BETA-LACTAM ANTIBIOTIC RESISTANCE IN ENTEROBACTER CLOACAE
ISOLATED FROM GROOTE SCHUUR HOSPITAL INPATIENTS

G L SAUNDERS
DEPARTMENT OF MEDICAL MICROBIOLOGY
MEDICAL SCHOOL
UNIVERSITY OF CAPE TOWN

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INTRODUCTION AND AIMS OF THE DISSERTATION

*Enterobacter cloacae* is an opportunistic pathogen that is recognised increasingly in patients with nosocomial infection. Although *E. cloacae* is notorious for its involvement in common-source outbreaks (Maki 1976), most hospital isolates are from sporadic infections, or colonisations, of patients. An increase in the rate of isolation of *E. cloacae* need not identify a common-source outbreak, but rather may be the result of coincidental isolations, each isolate having been selected from a patient’s flora by the use of a third-generation cephalosporin (Gaston 1988).

Resistance may emerge in *E. cloacae* to previously effective beta-lactam antibiotics, including second- and third-generation cephalosporins, monobactams and broad-spectrum penicillins. The spread amongst hospital patients of such resistant *E. cloacae* and the therapeutic failures associated with the emergence of resistance are of grave concern (Sanders 1982, 1987).

The percentage of isolates of *E. cloacae* that are resistant to third-generation cephalosporins is higher when the isolates are from patients in a tertiary-level hospital, as opposed to a community hospital (15% and 4%, respectively) (Ellner 1987). Amongst patients in similar units of tertiary-level hospitals, isolates from patients in the hospital that dispenses the greater amount of cephalosporins are more commonly (10% versus 1%) resistant to third-generation cephalosporins (Benn 1984). Control of the use of cephalosporins in tertiary-level hospitals has been advocated to prevent the emergence and spread of resistant *E. cloacae* (Sanders 1985, 1987).

Groote Schuur Hospital is a tertiary-level hospital with an antibiotic policy that includes restricted access to cephalosporins. Surveillance in the Medical
Microbiology Laboratory of Groote Schuur Hospital suggests that less than 10% of isolates of *E. cloacae* from patients in Groote Schuur Hospital are resistant to third-generation cephalosporins.

The aims of the dissertation are

(1) to determine the proportion of isolates of *E. cloacae* from Groote Schuur Hospital inpatients that are resistant to third-generation cephalosporins

(2) to determine this proportion using methods suitable for continued use in the routine laboratory.

A review of the relevant literature is followed by a report of the experimental work performed.
LITERATURE REVIEW

1. INTRODUCTION TO LITERATURE REVIEW

Investigation of the resistance to cefamandole and to cefotaxime that may emerge in *Enterobacter cloacae* has contributed to an increased understanding of the roles of bacterial cell wall permeability and of beta-lactamase activity in the resistance of Gram negative bacilli to beta-lactam antibiotics (Vu 1985).

Cefamandole, a second-generation cephalosporin, was found to be more active than ampicillin (a broad-spectrum penicillin) and cephaloridine (a first-generation cephalosporin) against *Enterobacter* species (Eykyn 1973). However, discrepancies in determinations of the minimum inhibitory concentration (MIC) of cefamandole for *Enterobacter* species suggested that resistance could emerge and that this resistance was enzyme-mediated (Findell 1976).

Although induction of increased beta-lactamase activity by benzylpenicillin was known to occur in *Enterobacter cloacae* (Hennessey 1967), cefamandole could not be shown to induce increased beta-lactamase activity (Ott 1979). Sanders (1979) showed that resistance to cefamandole emerged in *E. cloacae* cultured in the presence of cefoxitin, and was due to induction of increased beta-lactamase activity. Clinical failures of therapy were identified and were accompanied by the isolation of cefamandole resistant *Enterobacter* species (Sanders 1982), but in these isolates increased beta-lactamase activity followed a genetic mutation and persisted in the absence of an inducer (Gootz 1982).
Increased beta-lactamase activity was considered to be the sole mechanism for the resistance to cefamandole of Enterobacter species. Cefotaxime, a third-generation cephalosporin, was thought not to be hydrolysed by the beta-lactamase from E. cloacae, so that when cefotaxime resistant E. cloacae were identified, a novel form of resistance seemed likely (Then 1982).

2. BETA-LACTAM ANTIBIOTIC ACTION

Penicillin-binding proteins (PBPs) are located in the periplasmic space of Gram negative bacteria and include enzymes that are essential for cell wall assembly. PBPs catalyse the transpeptidase reaction that cross-links cell wall peptidoglycan subunits. The D-alanyl-D-alanine bond, the target for this reaction, and the amide bond of the beta-lactam ring are similar in structure. This allows the active site of the PBPs to bind to beta-lactam antibiotics (Chambers 1986) and results in inactivation of both beta-lactam antibiotic and PBPs (Nayler 1987). When sufficient essential PBPs are inactivated then cell wall assembly is prevented. Thus, to be effective, beta-lactam antibiotics not only must reach the periplasmic space, but also must accumulate there to achieve a minimum inhibitory concentration (Nikaido 1987).

3. OUTER MEMBRANE PERMEABILITY

The outer membrane of Gram negative bacilli is composed of an outer leaflet of lipopolysaccharide and an inner leaflet of phospholipids. This structure is hydrophobic and diffusion of hydrophilic substances through the
outer membrane is slow. General diffusion porins, such as outer membrane protein F (OmpF), are water-filled protein channels that traverse the outer membrane and allow rapid diffusion of hydrophilic substances, with molecular weights below 650 daltons, between the exterior of the cell and the periplasmic space (Benz 1988).

3.1 Cephalosporin diffusion

Cephalosporin entry into the periplasmic space is most rapid when diffusion across the outer membrane is via the general diffusion porins. The rate of diffusion through these porins is reduced when the cephalosporin is negatively charged or hydrophobic or has bulky side chains (Nikaido 1983, Yoshimura 1985).

(The following discussion is summarised, in part, in Figure 1.)

Monoanionic beta-lactam antibiotics (in order of increasing hydrophobicity) cephacetrile, cefazolin, cefmetazole, cefoxitin and cefamandole show a linear (inverse) relationship between hydrophobicity and rate of diffusion. The rate of diffusion of cefamandole is about one fifth of that of cephacetrile.

Monoanions with an oxime group (cefotaxime, ceftizoxime and cefuroxime) or an ureido group (cefoperazone) in the position 7 side chain are as hydrophilic as cephacetrile but diffuse as slowly as cefamandole.

Dianionic cephalosporins (moxalactam and ceftriaxone) and aztreonam are hydrophilic but diffuse at rates similar to cefoxitin and cefamandole.
Compounds with one positive and two negative charges that are hydrophilic (cephalosporin c and cefsulodin) have rates of diffusion similar to hydrophilic monoanions. Ceftazidime, which is hydrophobic and has an oxime group in the position 7 side chain, has a rate of diffusion similar to cefamandole.

Cephalosporins that are zwitterionic (cephaloridine, cephaloglycin, cephalalexin and cefaclor) still have higher rates of diffusion than cefacetrile, even though they are hydrophobic.

Dipolar ionic methoxyimino-cephalosporins (cefpirome, cefepime and E-1040) are hydrophobic and bulky, and are predicted to diffuse at rates similar to cefoxitin (Nikaido 1990).

Imipenem is zwitterionic, hydrophilic (hydrophilicity is similar to azthreonam) and has a high rate of diffusion, at least twice that of cefacetrile.

3.2 Diffusion of broad-spectrum penicillins

Broad-spectrum penicillins (ampicillin, amoxycillin, piperacillin and apalcillin) are as hydrophobic as cephaloridine and cefazolin. However, diffusion rates for these penicillins in the absence of porins are higher than for cephalosporins of comparable hydrophobicity (Yamaguchi 1983, 1985). Absence of OmpF raises the MICs of cephalosporins for Escherichia coli two- to three-fold, but there is no comparable increase in resistance to
ampicillin and piperacillin. These penicillins appear to have a reduced dependency on the OmpF pathway for entry into the cell (Hiraoka 1989).

4. BETA-LACTAMASES

Beta-lactamases are enzymes that inactivate beta-lactam antibiotics. The beta-lactamases of most Gram negative bacilli, including *E. cloacae*, have serine at the active site of the enzyme. Formation of a covalent bond between this serine and the ester group of the beta-lactam ring inactivates the beta-lactam antibiotic (and the beta-lactamase). The antibiotic either remains "trapped" as an enzyme-substrate complex (Graham 1989) or the serine-ester bond is broken, with beta-lactamase regeneration and antibiotic hydrolysis (Nayler 1987).

The beta-lactamases of Gram negative bacilli are located in the periplasmic space. If the rate that they inactivate beta-lactam antibiotic entering the periplasmic space is sufficient to maintain the periplasmic concentration of the antibiotic below the MIC for the essential PBPs, then the bacterium is resistant to the antibiotic. Thus the protective effect to the bacterium is a synergy between the activity of the periplasmic beta-lactamase and the diffusion barrier provided by the cell wall (Zimmermann 1977, Vu 1985).

4.1 Hydrolytic activity

The hydrolytic activity of an enzyme (beta-lactamase) is measured as a rate, the number of millimoles of substrate (beta-lactam antibiotic) hydrolysed per minute at stated conditions of pH and temperature. The rate of hydrolysis by a fixed amount of enzyme increases with increasing
substrate concentration until the substrate saturates the enzyme, when the rate of hydrolysis becomes proportional to the amount of enzyme. The choice of an initial substrate concentration ($S_0$) appropriate to the experiment is, therefore, important (Vu 1985, Wharton 1981, Bush 1986 and 1989a).

Jack (1970), choosing $S_0$ to obtain enzyme saturation, found that beta-lactamases from Gram negative bacilli could be distinguished by their rates of hydrolysis of benzylpenicillin, ampicillin and cephaloridine. (These "substrate profiles" were the foundation of the Richmond and Sykes classification (Sykes 1982) of beta-lactamases.)

The term "cephalosporinase" was used to describe the beta-lactamase from *E. cloacae* which, with $S_0$ 0,1 to 6 mM, hydrolysed cephaloridine 80 times faster than benzylpenicillin (Jack 1970). Results of subsequent experiments with this beta-lactamase, designed to determine the Michaelis-Menten parameters of the hydrolytic reaction suggested that substrate inhibition of the beta-lactamase occurred at $S_0$ 0,1 mM (Minami 1980b, Seeberg 1983) and drew attention to the importance of the $S_0$ chosen.

Vu and Nikaido (1985) measured rates of hydrolysis using $S_0$ of 1,0 to 100 micromoles. They concluded that Michaelis-Menten parameters based on the results of measurements with $S_0$ above 2,0 micromoles were inaccurate (possibly due to substrate inhibition of enzyme). Michaelis-Menten parameters based on rates of hydrolysis with $S_0$ 1,0 micromole showed that with $S_0$ 0,1 and 1,0 micromoles the differences between the rates of hydrolysis calculated for cefazolin and cefotaxime were small, compared to the differences at higher $S_0$. 
Furthermore, as the MICs of cephalosporins for PBPs are thought to be between 0.1 and 1.0 micromoles, the rates of hydrolysis at lower $S_0$ are those that are physiologically relevant (Vu 1985, Nikaido 1989).

These were important findings: Figure 2 shows the rates of hydrolysis of cefamandole, cefoxitin, cefotaxime and cefpirome at $S_0$ 0.1 to 100 micromoles by the beta-lactamase of *E. cloacae*.

### 4.2 Genetic control of beta-lactamase expression

The structural gene for beta-lactamase, *ampC*, is highly conserved amongst members of the *Enterobacteriaceae* (Bergstrom 1982).

In *E. coli* and *Shigella sonnei*, *ampC* is located adjacent to *frdD*, one of the genes for the fumarate reductase complex (Normark 1986, Linderberg 1986).

In *Citrobacter freundii*, the 1.2 kb region which separates *frdD* and *ampC* is required for induction of beta-lactamase overproduction (Bergstrom 1983). The region contains *ampR*, which codes for a regulatory protein, AmpR (Lindberg 1985). Honore (1986) sequenced *ampR* from *E. cloacae* and located the promotoirs for *ampR* and *ampC*.

In *C. freundii*, AmpR activates *ampC* transcription and suppresses *ampR* transcription. AmpR binds to a 38 bp region that, in *E. cloacae*, would precede the promotor for *ampC* and overlap the promotor for *ampR* (Lindquist 1989). Gene sequences predict that the AmpR has an amino
terminal helix-turn-helix DNA binding motif and places AmpR in the Lys R family of activator proteins (Henikoff 1988).

Inactivation of a distinct gene, \(ampD\), is followed by constitutive overproduction of beta-lactamase, provided \(ampR\) is present (Lindberg 1987). Another gene, \(ampG\), is required for both constitutive and induced overproduction of beta-lactamase, and may process a signal that activates \(ampR\) (Korfmann 1989).

5. CEPHALOSPORIN RESISTANCE OF E. CLOACAE

A minimum concentration of cephalosporin in the periplasmic space is required to inhibit essential PBPs. If accumulation of cephalosporin in the periplasmic space is prevented, then the bacterium will be resistant to the antibiotic.

In \textit{E. cloacae} two factors interact to prevent accumulation of second- and third-generation cephalosporins:-

(1) The outer membrane of \textit{E. cloacae} is 10-14 times less permeable to cephalosporins than that of \textit{E. coli} (Nikaido 1983, Yoshimura 1985, Nayler 1987).

(2) The beta-lactamase of \textit{E. cloacae} hydrolyses second- and third-generation cephalosporins with equal rapidity at the low concentrations found in the periplasmic space (Vu 1985, Yang 1988).
The chromosomally-encoded beta-lactamase of *E. cloacae* is not inhibited by clavulanate (10 mg/L) and has a pI of 7.8-8.8 on isoelectric focusing (Matthew 1975, Bush 1989a,b).

Resistance to cephalosporins emerges in *E. cloacae* when beta-lactamase activity increases following induction or mutation (Gootz 1982) and is due to overproduction of the beta-lactamase (Graham 1989). The amount of beta-lactamase in the bacterial cell increases 2-700 fold with induced overproduction and 1 000-30 000 fold with constitutive overproduction (Then 1986 and 1987, Graham 1989).

Strength of induction and stability to beta-lactamase determine the form of resistance that is most likely to emerge with use of a particular beta-lactam antibiotic (Livermore 1987a,b).

Strong inducers that are labile, such as cefoxitin and ampicillin, promote their own destruction. In contrast, weak inducers that are labile will remain effective until a mutant arises that constitutively overproduces beta-lactamase. Thus the survival advantage to the bacterium of this mutation is high with exposure to this group of antibiotics, which includes cefamandole, cefuroxime, third-generation cephalosporins and ureidopenicillins (Minami 1980a, Then 1987, Yang 1988).

Decreases in outer membrane permeability that coincide with increased beta-lactamase production may contribute to the resistance that emerges.

The contribution to resistance of a four fold increase in permeability is difficult to assess when there is constitutive overproduction of beta-
lactamase (Marchon 1987, Graham 1989) but might increase the cefotaxime MIC from 32 to 64-128 mg/L (Hopkins 1990).

In mutants that constitutively produce low levels of beta-lactamase, changes in permeability raise the cefotaxime MIC from 0.12 to 1.0 mg/L (Werner 1985, Then 1986).

6. SCREENING TESTS

Tests suited to the routine laboratory that will identify induced and constitutive overproduction of beta-lactamase by *E. cloacae* are discussed.

6.1 Induction tests

Sanders (1979) selected three isolates of *Enterobacter* species to define criteria that identified induction in a disc approximation test. Crude extracts of these isolates showed hydrolysis of cefamandole when growth had taken place in the presence of cefoxitin, but no hydrolysis when the cefoxitin had been omitted from the growth medium. In the disc approximation test, discs containing antibiotics were placed on an agar plate inoculated with a suspension of the isolate of *Enterobacter* species. A disc containing cefoxitin (30 microgrammes) was positioned to be at the edge of the zone of inhibition about a disc containing cefamandole (30 microgrammes). The radius of the zone of inhibition about the cefamandole disc was reduced by at least 4 mm for the three isolates selected, and this reduction was attributed to induction of increased beta-lactamase activity. Using 4 mm as a minimum reduction, induction was identified in 29/33 isolates of *Enterobacter* species resistant to cefoxitin.
Mulgrave (1985) determined the agar dilution MIC of cefotaxime for Enterobacter species with and without sub-inhibitory concentrations of cefoxitin in the agar, and identified induction if the cefotaxime MIC was 4 fold higher when cefoxitin was present. Chandler (1987) found improved correlation between this test and a cefotaxime-cefoxitin disc approximation test when induction was identified by any reduction in the inhibition zone.

Ashby (1987) demonstrated beta-lactamase activity by an additional method. In their disc approximation test, colour change in a drop of nitrocefin solution placed on growth near the reduced part of the zone of inhibition was compared to that obtained on growth in an antibiotic-free area. The inducer used was either imipenem (10 microgramme disc) or cefoxitin (30 microgramme disc). Both imipenem and cefoxitin are potent inducers (at 1 and 10 mg/L concentrations, respectively) that increase beta-lactamase activity 100 - 300 fold (Yang 1988).

Thore (1989) altered conditions of the disc approximation test until the number of isolates that showed a reduction in the inhibition zone reached a maximum, but concluded that sensitivity, specificity and appropriate criteria for the interpretation of disc approximation tests of induction are not known.

6.2 Detection of expanded-spectrum beta-lactamases

Plasmid-encoded beta-lactamases that are common in Gram negative bacilli, such as TEM-1 beta-lactamase, may be found in E. cloacae but do not confer resistance to cefotaxime (Seeberg 1983).
Plasmid-encoded expanded-spectrum beta-lactamases, identified in *Klebsiella ozaenae* in 1983, on the evidence of nucleotide sequencing, are derived from TEM-1 and TEM-2 or from SHV-1 beta-lactamases. These beta-lactamases confer moderate levels of resistance to third-generation cephalosporins (Philippon 1989). In *Klebsiella pneumoniae* the cefotaxime MIC may be raised from 0.1 mg/L to between 2 and 64 mg/L (Jarlier 1988).

Clavulanic acid (10 mg/L) inhibits the hydrolytic activity of the extended-spectrum beta-lactamases (Bush 1989b). A clavulanate-cefotaxime disc approximation test ("the clavulanate inhibition test") has been used to detect these beta-lactamases (Brun-Buisson 1987, Ben Redjeb 1988).
EXPERIMENTAL WORK

MATERIALS AND METHODS

Bacterial isolates

*Enterobacter cloacae* isolates were obtained from the Medical Microbiology Laboratory at Groote Schuur Hospital. Isolates (41) from diagnostic specimens had been collected at random in the Routine Laboratory (during February-April 1989) and in the Emergency Laboratory (during January-June 1989). Isolates (91) from surveillance cultures of gastro-intestinal and respiratory tract flora were collected sequentially (from February to August 1989).

Other bacteria were *Escherichia coli* (NCTC 10418) and isolates of *Salmonella typhimurium, Serratia marcescens, Proteus vulgaris, Shigella flexneri, Pseudomonas aeruginosa* (ceftazidime and piperacillin susceptible and resistant), *Haemophilus influenzae* (amoxycillin susceptible, and resistant) and *Klebsiella pneumoniae* (resistant and moderately susceptible to cefotaxime) (collected by R Bohmer) from diagnostic specimens submitted to the Medical Microbiology Laboratory.

**Identification of Enterobacter species**

Standard methods of identification were used (Cowan 1974, Farmer III 1985a,b, Brenner 1986, Ewing 1986). Ornithine and lysine decarboxylase and arginine decarboxylase/dehydrolase tests were incubated for three days (Pilsucki 1979).

Details of the identification procedure are given in Figures 3 and 4.
Part of the colony used for identification was inoculated onto a Dorset egg slope and was the source for subsequent or repeated tests.

**Antibiotics**

Commercially prepared discs containing antibiotic (Mast Laboratories, Merseyside, UK), stored dessicated at 4-8°C, were used in tests of susceptibility, induction and inhibition. Contents of discs used in susceptibility and induction tests are given in Table 1.

Nitrocefin powder reconstituted with buffer (Oxoid Laboratories, Basingstoke, Hampshire, UK) was diluted to 0.5 mg/ml with distilled water. Cefotaxime sodium for parenteral use BPC (Roussel Laboratories, Santon, RSA) powder was made up to 2mg/ml with distilled water. Stock solutions of nitrocefin and cefotaxime were stored at -20°C.

**Plate inoculation**

Isosensitest agar (Oxoid Laboratories) plates were inoculated uniformly as follows; a suspension of bacteria in peptone water was adjusted to give an inoculum that, when spread with a dry swab, gave semi-confluent growth after 18 hours incubation at 37°C. This method is the same as that described for Stokes’ comparative method (Stokes 1980).

**Susceptibility tests**

Discs containing antibiotic were applied to the surface of the agar. Peripheral inoculation of *E. coli* (NCTC 10418), or in some instances *S. typhimurium* was used
to control disc potency. After 18 hours incubation, the diameter of the zone of growth inhibition about the disc was measured with a microcaliper.

To estimate susceptibility, the zone diameters obtained using this method were compared to limits defining resistance ($\leq n$) and susceptibility ($\geq n$) prescribed in the Medical Microbiology Laboratory, Groote Schuur Hospital (Table 1).

Validation of this method was sought, for cefotaxime, by determining the minimum inhibitory concentration (MIC) of cefotaxime for 8 isolates of *E. cloacae* using a macro-method (Sanders 1985) with dilution of cefotaxime in Mueller-Hinton broth (Reeves 1978).

**Induction tests**

A disc approximation test (Sanders 1979) was used, with the following modifications (Chandler 1987, Thore 1989):

With cefoxitin and imipenem as inducers and cefamandole as the indicator, flattening of the zone of growth inhibition about the indicator disc, seen adjacent to an inducer disc, was interpreted as a positive test, and as suggesting the induced overproduction of beta-lactamase.

Preliminary tests, done to select the distance between the discs containing inducer and indicator, used eight isolates of *E. cloacae* chosen at random. Zone reduction was present when the cefamandole disc was (disc center to disc center, 6 mm diameter discs) 22-25 mm from the cefoxitin disc and 26-30 mm from the imipenem disc. Distances of 22 mm (cefotaxin-cefamandole) and 28 mm (imipenem-
cefamandole) gave maximum zone reductions for the eight isolates and were used in all subsequent induction tests.

Determination of isoelectric points

(a) Extraction of beta-lactamase

Overnight peptone water culture (5 ml) of *E. cloacae* or *P. aeruginosa* was added to 25 ml of Luria broth in a 250 ml bottle and incubated at 37°C, with aeration, for 4 hours (Minami 1980b). For *H. influenzae*, overnight growth on a boiled-blood agar plate was added to boiled-blood broth and incubated in 5% CO₂ in air, with intermittent shaking.

Bacterial cells were harvested by centrifugation at 2000 rpm at 4-8°C for 45 minutes in a Beckman J2-21 centrifuge with a JA-20 fixed-angle rotor. The pellet was washed by suspension in 10 ml of 0,1 M phosphate buffer pH 7,0 and centrifugation as described before (Yang 1988).

Cells, suspended in 5,5 ml of the phosphate buffer, were held on melting ice during sonication. A Virsonic cell disruptor (Model 16-850, Virtis, Gardiner, N.Y., U.S.A.) was used, with the medium tip at 60% of maximum output, for three cycles of sonication for 30 seconds, followed by a 30 second interval to allow for cooling of the sample.

Cell debris was removed by centrifugation at 34 000 rpm at 4-8°C for 30 minutes in a Beckman Ultracentrifuge with a VTi fixed-angle or a SW 50 swing-out rotor.
Supernatant, containing a crude extract of beta-lactamase, was stored at 4°C for up to two weeks.

(b) **Estimation of beta-lactamase activity**

A rapid estimate of beta-lactamase activity (Sanders 1986, Huovinen 1988) was obtained by adding 0,05 ml of supernatant to 0,15 ml of 50 mg/ml nitrocefin (both pre-warmed to 37°C) in the well of a microtitre plate. Colour change was noted at 15 second intervals during incubation at 37°C for 15 minutes. Control wells contained nitrocefin and water or supernatant (as above) from an amoxycillin susceptible isolate of *H. influenzae*.

(c) **Preparation of polyacrylamide gels**

Stock solution of 25 gm acrylamide and 725 mg of N,N'-methylene-bis-acrylamide was made up to 75 ml with distilled water, paper-filtered and then stored in the dark at 4°C.

Stock solution (3,6 ml) and 0,8 ml of 40% ampholines (pI 3,5 to 9,5) were added to 10 ml distilled water and degassed under vacuum.

N,N,N',N'-tetramethylethlenediamine (0,08 ml) and fresh ammonium persulphate (1,44 mg in 0,6 ml distilled water) were added (Seeburg 1983).

The gel was cast onto a sheet of Gel-bond (FMC, Rockland, Maine, U.S.A.) between glass plates (250x100 mm) separated by a plastic gasket 1 mm
thick. A row of plastic tabs, stuck to the inner surface of one glass plate, were used to indent the upper face of the gel and form application wells.

The gel was stored in the dark at 4°C for 18 hours before use.

(d) **Isoelectric focusing**

Isoelectric focusing took place at 4°C in a flat-bed tank. The anode well contained 1 M H₃PO₄ and the cathode well was 1 M NaOH. Crude extract of beta-lactamase (0.02 ml) was pipetted into each application well. Constant power of 10 watts was applied for two hours.

(e) **Visualisation of beta-lactamase**

After 15 minutes at 37°C the gel was overlaid to a depth of 1 mm with 2% agar containing 0.05 mg/ml nitrocefin (added at 42°C) and then incubated at 37°C. During the next hour the positions of beta-lactamases were revealed as a pink bands against a yellow background (Matthew 1975, Sanders 1986). The positions of the bands were marked on a sheet of transparent plastic laid over the agar.

(f) **Estimate of pI gradient**

A 1 cm wide strip was cut from the edge of the gel and divided into ten pieces. Each piece was immersed overnight in 2 ml of glass distilled water and the next day the pH of the eluate was measured with a glass-calomel-electrodes probe pH meter.
Clavulanate inhibition test

A disc approximation test (Ben Redjeb 1988) was used with the following modification:-

A disc containing either amoxycillin (25 microgrammes) or amoxycillin (20 microgrammes) plus clavulanate (10 microgrammes) (Augmentin) was placed 20 mm (disc center to disc center, 6 mm diameter discs) from a disc containing cefotaxime (30 microgrammes). The largest radius of the zone of growth inhibition about the cefotaxime disc was measured with a microcaliper.

A greater increase in this radius with amoxycillin and clavulanate present, compared to that with amoxycillin, indicated clavulanate inhibition of beta-lactamase activity.

RESULTS

Selection from collected isolates

The total of 93 isolates studied included 63 isolates from two defined samples:

(1) Inpatient sample

Isolates (29) selected at random from those collected were a 30.5% sample of the isolates of Enterobacter species reported in diagnostic specimens from Groote Schuur Hospital inpatients (Table 2).
Surveillance sample

Isolates (34) were from nine Groote Schuur Hospital inpatients who had, in each case, *Enterobacter* species isolated on three or more different occasions.

Additional isolates studied were either not reported (24) or were from patients in Woodstock Hospital (6). The latter included four isolates of *E. cloacae* that were amoxycillin susceptible.

Identification of isolates

For most isolates the identification made at the time of collection was confirmed. (Figures 5 and 6). The usual identification procedures used in the Medical Microbiology Laboratory do not distinguish *E. taylorae* and *E. hormenchei* from *E. cloacae*.

The isolate of *Serratia* species was yellow pigmented after four days at room temperature on 2% blood agar. It was distinguished from *E. sakazakii* by sorbitol fermentation and decarboxylase reactions and from *E. aerogenes* by overnight production of DNAse at 37°C and anaerogenic fermentation of glucose.

The results of most tests in the identification procedure were readily reproduced:

O'Meara's Voges-Proskauer test gave consistent results on broth cultures incubated for 48 hours, but not on those incubated for 24 hours.
Most isolates of *E. cloacae* had given an alkaline reaction in Moeller's test for arginine decarboxylase/dehydrase at 48 hours, but only 17/69 had given an alkaline reaction at 24 hours.

Ten isolates of *E. cloacae* showed no evidence of motility in hanging drop preparations after 4, 8, 12 and 18 hours in peptone water at 37°C. Seven of these isolates came from one patient.

Seven isolates identified as *E. asburiae* showed frequent tumbling and infrequent running motility after eight hours in peptone water at 37°C. After 18 hours motility was absent.

**Susceptibility testing**

All isolates of *Enterobacter* species from the inpatient sample and the surveillance sample were susceptible to imipenem, co-trimoxazole, gentamicin and amikacin.

Table 3 gives the results of disc diffusion susceptibility tests with beta-lactam antibiotics.

The results are in groups that became evident during the study. The results for the isolates of *E. taylorae* and *E. hormanchei* are included with those for *E. cloacae*: Both were resistant to amoxycillin and cefoxitin only. Isolates of *E. cloacae* (2) and *E. asburiae* (7) that were more susceptible to amoxycillin than *E. coli* (NCTC 10418) were as susceptible to amoxycillin as *Salmonella typhimurium*.

The limit prescribed for cefotaxime resistance in the disc diffusion test was incompatible with the diameters obtained and their bimodal distribution. (Data not shown).
This incompatibility was resolved with minimum inhibitory concentration (MIC) determinations.

Cefotaxime minimum inhibitory concentration

Figure 7 compares the cefotaxime MICs found by broth dilution and the diameters of the inhibition zones given by cefotaxime in disc diffusion tests for selected isolates. Both methods separated isolates of *E. cloacae* that were resistant to cefotaxime (6) from those that were susceptible (2).

The MIC determination was repeated with two isolates. Cefotaxime resistance was confirmed (MIC $\geq 128$ mg/L) in one isolate. Interestingly, the other isolate, which had been cefotaxime susceptible (MIC 0.25 mg/L) showed turbid broth in tubes containing 0.5, 2, 4, 16 and 32 mg/L cefotaxime but not in the intervening 1 and 8 mg/L tubes. No growth was obtained in subcultures from the 1 and 8 mg/L tubes. *E. cloacae* that were isolated in subcultures from the other tubes remained resistant (with growth up to the disc, in disc diffusion tests) to cefotaxime after growth on an antibiotic-free medium.

Induction tests

Results of the induction tests on isolates of *E. cloacae* are summarised in Table 4.

The mean reduction in the radius of the inhibition zone about the cefamandole disc adjacent to cefoxitin was 2.87 mm (range 1-5 mm) and adjacent to imipenem was 3.10 mm (range 1-5 mm).
Growth of a colony of *E. cloacae* within the inhibition zone about the cefamandole disc was observed in two cefoxitin induction tests. One isolate showed cefotaxime resistance on direct disc diffusion susceptibility testing but after growth on an antibiotic-free medium this isolate was cefotaxime susceptible (and cefoxitin induction test positive). The other isolate retained cefotaxime resistance after subculture on an antibiotic-free medium. This isolate and the isolate that showed 'skipped tubes' in the cefotaxime MIC determination (above) gave positive imipenem induction tests but negative cefoxitin induction tests.

With both cefoxitin and imipenem, isolates of *E. asburiae* (7) were induction test negative whilst isolates of other *Enterobacter* species (4) were induction test positive.

Loss of disc strength was detected on two occasions and caused 13 tests to be repeated. Defects in the discs were associated with improper use (imipenem left undesiccated and at room temperature and cefoxitin used six months past the manufacturer’s expiry date). Once disc failure was excluded, the induction test as defined was readily reproduced.

**Isoelectric focusing and zymography**

Supernatants that developed a red colour within ten minutes in the rapid estimate of beta-lactamase activity were used.

The direction of the zone gradient on the gel was confirmed by pH measurements and by the position of the bands given by supernatants from beta-lactam resistant isolates of *H. influenzae* and *P. aeruginosa* (illustrated in Figure 8).
The supernatant from the cefotaxime resistant isolate of *E. cloacae* showed a single band on zymography. The isoelectric point was calculated to be 8.0 at this position. The pH of the eluate from the gel adjacent to the band was 7.9.

**Clavulanate inhibition test**

Isolates of *K. pneumoniae* (broth dilution cefotaxime MICs 16-64 mg/L) and of amoxycillin susceptible *E. cloacae* controlled the clavulanate inhibition test.

The nine isolates of *E. cloacae* tested were from the nine patients who had cefotaxime resistant *E. cloacae* isolated, and included the isolate examined by isoelectric focusing. None of these isolates showed the marked augmentation of cefotaxime inhibition zones by clavulanate that was evident in the controls (Table 5).

**Statistical analysis**

Of 83 inpatients who had *E. cloacae* reported in diagnostic specimens during the collection period and for whom work-sheets were available, eight had cefotaxime (or ceftriaxone) resistance recorded. In the inpatient sample of 29, there were five patients with cefotaxime resistant *E. cloacae*.

Using the Fisher exact probability test, there is a reasonable chance (1 in 10) that in these two groups of patients the proportions of cefotaxime resistance are identical (p = 0.2296 alpha = 0.05).
95% confidence limits for the proportion of cefotaxime resistance obtained from work-sheets are 3.7% to 15.5%. A sample size of 400 would be needed to find, for example, 7% to 13% at a 95% probability level (Ractliffe 1967).
DISCUSSION

The aims of the study were (1) to determine the proportion of isolates of Enterobacter cloacae from patients in Groote Schuur Hospital that are resistant to third-generation cephalosporins and (2) to determine this proportion using methods suitable for continued use in the routine laboratory.

Resistance to cefotaxime was examined: Resistance that was permanent and due to constitutive hyperproduction of beta-lactamase had to be distinguished from that which was transient and due to induced hyperproduction of beta-lactamase (Livermore 1987a, Yang 1988).

Constitutive hyperproduction of beta-lactamase was identified in isolates of E. cloacae that remained resistant to cefotaxime after growth in an antibiotic-free medium and by excluding the presence of extended-spectrum beta-lactamases (Phillipon 1989) using a disc approximation test of clavulanate inhibition (Brun-Buisson 1987, Ben Redjeb 1988). A cefotaxime resistant isolate of E. cloacae, shown to have a characteristically basic beta-lactamase (Matthew 1975, Bush 1989b), cefotaxime resistant isolates of K. pneumoniae and amoxycillin susceptible isolates of E. cloacae controlled the test.

Clavulanate inhibition was absent in all the cefotaxime resistant isolates of E. cloacae tested. The test could fail in the presence of multiple beta-lactamases: Amoxycillin-clavulanate inhibition may be diminished in Klebsiella species and E. coli producing PSE-1 or high levels of TEM-1 or SHV-1 beta-lactamas (Thompson 1990).
The pattern of cefotaxime resistance and cefoxitin susceptibility distinguished the three isolates of *K. pneumoniae* from the cefotaxime and cefoxitin resistant isolates of *E. cloacae*. However, reduced cefoxitin susceptibility, described for OmpF deficient *E. coli* producing other TEM-3 or TEM-9 beta-lactamase (Jacoby 1990) and a porin deficient *K. pneumoniae* producing TEM-3 beta-lactamase (Pangon 1990), might obscure this distinction.

Ideally, beta-lactamase from every isolate studied should have been examined by isoelectric focusing (Huovinen 1988).

The complete correlation between cefamandole resistance and cefotaxime resistance was to be expected (Vu 1985, Nikaido 1987), and justifies the use of cefamandole as the indicator in the induction test.

Interpretation of the induction test is problematic (Chandler 1987, Thore 1989): Incomplete agreement between disc approximation tests using cefoxitin as the inducer and those using imipenem (Thore 1989) prompted the use of both inducers in this study.

Isolates with intermediate susceptibility to cefoxitin have either had induction identified with a modified disc approximation test (that uses a low content cefoxitin disc) (Sanders 1979) or have been excluded from analysis (Chandler 1987).

In this study, the eight cefotaxime susceptible isolates that did not give a clear correlation between the results of cefoxitin susceptibility testing and the cefoxitin induction test were all imipenem induction test positive. Permanent resistance to cefotaxime emerged in two of these isolates, one during a disc diffusion susceptibility test and the other during a broth dilution test. This latter result
reproduces that which led to the suggestion that cefotaxime resistance might arise by mutation (Findell 1976).

If the receptor that initiates induction is periplasmic (Korfmann 1989), then limited access to the periplasmic space would reduce cefoxitin induction of beta-lactamase production, but imipenem might still have access (Hiraoka 1989) to this receptor. Perhaps changes in cell wall permeability (Werner 1985, Then 1986, Graham 1989, Hopkins 1990) might account for some of the difficulties in the interpretation of the induction test.

The majority of cefotaxime susceptible isolates of *E. cloacae* presented no such difficulties. They were cefoxitin resistant and gave positive induction tests with both cefoxitin and imipenem. Considering that induction is part of the mechanism of resistance (Livermore 1987a,b, Gatus), the association between cefoxitin resistance and a positive cefoxitin induction test was to be anticipated and has been noted previously (Moritz 1986).

In isolates of *E. cloacae* that were as susceptible to amoxycillin, cefoxitin and cefotaxime as *E. coli* (NCTC 10418), *ampG* (Korfmann 1989) may be absent. Amoxycillin is a strong inducer (Minami 1980a, Then 1987, Yang 1988) that, at periplasmic concentrations, is hydrolysed by the beta-lactamases of *E. cloacae* at rates similar to cefoxitin (Vu 1985, Galleni 1988a,b). Amoxycillin resistance does not imply induction since, for example, TEM-1 beta-lactamase may be present (Seeberg 1983). In contrast, amoxycillin susceptibility suggests absence of inducibility.
Absence of ampC seems likely in isolates of *E. cloacae* and *E. asburiae* that were as susceptible to amoxycillin, cefoxitin and cefotaxime as *Salmonella typhimurium* (Lindberg 1986).

The discussion above is summarised, in part, in Table 6, which shows the distribution of proposed phenotypes and genotypes for the isolates studied.

The routine laboratory could use the inhibition and induction tests used in this study but, probably, they would be used as supplementary tests that followed identification of an isolate as *E. cloacae*. The incorporation of the inhibitor or inducer in the agar of plates inoculated during multipoint susceptibility testing is an attractive alternative. However, the inhibition or induction test must be clearly separated in concept from that of a susceptibility test: For example, the concentration of clavulanate required for inhibition tests may be inappropriate for susceptibility tests (Bush 1989a, Thompson 1990, Jacoby 1990). Furthermore, imipenem may be too unstable at low concentration for use as an inducer (European Study Group on Antibiotic Resistance 1987).

A conclusion of this study is that patterns of susceptibility could give useful information about the cephalosporin resistance of *E. cloacae* provided borderline susceptibility results in the disc diffusion test are checked by MIC determinations or, at least, provide a starting point for further investigation:

Cefotaxime and cefoxitin resistance suggests constitutive overproduction of beta-lactamase
Cefotaxime susceptibility and cefoxitin resistance suggests inducible overproduction of beta-lactamase

Cefotaxime and cefoxitin susceptibility suggests uninducible beta-lactamase

This study confirms that a low proportion (<16%) of the isolates of *E. cloacae* from Groote Schuur Hospital inpatients show permanent resistance to third-generation cephalosporins. The sample number for a confident estimate of this proportion has been obtained, and highlights the danger of drawing conclusions based on small numbers of isolates, as has been done (Benn 1984, Ellner 1987).

Perhaps of more importance is the observation, based on the results of the induction tests, that most (48/76) of the cefotaxime susceptible isolates of *E. cloacae* from Groote Schuur Hospital inpatients have the genetic potential for resistance to third-generation cephalosporins.

This aspect of the cephalosporin resistance of *E. cloacae* and the relative contributions of changes in cell wall permeability and of induced overproduction of beta-lactamase to this resistance warrant further investigation.

For the present, the clinical implications of the results of this study are uncompromising: Unnecessary use of antibiotics that select constitutive overproducers of beta-lactamase should be avoided (Sanders 1987). Whenever these antibiotics are used, provision for their failure will have to be made (Follath 1987, Spritzer 1990). Furthermore, any source of *E. cloacae* within the hospital,
such as contaminated enteral feeds (Levy 1989) and milk powders (Biering 1989),
must be controlled.
REFERENCES


Honore N, Nicolas M-H, Cole S T (1986). Inducible cephalosporinase production in clinical isolates of Enterobacter cloacae is controlled by a regulatory gene that has been deleted from Escherichia coli. EMBO J. 5, 3709-3714.


TABLE I - ANTIBIOTIC DISCS

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Inhibition zone limits prescribed in the Medical Microbiology Laboratory, Groote Schuur Hospital</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
</tr>
</tbody>
</table>

Inhibition zone limits in mm.
<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>ISOLATES COLLECTED</th>
<th>IN-PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-stream urine</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>IV Catheter Tip</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Catheter Specimen</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Urine</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Peritoneal Swab</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Blood</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tracheal Aspirates</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Dialysis Fluid</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 - Inpatient Sample

Enterobacter species reported in diagnostic specimens

Table 3- Susceptibility of *Enterobacter* species isolates to Beta-lactam Antibiotics

<table>
<thead>
<tr>
<th>Susceptibility</th>
<th>Number (%) of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>13(14)</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>8(9)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>44(48)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>26(29)</td>
</tr>
</tbody>
</table>

S Susceptible  I Intermediate  R Resistant

A Enterobacter species (n=91)  
B Enterobacter cloacae (n=76)  
C Inpatient sample E. cloacae (n=25)
Table 4- Induction Tests and Cefoxitin Susceptibility of *Enterobacter cloacae* Isolates

<table>
<thead>
<tr>
<th>Cefoxitin Susceptibility</th>
<th>Induction Test Positive [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cefoxitin Test</td>
</tr>
<tr>
<td>Susceptible (n=6)</td>
<td>0</td>
</tr>
<tr>
<td>Intermediate (n=4)</td>
<td>4</td>
</tr>
<tr>
<td>Resistant (n=40)</td>
<td>40</td>
</tr>
</tbody>
</table>

[1] See text for a description of the method and the criteria for a positive test.
<table>
<thead>
<tr>
<th>Antibiotic discs</th>
<th>Zone of inhibition (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOXICILLIN + CLAVULANATE</td>
<td>6-9</td>
</tr>
<tr>
<td>CEFOTAXIME</td>
<td>9-12.5</td>
</tr>
<tr>
<td></td>
<td>5-7.5</td>
</tr>
<tr>
<td>AMOXICILLIN + CEFOTAXIME</td>
<td>7.5-9.5</td>
</tr>
<tr>
<td>CEFOTAXIME</td>
<td>11-14</td>
</tr>
<tr>
<td>AMOXICILLIN</td>
<td>12-15</td>
</tr>
<tr>
<td>CEFOTAXIME</td>
<td>15-18</td>
</tr>
<tr>
<td>AMOXICILLIN</td>
<td>18-21</td>
</tr>
<tr>
<td>AMOXICILLIN</td>
<td>21-24</td>
</tr>
<tr>
<td>AMOXICILLIN</td>
<td>24-27</td>
</tr>
</tbody>
</table>

B Cefotaxime resistant and moderately susceptible K. pneumoniae
A Cefotaxime resistant E. cloacae

Radial distance, disc edge to zone edge

n=9
n=8
n=2

See text for method

Table 5 - Clavulanate Inhibition Test
<table>
<thead>
<tr>
<th>PROPOSED GENOTYPE</th>
<th>PROPOSED PHENOTYPE</th>
<th>All Isolates</th>
<th>Inpatient Sample [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{ampR}^+\text{ampC}^+\text{ampD}^+\text{ampG}^+ )</td>
<td>constitutive overproduction of beta-lactamase</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>( \text{ampR}^+\text{ampC}^+\text{ampD}^+\text{ampG}^+ )</td>
<td>inducible overproduction of beta-lactamase</td>
<td>36</td>
<td>19</td>
</tr>
<tr>
<td>( \text{ampR}^+\text{ampC}^+\text{ampD}^+\text{ampG}^+ )</td>
<td>cefoxitin susceptible and imipenem inducible (permeability change?)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>( \text{ampR}^+\text{ampC}^+\text{ampD}^+\text{ampG}^+ )</td>
<td>constitutive low production of beta-lactamase</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>( \text{ampC}^- )</td>
<td>absent beta-lactamase production</td>
<td>13</td>
<td>0</td>
</tr>
</tbody>
</table>

[1] See text for the definition of this sample
Fig. 1 - Diffusion Rates of Cephalosporins.

Aztreonam and Imipenem


Fig. 2 - Hydrolysis of Cephalosporins by Beta-lactamase from E. cloacae

Relative Rates of Hydrolysis

Data from Nikaido 1987, 1990 and Galleni 1988
Fig. 3- Identification of Enterobacter species

GROWTH ON MACConKEY AGAR
GRAM NEGATIVE BACILLUS
OXIDASE NEGATIVE
ACID AND GAS FROM GLUCOSE FERMENTATION
SIMMONS CITRATE POSITIVE
ONPG POSITIVE
INDOLE NEGATIVE
PHENYLALANINE DEAMINASE NEGATIVE
HYDROGEN SULPHIDE PRODUCTION NEGATIVE
Dnase NEGATIVE
HANGING DROP MOTILITY PRESENT
OMERAS YOGES-PROSKAER POSITIVE
MOLLERS TESTS READ AT 1, 2 AND 3 DAYS:
ORNITHINE DECARBOXYLASE POSITIVE
ARGININE DECARBOXYLASE/ DEHYDROLASE POSITIVE
LYSINE DECARBOXYLASE NEGATIVE
SORBITOL FERMENTATION
NO DNASE AT ROOM TEMPERATURE AT 2 AND 7 DAYS
NO PIGMENT PRODUCTION AT ROOM TEMPERATURE

12 hours incubation at 37 C, unless indicated
**Fig. 4 - Identification of *Enterobacter* species**

<table>
<thead>
<tr>
<th><strong>Species</strong></th>
<th><strong>MOT</strong></th>
<th><strong>SORB</strong></th>
<th><strong>VP</strong></th>
<th><strong>ARG</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. cloacae</strong></td>
<td>MOT-</td>
<td>SORB+</td>
<td>VP+</td>
<td>ARG-</td>
</tr>
<tr>
<td><strong>E. cloacae</strong></td>
<td>Non-motile</td>
<td>MOT-</td>
<td>SORB+</td>
<td>VP+</td>
</tr>
<tr>
<td><strong>E. aeroburan</strong> (few)</td>
<td>MOT-</td>
<td>SORB+</td>
<td>VP-</td>
<td>ARG+</td>
</tr>
<tr>
<td><strong>E. aeroburan</strong> (most)</td>
<td>MOT-</td>
<td>SORB+</td>
<td>VP-</td>
<td>ARG-</td>
</tr>
<tr>
<td><strong>E. hormaechaei</strong></td>
<td>MOT-</td>
<td>SORB-</td>
<td>SUC+</td>
<td></td>
</tr>
<tr>
<td><strong>E. hormaechaei</strong></td>
<td></td>
<td>MOT+</td>
<td>SORB-</td>
<td>SUC+</td>
</tr>
<tr>
<td><strong>E. taylorae</strong></td>
<td>MOT+</td>
<td>SORB-</td>
<td>SUC-</td>
<td></td>
</tr>
<tr>
<td><strong>E. sakazakii</strong></td>
<td>MOT+</td>
<td>SORB-</td>
<td>delayed DNAase and pigment</td>
<td></td>
</tr>
<tr>
<td><strong>E. agglomerans</strong></td>
<td>ORN-</td>
<td>ARG-</td>
<td>LYS-</td>
<td></td>
</tr>
<tr>
<td><strong>E. aerogenes</strong></td>
<td>ORN+</td>
<td>ARG-</td>
<td>LYS+</td>
<td>MOT+</td>
</tr>
<tr>
<td><strong>E. gergoviae</strong></td>
<td>ORN+</td>
<td>ARG-</td>
<td>LYS+</td>
<td>MOT+</td>
</tr>
</tbody>
</table>

**MOT** = Motility  
**SORB** = Sorbitol fermentation  
**VP** = Voges-Proskauer  
**ARG** = Arginine decarboxylase/ dehydroase [1]  
**SUC** = Sucrose fermentation  
**ORN** = Ornithine decarboxylase [1]  
**LYS** = Lysine decarboxylase [1]

Read at [1] 1, 2 and 3 and [2] 2 and 7 days
Fig. 5 - Identification of Isolates
Inpatient Sample

- E. cloacae: 22
- Serratia sp.: 1
- C. diversus: 1
- E. hormaecheii: 1
- E. taylorae: 1
- E. cloacae non-motile: 1
- E. aerogenes: 2

29 isolates collected from 29 inpatients
Fig.6- Identification of isolates
Surveillance Sample

- E. cloacae: 19
- E. agglomerans: 1
- E. asburiae: 5
- E. cloacae non-motile: 9

34 isolates from 9 patients
Fig. 7 - Cefotaxime Susceptibility

Minimum Inhibitory Concentrations Versus Diameter of Inhibition Zones

8 isolates of Enterobacter cloacae
Fig. 8

Lane 1: Beta-lactamase from (1) amoxycillin resistant P. aeruginosa and (2) cefotaxime resistant E. cloacae

Lane 2: Beta-lactamase from (3) H. influenzae, (2) cefotaxime and piperacillin resistant P. aeruginosa and (3) cefotaxime resistant E. cloacae

Lane 3: Beta-lactamase from (1) amoxycillin resistant H. influenzae, (2) cefotaxime and piperacillin resistant P. aeruginosa and (3) cefotaxime resistant E. cloacae

Lane 1-3: Beta-lactamase from (1) amoxycillin resistant P. aeruginosa and (2) cefotaxime resistant E. cloacae

Lane 2: Beta-lactamase from (3) H. influenzae, (2) cefotaxime and piperacillin resistant P. aeruginosa and (3) cefotaxime resistant E. cloacae

Lane 3: Beta-lactamase from (1) amoxycillin resistant H. influenzae, (2) cefotaxime and piperacillin resistant P. aeruginosa and (3) cefotaxime resistant E. cloacae

Fig. 8 - Beta-lactamase Focusing of Lane 1-3: Beta-lactamase from (1) amoxycillin resistant P. aeruginosa and (2) cefotaxime resistant E. cloacae.