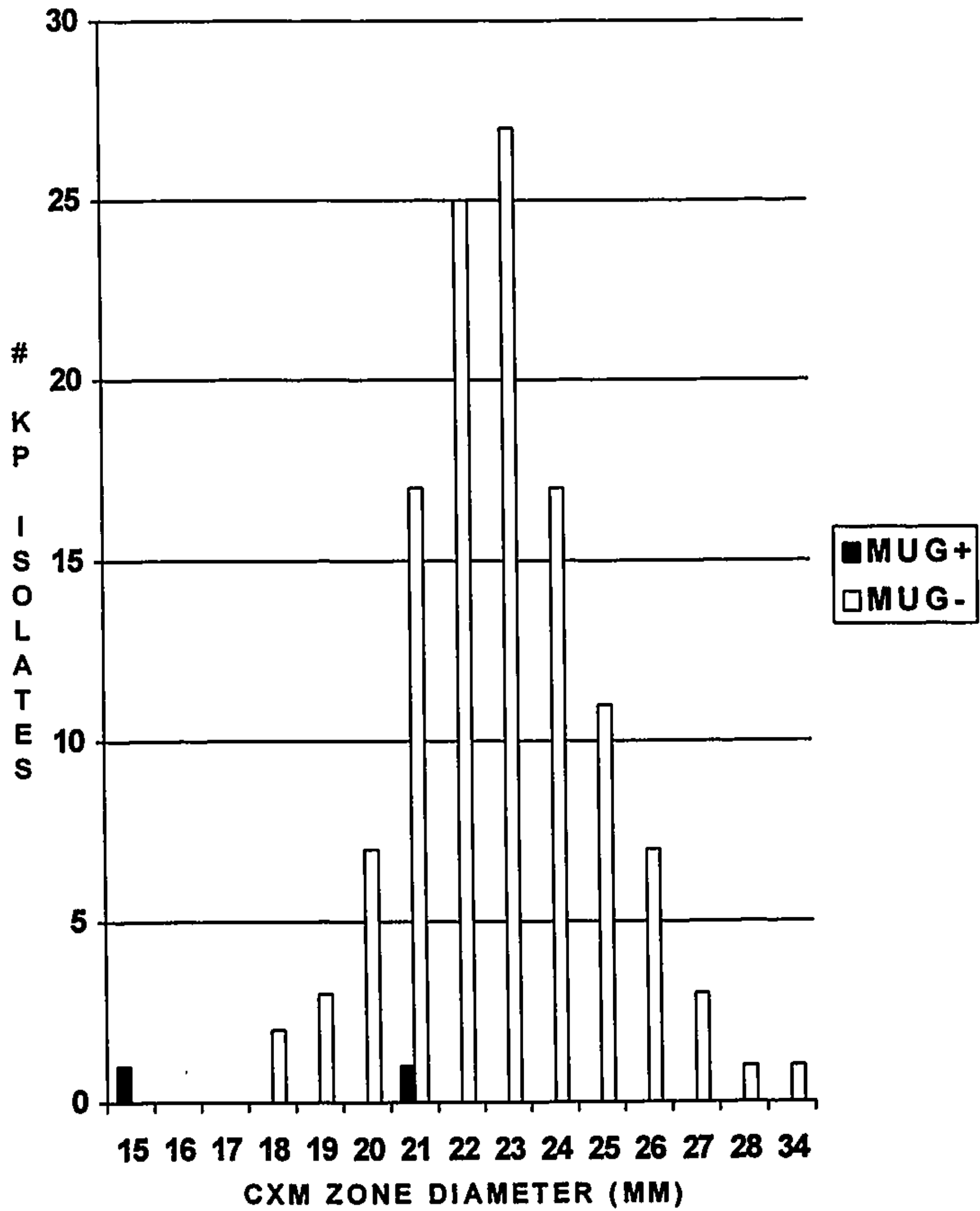


Figure 22: All the Series II *K. pneumoniae* isolates were moderately susceptible with zone diameters 16 - 20 mm or susceptible with zone diameters ≥21 mm to CFP. All the isolates with zone diameters between 19 and 23 mm were positive on fluorescence. One isolate with a zone of 26 mm was also positive on fluorescence. The remainder of the isolates with zone diameters ≥24 mm were all fluorescence negative.

Figure 22

CEFOPERAZONE SERIES II

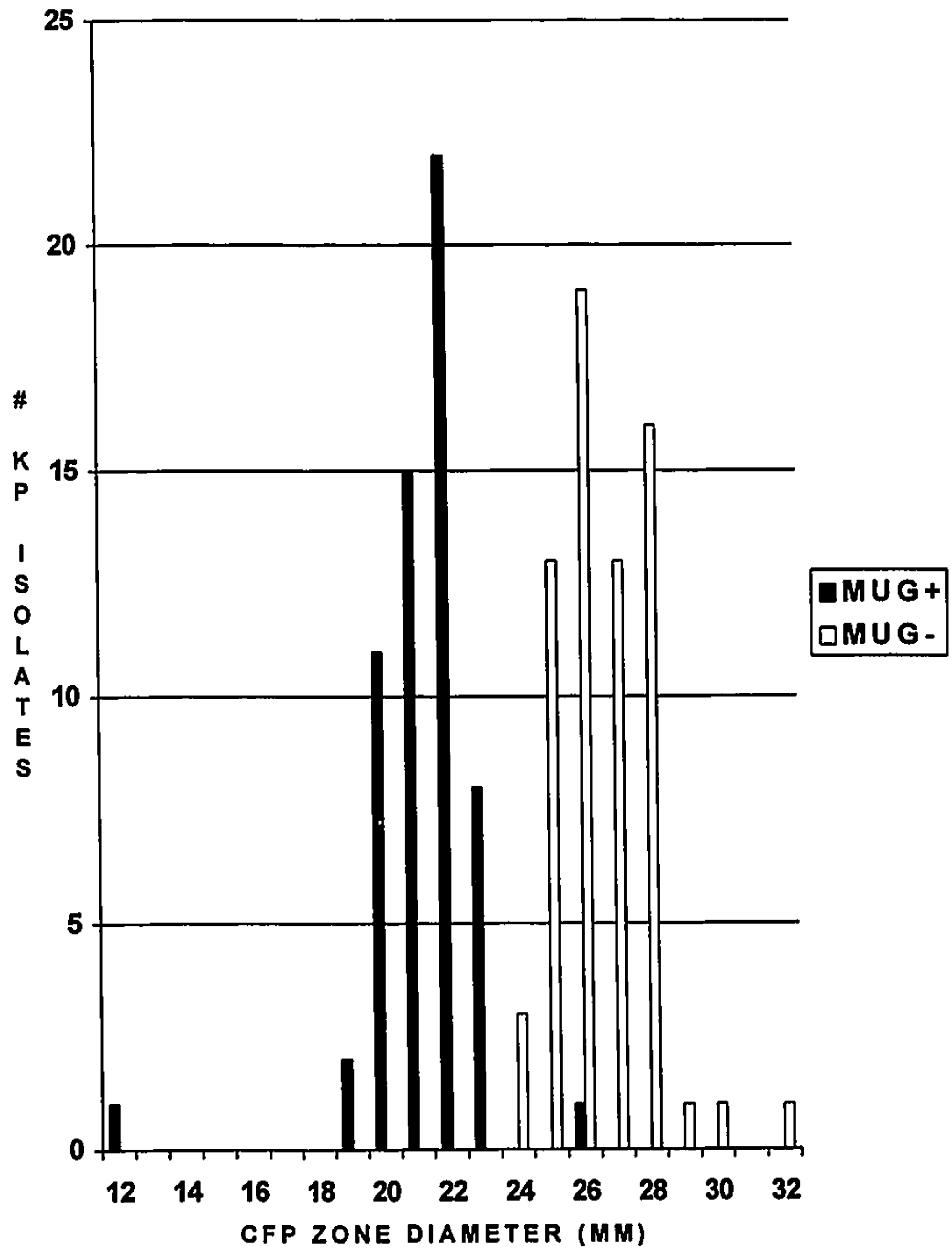


Figure 23: The incidence of enzymatic activity against CAZ was low in the Series II isolates with 2/127 having positive fluorescence. Although all the isolates were susceptible to CAZ with zone diameters ≥18 mm. Both the positive isolates had small zones of 21 and 23 mm respectively.

Figure 23

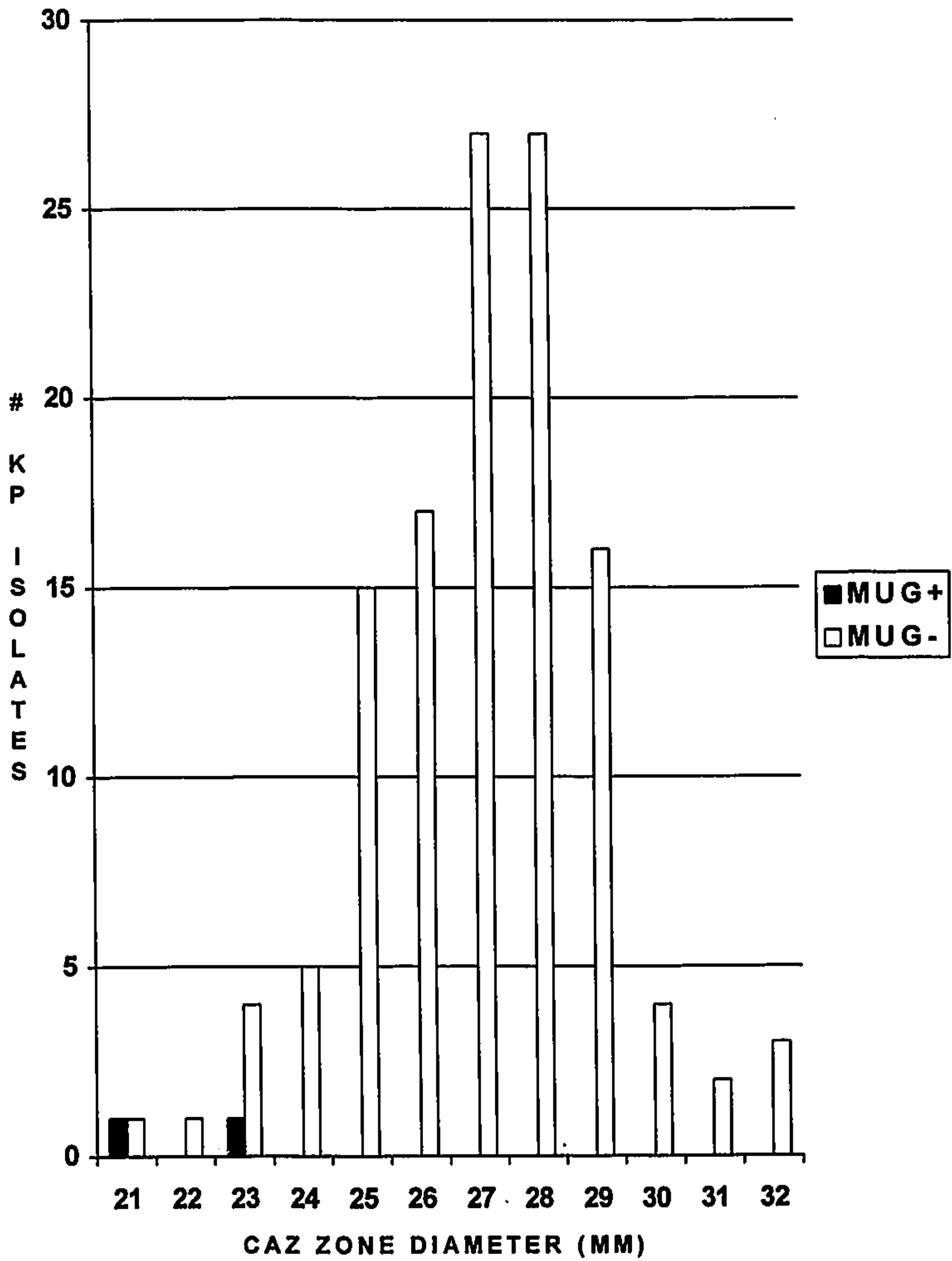
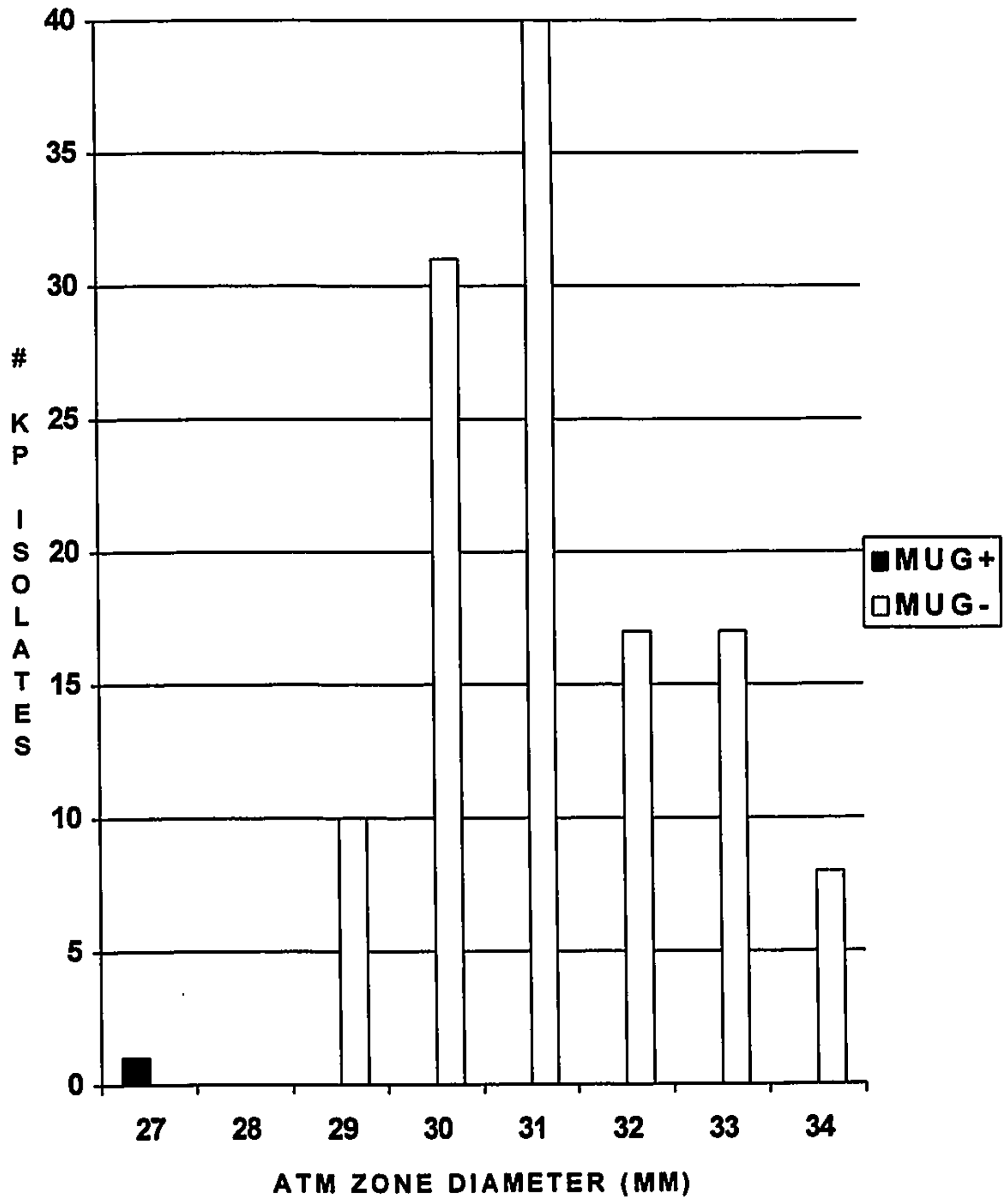


Figure 24: All the Series II isolates were susceptible to ATM with zone diameters ≥22 mm. One isolate with a zone of 27 mm was positive by fluorescence. The incidence of enzymatic activity against ATM is low at 1/127 (0.8%).

Figure 24

AZTREONAM SERIES II



DISCUSSION

1. INTRODUCTION

In the early 1980's after the development of third the generation cephalosporins, (CAZ, CTX and CRO) and the monobactam (ATM) which were designed to resist the action of known plasmid-mediated ß-lactamases, use of these agents to treat gram negative infections became widespread. Ceftazidime, which was initially utilized in the treatment of *Pseudomonas aeruginosa* infection in cystic fibrosis, became frequently used for other gram negative infections. By 1983 in Europe there were reports of plasmid-mediated ß-lactamases that hydrolysed these new third generation ß-lactams which were called extended-spectrum ß-lactamases (ESBL's) (82). Reports surfaced of single *K. pneumoniae* isolates resistant to CAZ and CTX (38,39,40,78,86,97,98,99,100,101,102,103). As these ESBL's were isolated and characterized by molecular studies it was found that these enzymes had amino acid substitutions at particular sites of the original TEM and SHV-type enzymes. These alterations occurred at the active site of the enzyme and extended the substrate spectrum of the plasmid-mediated ß-lactamase (33,34,36,37,38,40,43, 47,79,82,103-118).

In 1992 Saunders using the DDST reported that small decreases in susceptibility to third generation cephalosporins which were not sufficient to raise the MIC above the NCCLS breakpoint in *K. pneumoniae* isolates may indicate the presence of a ß-lactamase with an extended spectrum (83). It is alarming that these organisms harbouring these enzymes for extended-spectrum ß-lactams were being reported as susceptible and that these agents were utilized in treatment. Clinically, it would be useful to have a flexible, sensitive, and reliable screening test for ß-lactamase which could be reported and indicate

to the clinician which antibiotic to use. The precedent for the use of such a test has already been set. In 1974 the reports of ampicillin resistant *H. influenzae*, due to the possession of a TEM-1 enzyme, resulted in the development of a screening test utilizing the chromogenic cephalosporin, nitrocefin. Any *H. influenzae* isolate that was susceptible by zone diameter measurement but was positive for ß-lactamase by nitrocefin testing was reported as resistant to the clinician. Although nitrocefin testing is valuable to the clinician, it does not indicate the antibiotic spectrum for enzymatic activity. A screening test for the detection of plasmid-mediated extended-spectrum ß-lactamases in *K. pneumoniae* isolates was developed in the belief that this might pave the way for a clinically useful test.

2. SUSCEPTIBILITY TESTING

Susceptibility reporting guidelines are controlled by a regulating body (NCCLS) in Canada and United States that determines the zone diameter or the minimal inhibitory concentration that categorizes an organism as susceptible, moderately susceptible or resistant to an antimicrobial agent. Antimicrobial susceptibility is routinely done in a clinical microbiology laboratory by the disc diffusion (94c) or by automated microdilution minimal inhibitory concentration (MIC's). This arbitrary nature of interpretive criteria is exemplified by discrepancies between countries. Reports of resistant isolates from European countries must be considered with these differences in mind. For example with CTX the break point indicating susceptibility in Canada is a zone of ≥23 mm (defined by NCCLS) while the break point for susceptibility to CTX in France is ≥21 mm (defined by

a different group) (118). In a clinical situation a *K. pneumoniae* isolate in Canada with a zone diameter of 22 mm would be reported as moderately susceptible in Canada but would be susceptible in France. The first reports of ESBL's were from Europe and later in the United States but have not been numerous in Canada. This fact makes the literature confusing. Could this be due to the variability in zone diameter break points in susceptibility testing between countries? In order to compare the incidence of resistance between countries it is necessary to standardize zone diameter measurements and experimental procedures including type of media, depth of media in the plates, and inoculum concentration to universal standards.

3. DDST EVALUATION

The Series I isolates were closely studied with the DDST against a broad-spectrum penicillin (PRL), a first (KZ), and third (CAZ and CFP) generation cephalosporins (see Table 4). When the DDST was first described by Vatopolous et al (80), it was considered to be a reliable test to determine if a gram negative bacilli harboured a ß-lactamase that was inhibited by clavulanic acid. The DDST depends on the diffusion of antibiotic into the culture media and the synthesis of ß-lactamase by the organism. We found that the placement of the antibiotic discs was critical (see Figures 1 and 2). We devised a calculation for the distance between the ß-lactam and the AMC discs that was applied for each isolate. This has been described in Materials and Methods. Shortly after this calculation was devised Saunders et al reported difficulties with the DDST as described by Vatopolous (80) and suggested placement of the discs at 20 mm apart (83). On

reviewing the *K. pneumoniae* isolates in this study that were DDST positive with the revised calculation for disc placement, the distance between the AMC and ß-lactam discs was on the average 20 mm. A distance of 30 mm between the discs is too far and results in false negatives.

For all the antibiotics tested with the DDST, this test was sensitive in detecting enzyme activity with calculated disc distances (see Figures 1 and 2). Susceptibility testing by zone diameter or MIC and DDST at 30 mm spacing may not detect organisms that possess a ß-lactamase (see Tables 3 and 4). By trial and error it is evident that disc placement is important and appears to enhance the sensitivity of this test (83). The DDST is time-consuming in that it could take at least 48 hours for a result depending on isolation of the organism and zone diameter result. Although the DDST is more sensitive than susceptibility testing in detecting ß-lactamase activity, the time required for a result makes this test unsuitable for a screening test in a clinical laboratory.

4. FLUORESCENCE TEST

The sensitivity of the fluorescence test was established with the Series I *K. pneumoniae* isolates. All the isolates that were DDST positive were fluorescence positive and none were DDST positive and fluorescence negative (see Table 8). With all the ß-lactams tested, there was a higher incidence of positives with the fluorescence than positives with the DDST (see Tables 3 and 5 and Figures 12 to 24). The isolation of the *K. pneumoniae* was all that was necessary. Unlike the DDST and Masuda, further testing or manipulations were not required. This shortened the time required for results.

The Series II *K. pneumoniae* isolates were studied to determine the actual incidence of ESBL's in Ottawa. All the isolates were susceptible to third generation cephalosporins by zone diameter measurements (see Figures 23 and 24). From the results in Series I, it was determined that some of these isolates could harbour an ESBL which was determined by the fluorescence test (see Figures 15). From the fluorescence test results in Series II with the third generation cephalosporins, the actual incidence *K. pneumoniae* isolates harbouring ESBL's was low in Ottawa (see Figures 23 and 24).

The specificity of the fluorescence test is demonstrated by the neutralization of the ß-lactamase activity by CA in both Series I and II. In the majority of fluorescence positive isolates the enzymatic activity was neutralized by CA indicating that the ß-lactamase was a plasmid-mediated CA-susceptible enzyme (see Table 7). In the isolates that the enzymatic activity was not inhibited by CA, it is possible that these organisms could harbour a chromosomally-mediated ß-lactamase or a plasmid-mediated enzyme neither of which was inhibited by CA (92).

The specificity of this fluorescence test was also investigated by testing a known ciprofloxacin resistant *K. pneumoniae* isolate. This *K. pneumoniae* isolate that grew on the MHA-MUG plates incorporated with ciprofloxacin was negative by fluorescence. Ciprofloxacin resistance due to enzyme activity has not been described, so that the absence of fluorescence in this experiment is additional evidence that the fluorescence phenomenon is enzyme mediated.

One of the disadvantages of the DDST is that it will not detect ß-lactamases that are not inhibited by CA and theoretically, the fluorescence test should work for CA-resistant

enzymes. Although the majority of plasmid-mediated ß-lactamases are inhibited by CA, enzymes such as MIR-1, TRI-1, and TRC-1 are not. The chromosomally Class 1 mediated enzymes and MIR-1, TRI-1 and TRC-1 plasmid medicated enzymes are inhibited by OX. In the plates containing OX these enzymes should be inhibited but in practice this was not feasible.

The concentration of CA and OX incorporated in the plates was 1/8 MIC of *E. coli* for OX which was 30 μg/mL and 1.0 μg/mL for CA. Attempts were made to use OX as an inactivating agent in the fluorescence test. At a concentration 30 μg/mL the growth of the *E. coli* at 1/2 MIC of the ß-lactam was decreased so that the lawn was nonexistent and growth of the *K. pneumoniae* was inhibited. In plates with the OX concentration less than 30 μg/mL, the enzyme was not inhibited. On the MHA-MUG plates containing ß-lactam, CA, and OX growth of the indicator *E. coli* was inhibited which was likely due to the additive effect of the three antibiotics in the plate. The concentration of CA at 1.0 μg/mL did not inhibit growth of the *E. coli* but was sufficient to inhibit the ß-lactamase. Therefore, this fluorescence test will show the presence of an enzyme inhibited by CA. At this point we do not know the significance of the results on those few isolates whose enzyme activity was not neutralized by CA and this test does not permit the characterization of an enzyme not inhibited by CA. (See Table 7).

In both the Series I and Series II isolates, all the *K. pneumoniae*'s resistant to any of the ß-lactams by zone diameter or by MIC were fluorescence positive. There is no question that an organism would not be treated with an antibiotic to which it is resistant by established criteria. The problem lies with those isolates that have a lesser degree of

enzymatic activity and are reported as susceptible or moderately susceptible. The phenomenon in which HI and PPNG possess enzymatic activity but are susceptible has been reported (2). Some *K. pneumoniae* isolates that are susceptible to extended-spectrum ß-lactams have enzymatic activity against them (see Figures 15, 23, and 24). It would be useful for the clinician to know this fact and to avoid using the antibiotic for treatment. It is with this fact in mind that the development of a sensitive, reliable, and flexible screening test for ß-lactamase would be beneficial in a clinical laboratory. This study not only developed an novel test for ESBL's but also looked at a population to indicate the prevalence of enzymatic activity.

5. POSSIBLE CLINICAL CORRELATION

To overcome the problem of plasmid-mediated ß-lactamases, ß-lactamase inhibitors (clavulanic acid, sulbactam or tazobactam) were combined with amoxycillin and ticarcillin to extend the activity of these penicillins by inhibiting the ß-lactamase activity that might be present in a gram negative organism causing an infection (86). The first report of resistance to ß-lactam-inhibitor combinations came in 1987. In 1991 strains of *E. coli* harbouring clavulanate-resistant TEM-type ß-lactamases were genetically characterized and called TRI-1 and TRI-2 (Tem-Resistant to Inhibitor) (15,50). As these enzymes become more prevalent within the gram negative population, specifically with *K. pneumoniae* infections that are the source of morbidity and mortality in immunocompromised patients in neonatal, intensive care and chronic care facilities, treatment becomes problematic.

Humans with serious gram negative infections differ in severity of disease and other

supportive treatment is rarely identical which makes comparisons between antibiotic therapy difficult. Animal studies permit the standardization of experimental procedures and comparisons. Animal experiments simulate more closely the characteristics of human infections than in vitro models and provide end points which allow comparisons between different antibiotic regimens. These end points include the number of CFU of bacteria/unit weight of tissue, the frequency of emergence of resistance, death vs survival, and the frequency of relapses once treatment has been stopped (42,119,120). These in vivo experiments showed that in the case of *K. pneumoniae* isolates possessing an ESBL there was a marked inoculum effect. It was also shown that neutralization of the ß-lactam even where the ß-lactam MIC in the absence of sulbactam gave a susceptible result (42,119). In view of this inoculum effect in vitro susceptibility tests which use a standard inoculum less than numbers encountered clinically could indicate false susceptibility (120). Susceptibility tests alone do not indicate what ß-lactams may be inactivated by enzymatic activity. The use of this fluorescence test might enable the physician to chose antimicrobial therapy that would be less likely to result in treatment failure. The detection of ß-lactamase activity by this fluorescence test would be an important adjunct to susceptibility tests in a routine laboratory.

CONCLUSIONS

The original data resulting from this study are (1) The improved disk spacing for the DDST was reached before the paper by Saunders was published. (2) The fluorescence test developed in this work has not been described elsewhere.

The conclusions established from this study in developing a screening test for ß-lactamase are as follows:

1. The Fluorescence Test is more sensitive than the DDST in screening for ESBL production in *K. pneumoniae* isolates. This test can be done within 18 hours after isolation of the organism and does not depend on susceptibly testing as with the DDST. Any antibiotic can be tested which increases the flexibility of this test. Neutralization by inhibitors can be tested at the same time by incorporation of the inhibitor into a second series of MHA-MUG plates. With the Steer's Replicator 37 organisms can be tested at the same time.
2. The many *K. pneumoniae* isolated probably have a TEM or a SHV-type ß-lactamase which inactivates the first generation cephalosporin, KZ, but would be still reported as susceptible on routine laboratory susceptibility tests.
3. Using the Fluorescence Test, it was evident that there are a small number of *K. pneumoniae* isolates in Ottawa that do show a decrease in susceptibility to some third generation cephalosporins on MIC determinations but are susceptible on disc diameter measurements. This does not appear to be a problem in the Ottawa area, yet. However, if the use of third generation cephalosporins for treatment increases, we may see an increase of ESBL production in *K. pneumoniae*. During this time it

may be useful to screen organisms for ESBL.

4. The Fluorescence Test could be used as an epidemiological tool to track the incidence of resistance to a variety of antimicrobial agents and could also be used as a supplement to routine susceptibility testing in a laboratory.

APPENDIX 1

ISOLATES UTILIZED IN THIS STUDY

ORGANISM	ORIGIN	CHARACTERISTICS
E. coli	ATCC 25922	NCCLS Standard
K. pneumoniae	Toronto	Possesses a TEM-10 enzyme which confers resistance to ceftazidime
KP-1212	Ottawa Civic Hospital	Decreased susceptibility to ceftazidime
KP-1409 KP-1730 KP-7538 KP-6 KP-7 KP-11 to Kp-75 KP-40 KP-67 KP-68 KP-74 toKP-91	Ottawa Civic Hospital	
KP-1 to KP-3 KP-5 KP-8 to KP-10 KP-16 to KP-28 KP-41 to KP-44 KP-69 to KP-73	Children's Hospital of Eastern Ontario	Selected *K. pneumoniae* strains to develop the fluorescence test to detect beta-lactamase.
K-29 to K-39 KP-45 to KP-66	Oshawa General Hospital	
OCH-1 to OCH-127	Ottawa Civic Hospital	Consecutive *K. pneumoniae* isolates to test and compare the zone diameter and fluorescence test.

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