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" PREVALENCE OF EXTENDED-SPECTRUM ß-LACTAMASES (ESBLs) IN THE ACADEMISCH ZIEKENHUIS MAASTRICHT – HOLLAND"

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Por medio de la presente ponemos en conocimiento para los archivos del Programa de Medicina del Colegio de Ciencias de la Salud de la Universidad San Francisco de Quito que el Comité de Tesis de la estudiante María Cristina Viteri Quincha No. 6972 conformado por el Dr. Marco Fornasini (director principal), Dr. Gonzalo Mantilla aprobaron la versión final de la tesis de la mencionada estudiante: **"Prevalence of Extended-Spectrum B-Lactamases (ESBls) in the Academisch Ziekenhuis Maastricht-Holland".**

Para constancia firman los integrantes del Comité.

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STATEMENT

L.S.,

Herewith I declare that Mrs. Christina Viteri is a very good motivated student. During her stay at our department of Medical Microbiology she became very rapidly familiar with both the lab work and the theoretical background of the project.

Sincerely Yours,

Ellen E. Stobberingh, PhD
Associate Professor of Medical Microbiology

ACKNOWLEDGEMENTS

I would like to thank doctor Ellen Stobberingh for all of her help during the realization of this project and the laboratory team for their patience and friendship.

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TABLE OF CONTENTS

" PREVALENCE OF EXTENDED - SPECTRUM ß - LACTAMASES (ESBLs) IN THE ACADEMISCH ZIEKENHUIS MAASTRICHT – HOLLAND"

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THESIS SUMMARY

The prevalence of ESBLs was studied in a group of 50 isolates members of the family enterobacteriaceae which were found to be unusually resistent to an extense antibiotics battery. The samples were collected in the Academic Hospital of Maastricht (Holand) during the period of time from July 2002 to March 2003.
Samples from all the hospital departments were included.

Three different phenotypic tests were performed to detect the ESBLs producing strains (standard disk diffusion method, double disc test and E-test).

Six (12%) out of the 50 strains were confirmed to be positive for at least 1 of the ESBLs phenotypic tests and one (2%) was non determinated.

INTRODUCTION

Extended-spectrum ß-lactamases (ESBLs) are enzymes that hydrolize the extended spectrum cephalosporins (Ex. Ceftazidime, Cefotaxime) and / or the monobactams (Aztreonam)**(4,7,9)**. Generally , they confer resistance to most β- lactam with the exception of the cephamycins (cefoxitin, cefotetan and cefmetazole) and carbapenems (Imipenem, Meropenem), while generally retaining susceptibility to the β- lactamase- inhibitors (Ex. Clavulanic acid) **(2,7,20)**.

These enzymes, usually plasmid mediated, are the results of point mutations in a few amino-acid substitutions in gene sequence, frequently though not always, derived from either a TEM or a SHV-related ß-lactamase. As a result, MICs of the newer cephalosporins and aztreonam are elevated, although clinical resistance is not always evident **(5,9)**.

The first isolate resistant to extended spectrum cephalosporins was found in Germany in 1983 and produced a SHV-2 Beta lactamase. Two years later, the first outbreak of an ESBL producing organism was reported in France **(2,4,8)**.

Initially, the ESBL-producing organisms were all isolated from nosocomial infections, specially due to the use of extended-spectrum cephalosporins. By now, this problem which is associated with high morbidity and mortality is occurring worldwide and colonizing organisms in outpatient and nursing home populations are being found to produce ESBLs **(5,3,8)**.

These enzymes occur mainly in Klebsiella and E. coli species, but they may also be present in other members of the Enterobacteriaceae family such as Citrobacter, Serratia, Proteus, Salmonella, and Enterobacter **(3,7-9,11,12).**

Various TEM and SHV derivatives such as TEM-3, SHV-4, 5 and 12 have been reported as the dominant ESBL in Europe **(17)**. However, worldwide SHV-5 is more prevalent **(15)**.

Although its prevalence vary among countries, hospitals, UCIs and patient types, the incresase in prevalence has been associated with some risk

factors that include use of antibiotics (as it is the case for UCIs, hematology departments, burn units and infections that need a continious use of antibiotics). Also in the developing countries this is a worrying issue due to its medical and surgical advances which outpace infection control.

On the other hand, there are countries like Spain and Taiwan, where ESBLs producing strains are common in part because of the over-the-counter availability of antibiotics **(1)**.

Eventhough an early detection of ESBL-producing organisms is very important, there is the inconvenience of a difficult diagnosis of these strains, since the resistance to extended-spectrum cephalosporins of many strains may not be detected by routine susceptibility testing methods that follow current National Committee for Clinical Laboratory Standards (NCCLS) breakpoints **(4, 23)**.

Cephalosporin affinity of ESBLs varies widely by geographic location, being Cefotaxime and Ceftriaxone more affin than Ceftazidime in Asia-Pacific and Latin America; while, Ceftazidime is slightly more affin than Cefotaxime in USA, Canada and Europe **(7)**. For this reason, Cefotaxime and Ceftazidime need to be tested for a better screening and confirmation (as recommended by the **NCCLS)**.

ESBL producing bacteria may be multidrug resistant, which includes resistance to non Beta lactam antibiotics such as aminoglycosides, fluorquinolones, tetracyclines and trimethoprim-sulfametoxazole **(2,5,7, 19,NCCLS 1997)**. This fenomenom could be due to co-transfer of resistance factors on plasmids **(7)**. Therefore, resistance to these drugs could be used to predict the presence of ESBL **(2)**.

Since there are no up to date data on this topic in the University hospital and the frecuency of antibiotic resistance is a matter of worlwide worry, we investigated the prevalence of ESBLs producing organisms in the university hospital.

MATERIALS AND METHODS

Bacterial strains

Fifty strains, members of the family Enterobacteriaceae, of which the resistance pattern suggested the posibility of ESBL production, were collected during a 9 months period between July 2002 and March 2003.
The samples were chosen among all samples taken from in and out patients from all the departments at the Academic Hospital of Maastricht. These bacteria were found to be unusually resistent to some of the antibiotics from a battery of antibiotics when using zone diameter interpretive standards and equivalent minimal inhibitory concentration (MIC) breakpoints for enterobacteriaceae according to the **NCCLS** references.

The samples included the following species: *Escherichia coli* (n=19), 16 *Proteus mirabillis* (n=16), *Enterobacter cloacae* (n=9), *Citrobacter amnigenns* (n=2), 1 *Citrobacter freundii* (n=1), *klebsiella oxytoca* (n=2) and *Klebsiella pneumoniae* (n=1).
Only one strain of a given specie per patient was included.

Antimicrobial agents

Eleven antibiotic disks were tested with the disk diffusion susceptibility test. They included the penicilines amoxixillin (30μg) and piperacillin (100 μg), the second generation cephalosporin cefuroxime (60μg), the third generation cephalosporins ceftazidime (30μg), ceftriaxone (30μg) and cefpodoxime (30μg), the monobactam aztreonam (30μg), the fluorquinolone ciprofloxaxin (10μg) and the aminoglycoside gentamicine(40μg). Amoxicillin plus clavulanic acid (30/15μg) and piperacillin plus tazobactam (100/10μg) disks were used as a source of the β-lactamase inhibitors. The antibiotic disks were obtained from Rosco **(Neo-Sensitabs, Rosco).**

Rosco tablets instead of paper disks were used to performe the disk difussion susceptibility test and the double-disk synergy test because of the readily availability in our laboratory.
Ceftazidime and cefotaxime ESBL E-test strips were obtained from AB Biodisk.

Screening and Confirmatory Test for ESBLs

The standard disk diffusion susceptibility testing was carried out as the initial screening test for all the participating samples according to NCCLS methodology.

Two plates for 11 different antibiotics were used:

In the first plate, antibiotic disks containing gentamicine, aztreonam, ceftazidime, ceftriaxone and cefpodoxime were placed 20 mm edge to edge from an amoxixillin-clavulanic acid disk and from each other **(See figure # 1a).**

In the second plate, antibiotic disks containing **piperacillin-tazobactam, ciprofloxacin, cefuroxime, piperacillin and amoxicillin** were placed 20 mm edge to edge from each other **(See figure # 1b).**

For the performance of this test, an overnight culture was diluted to an optical density equal to that of a 0,5 Mc Farland turbidity standard. This suspension was then used to inoculate Muller Hinton II agar plates by swabbing them with a cotton swab. The results were interpreted after an overnight bacterial incubation at 37°C, by using the instructions of the disk manufacturer (Neo-Sensitabs, Rosco) **(See table 1)**.

The NCCLS recommendations for the screening and confirmatory test of ESBLs in *Klebsiella* and *E coli*, were applied:

1. The disk diffusion test must include at least one of the following antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone. *All of them but cefotaxime were included in this study.*
2. If the bacteria show resistance to at least one of these antibiotics, a putative ESBL producer shoud be reported and the confirmatory testing requires the use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. A $\supseteq$ 5 mm increase in a zone diameter for either one of the two beta lactams tested in combination with clavulanic acid versus its zone when tested alone will be indicative of ESBL. *In order to apply this recommendation, the ESBL E-test strips were also used in this study.*

On the other hand, the samples that in the first plate, had an enhanced zone of inhibition between any one of the betalactam disks and the disk containing clavulanic acid were also interpreted as presumptive evidence for the presence of an ESBLs producing organism and the double disk potentiation procedure was applied to confirm the hypothesis.

The clavulanate double-disk (DD) potentiation procedure was used as the confirmatory test to determine if there was or there was not a real interaction (an enhanced zone of inhibition) between the beta lactam and the beta-lactamase inhibitor in the samples tested by the disk diffusion susceptibility testing. For this test, the bacteria strains were also inoculated in Muller Hinton II agar plates in the same way as it was described before. Then, one disk containing clavulanic acid was placed in the center of the agar plate and several disks of the same beta lactam were placed around it to different distances according to the size of the inibition zone observed during the doble disk diffusion test.

A clear cut extension of the inhibition zone around the betalactam antibiotic disk toward the clavulanic acid-containing disk ($\supseteq$ 5 mm increase) was interpreted as a positive double-disk synergy test result with this particular betalactam **[as described by Vercauteren (11)].**

The ESBL screening Etest strip.

As it was mentioned before, the E-test strips were also used for the phenotypic determination of ESBL presence in the *Escherichia coli, Klebsiella pneumonia* and *Klebsiella oxytoca* strains.

The strip carries two gradients: on one of the ends, there is ceftazidime (with a concentration from 0,5 to 32 µg/mL) or cefotaxime (with a concentration from 0,25 to 16 µg/mL) and on the other end there is ceftazidime (from 0,064 to 4 µg/mL) or cefotaxime (from 0,016 to 1,0 µg/mL) plus a fixed concentration of

clavulanic acid (4 µg/mL). The strips were used according to the manufacturer's instructions.

After an overnight bacterial incubation at 37°C, the MICs on both ends of the strip were interpreted as the point of intersection of the inhibition ellipse with the Etest strip edge.

For the interpretation, the strains were considered as ESBL positive with either one of the next results: a cefotaxime MIC $\supseteq$ 0,5 and a ratio of cefotaxime MIC/cefotaxime-clavulanic acid MIC equal to or greater than 8; a ceftazidime MIC $\supseteq$ 1 and a ratio of ceftazidime MIC/ceftazidime-clavulanic acid MIC equal to or greater than 8; or when a phantom zone or deformation of the CT or TZ ellipse appeared.

When MIC values were below or above the test ranges, the interpretation was non determinable.

RESULTS

The results of the Standarized disk diffusion test are shown in **Table 2**.

Six (12%) out of the 50 strains were confirmed to be positive for at least 1 of the ESBLs phenotypic tests and one (2%) was non determinated: **(See Table 3)**

- Four samples had a positive interaction in the Double disk synergy test: **(See figure # 2)**
  In two *E. coli*: Aztreonam and Ceftriaxone disks showed an enhanced inhibition zone towards the amox + clavulanate disk. Ceftazidime and Cefpodoxime did it also in one of them.
  In two K. oxytoca strains there were also positive results. In both of them Cefpodoxime was positive. In one strain aztreonam shown the interaction and in the other Ceftriaxone did.

- A total of six strains had an ESBL E-test positive result and one strain had a non determinable result: **(See figure # 3)**
  Two E. coli strains were positive for both Ceftazidime and Cefotaxime strips; other two were positive only for the Ceftazidime strip and other strain was positive for the Cefotaxime strip and non determinable for the Ceftazidime strip.
  Both of the K. oxytoca strains were positive for the Cefotaxime strip.

❖ The four strains that were positive for the double disk (DD) test were also positive for the ESBL E-test. However, three of the strains that were positive for either the Ceftazidime strip, the Cefotaxime strip or both E-test strips did not have a positive DD test.

- ESBL-positive isolates were also resistant to other non-beta-lactam drugs. **(See Table # 4)**

In four Enterobacter cloacae isolates, a cephalosporinase induction occured, being detected as a blunting of the inhibition zone around the Beta lactam adjacent to the inducer disk (Augmentin). **(See figure # 4)**
In this case, the Beta lactamase induced by the Augmentin disk, protects the bacteria against the Beta lactam, truncating the zone of that disc **(6).**

During the disk diffusion test, Proteus mirabilis strains shown an unusual antibiotic resistance pattern. 94 % were resistent to gentamicine, 88% were resistent to piperacillin and amoxicilline, 47% were resistent to ciprofloxacine and 2 strains (12%) shown resistance to ceftriaxone and therefore were included as putative ESBL producers.

DISCUSION

Although other classes of ESBL exist (CTX-M and IBC type beta lactamases), most of them are derivated from either a TEM or an SHV enzyme **(13, 14, 16, 20)**. Unfortunately, it is not know if the breakpoints used to detect TEM and SHV derivatives are equally efficient in detecting strains producing the other B- lactamase types. However, these types appear to reduce significantly the susceptibility to at least 1 broad-spectrum cephalosporin and therefore the respective strains should be readily recognized as ESBL-producers **(17)**.

When performming the phenotypic tests, one of the principal problems in the detection of ESBL producers is the possibility of low level expression of the enzyme and of an inoculum effect, resulting in variable MIC values and zone diameters. In strains with low level of beta lactamase production, MIC of extended spectrum beta lactam agents may decrease only a small amount in the presence of an inhibitor due to the small contribution of the ESBL to microbiological resistance **(5)**. Consequently, isolates may be reported by the laboratory as sensitive to extended espectrum cephalosporins, but in vivo, treatment failure may occur with this group of antibiotics **(20)**.

On the other hand, a potential source of error, could have been the simultaneous production of an ESBL and a class C Beta-lactamase, since the β-lactamase inhibitors (clavulanate) can not inhibit these enzymes **(1)**, and therefore decreasing the sensitivity of these methods.
ESBL producers that show resistance or decreased susceptibility to at least 1 penicilline – inhibitor combination can be associated with the production of TEM-1 and or adquired AmpC Beta-lactamases **(15)**.

For all the strains except for the E. coli and Klebsiella species, the double disk synergy test was the only phenotypic confirmatory test used in this

study since the E-test could only be applied in both of the species mentioned above.

Fortunately, extended spectrum enzymes in the TEM or SHV families become even more susceptible than the parental types to inhibition by agents such as clavulanate or sulbactam, so that a double disk diffusion test provides a good technique to confirm the presence of these enzymes in the clinical laboratory **(18)**. However, eventhough the double disk sinergy test is considered reliable, it may not yield good results if the disks are not placed at an appropriate distance appart **(5, 10)**.

In some cases, the interpretation of this test was not easy to perform being necessary to repeat it for several times until the results were clear enough.

In some cases, the ESBL E-test strips were not so easy to read. However, this technique detected a 33% more ESBL producing strains than the Double disk synergy test.

Eventhough the presence of ESBL producers among Proteus mirabillis is not very common, it is important to have in mind that this is the second most common cause of urinary tract infections and is also an important cause of nosocomial infections. Therefore screening for ESBLs production should be routinely considered also for this species.

Most of the ESBL in Proteus species are TEM-type derivatives but also other enzymes from the group 2be have been reported **(12)**. However, in this study none of the isolates from this specie was found to be an ESBL producer.

Proteus mirabillis isolates (from the wild type) are usually susceptible to ampicillin and other β-lactams. Nevertheles, in the risent years a growing number of β-lactamase producer strains have been found and the hypothesis of the presense of a naturally occurring β-lactamase in this species has been stablished **(22)**. Most of the time, if there is resistance to cefoxitin, the

production of AmpC-like enzyles could be envolved. In this study, all the strains were susceptible to cefuroxime.

If the strain is resistant to amoxicillin-clavulanate but susceptible to cephalothin (1st GC), the production of an inhibitor-resistant TEM or oxacilin type enzymes could be the reason.

At the moment, many strains gentamycine resistent have been reported but there is no much data available on the resistance mechanisms. Perhaps this resistance patterns are due to the more common use of this drug in the clinical instances. In this study, 94% of the Proteus mirabillis strains were resistent to the aminoglycoside.

In this study, even when none of the Enterobacter cloacae isolates was found to be an ESBL producer, there was a high incidence of resistance to extended spectrum cephalosporines. Resistance to cephalosporins (including ceftazidime) in this species is mainly due to a chromosomal encoded and inducible cephalosporinase. In this species, there are several genes that interact in the induction process: an ampC gene, which encodes a β-lactamase; an ampR gene that encodes a regulatory protein which enhances ampC expression and an ampD gene that encodes a repressor. Mutations in the ampD allele have been associated with cephalosporinase overproduction, which facilitate the transition from the susceptible to the resistant state **(21)** .

On the other hand, in species such as *Enterobacter cloacae* the hyperproduction of a cephalosporinase in addition to an ESBL, results in high levels of resistance to Cefotaxime, Ceftazidime and Aztreonam that are responsible for a false-negative synergy test (because of reduced zone diameters). Synergy can be detected by a synergy test with a disk of cefepime or cefpirome which are less hydrolyzed than the others by the cephalosporinase **(2)**.

Most ESBLs are encoded by genes located on very large plasmids and these plasmids often carry genes for resistance to other antimicrobial agents as well (aminoglycosides, sulfonamide, tetracycline, etc) **(10, 15, 18)**. Therefore, resistance to these antibiotics could alert the production of ESBL in these organisms. This is the reason why in this study we included all the strains that were found to be unusually resistent to some antibiotics from a complete battery to which these strains are normally tested in the laboratory.

It is common for organisms to produce 3 or 4 beta lactamases if they produce a plasmid mediated Extended Spectrum Beta lactamase **(5)**. Detection of SHV-type beta lactamases can be problematic. Some studies have shown that using the ESBL E-test strips, only 50% of these were detected. However, genetic test may indicate the presence of an SHV-type beta lactamase, eventhough active enzyme is not produced **(IDEM)**.

It is important to keep in mind that even when many ESBL producing organisms do not appear resistant to newer cephalosporines and aztreonam in routine susceptibility tests, these drugs are usually unsuccessful in treating infections due to ESBL producind organisms **(15)**.

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TABLE # 1

Breakpoints for the interpretation of results according to the manufacturer (Neo Sensitab, Rosco)

	Genta (40µg)	Aztreo (30µg)	Ceftaz (30µg)	Ceftriax (30µg)	Cefpod (30µg)	Amx-clav (30/15µg)	Pip-taz (100/10µg)	Cefurox (60µg)	Ciproflox (10µg)	Piperac (100µg)	Amoxic (30µg)
R	< 23	< 22	< 20	< 32	<= 17	< 24	< 22	< 23	< 20	< 20	< 18
I	23 - 29	22 - 25	20 - 25		18 - 20		22 - 25	23 - 27	20 - 23	20 - 25	18 - 27
S	>= 30	>= 26	>= 26	>= 32	>= 21	>= 24	>= 26	>= 28	>= 24	>= 26	>= 28

TABLE # 2

Results of the standarized disk diffusion test (interpretation according to the manufacturer breakpoints)

N.	Gramnegative	Gentamicin	Aztreonam	Ceftazidime	Ceftriaxone	Cefpodoxime	Amoxi-clavul	Pipera-tazob	Cefuroxime	Ciprofloxaxine	Piperacillin
1	Enterobacter cloacae	R	R	R	R	R	R	R	R	I	R
2	Enterobacter cloacae	S	S	S	S	S	R	S	S	S	S
3	Enterobacter cloacae	I	R	R	R	R	R	R	R	S	R
4	Enterobacter cloacae	I	S	S	S	S	R	S	I	S	S
5	Enterobacter cloacae	I	R	R	R	R	R	R	R	S	R
6	Enterobacter cloacae	I	S	S	S	S	R	S	I	S	S
7	Enterobacter cloacae	I	R	R	R	R	R	R	R	S	R
8	Enterobacter cloacae	I	S	S	R	I	R	S	R	S	S
9	Enterobacter cloacae	R	S	S	R	S	R	S	I	S	S
10	Escherichia coli	R	S	S	S	S	S	S	S	R	I
11	Escherichia coli	R	S	S	S	S	R	R	I	R	R
12	Escherichia coli	I	S	S	S	S	R	R	I	R	R
13	Escherichia coli	I	S	S	S	S	R	I	I	R	I
14	Escherichia coli	R	S	S	R	S	R	R	R	R	R
15	Escherichia coli	I	S	S	S	S	S	I	I	R	R
16	Escherichia coli	R	S	S	S	S	S	I	I	S	R
17	Escherichia coli	R	I	R	R	R	R	R	R	R	R
18	Escherichia coli	S	S	S	S	S	S	R	S	R	R
19	Escherichia coli	R	S	S	R	S	R	R	I	R	R
20	Escherichia coli	R	S	S	S	S	R	S	I	S	R
21	Escherichia coli	R	S	S	S	S	S	R	R	R	R
22	Escherichia coli	R	S	S	R	S	S	I	R	R	R
23	Escherichia coli	R	S	S	S	S	S	S	I	S	R
24	Escherichia coli	R	S	R	R	R	R	I	R	R	R
25	Escherichia coli	R	I	R	R	R	S	I	R	R	R

N.	Gramnegative	Gentamicin	Aztreonam	Ceftazidime	Ceftriaxone	Cefpodoxime	Amoxi-clavul	Pipera-tazob	Cefuroxime	Ciprofloxaxine	Piperacillin
26	Escherichia coli	I	S	I	R	R	S	S	R	S	R
27	Escherichia coli	R	S	S	S	S	S	S	S	R	I
28	Escherichia coli	R	R	I	R	R	R	R	R	S	R
29	Proteus mirabilis	I	S	S	R	S	S	I	S	S	R
30	Proteus mirabilis	R	S	S	S	S	S	S	S	I	R
31	Proteus mirabilis	R	S	S	S	S	S	S	S	I	R
32	Proteus mirabilis	R	S	S	S	S	R	S	S	I	R
33	Proteus mirabilis	R	S	S	S	S	S	S	S	I	R
34	Proteus mirabilis	R	S	S	S	S	S	S	S	R	R
35	Proteus mirabilis	R	S	S	S	S	S	S	S	S	R
36	Proteus mirabilis	R	S	S	S	S	S	S	S	R	R
37	Proteus mirabilis	R	S	S	S	S	S	S	S	S	R
38	Proteus mirabilis	R	S	S	S	S	S	S	S	S	I
39	Proteus mirabilis	I	S	S	S	S	S	S	S	S	S
40	Proteus mirabilis	R	S	S	S	S	R	S	S	S	R
41	Proteus mirabilis	R	S	S	S	S	S	S	S	I	R
42	Proteus mirabilis	I	S	S	S	S	S	S	S	R	R
43	Proteus mirabilis	R	S	S	S	S	S	S	S	S	I
44	Proteus mirabilis	R	S	S	R	S	S	S	S	S	I
45	Citrobacter amn	S	S	S	S	S	S	S	S	S	S
46	Citrobacter amn	S	S	S	S	S	S	S	S	S	I
47	Citrobacter freu	I	I	R	R	R	S	R	R	S	R
48	Klebsiella pneumoniae	I	S	S	R	S	S	I	R	S	I
49	Klebsiella oxytoca	R	R	S	R	S	S	R	R	S	R
50	Klebsiella oxytoca	I	R	S	R	S	R	R	R	S	R

TABLE # 3

Comparisson of the results by each of the phenotypic methods used to detect ESBL.

Method	Disk diff. test (Resistant strains)								Double disk synergy test (positive results)								E-test (positive results)			
Antibiotics	Azt		Ctz		Ctx		Cpd		Azt-Aug		Ctz-Aug		Ctx-Aug		Cpd-Aug		Ctz/clav ac		Cftx/clav ac	
Enterobacteriaceae	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
E. cloacae (n=9)	4	44	4	44	6	67	4	44	0	0	0	0	0	0	0	0				
E. coli (n=19)	1	5	3	16	8	42	5	26	2	11	1	5	2	11	1	5	4	21	3	16
P. mirabillis (n=16)	0	0	0	0	2	13	0	0	0	0	0	0	0	0	0	0				
K. pneumoniae (n=1)	0	0	0	0	1	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. oxytoca (n=2)	2	100	0	0	2	100	0	0	1	50	0	0	1	50	2	100	0	0	2	100
C. amni (n=2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
C. freudii (n=1)	0	0	1	100	1	100	1	100	0	0	0	0	0	0	0	0				

Azt (aztreonam), Ctz (Ceftazidime), Ctx (Ceftriaxone), Cpd (cefpodoxime), Aug (Amox-clav), Cftx (Cefotaxime)
For the disk diffusion test only the Beta-lactams recommended by the NCCLS for the detection of ESBL were included in this table.
The E-test were only applied to the E. coli and Klebsiella strains as indicated in the manufacturer instructions.

TABLE # 4

Other antibiotics to which the ESBL producers were also resistant.

N.	Gramnegative	Gentamicin	Amoxi-clav	Pipera-tazo	Cefuroxime	Ciprofloxaxin	Piperacillin	Amoxicillin
24	Escherichia coli	R	R	I	R	R	R	R
25	Escherichia coli	R	S	I	R	R	R	R
26	Escherichia coli	I	S	S	R	S	R	R
28	Escherichia coli	R	R	R	R	S	R	R
49	Klebsiella oxytoca	R	S	R	R	S	R	R
50	Klebsiella oxytoca	I	R	R	R	S	R	R

Gramnegative	Gentamicin	Amoxi-clav	Pipera-tazo	Cefuroxime	Ciprofloxaxin	Piperacillin	Amoxicillin
Escherichia coli	75%	50%	25%	100%	50%	100%	100%
Klebsiella oxytoca	50%	50%	100%	100%	0%	100%	100%

Figure 1 (Standard disk diffusion susceptibility testing)

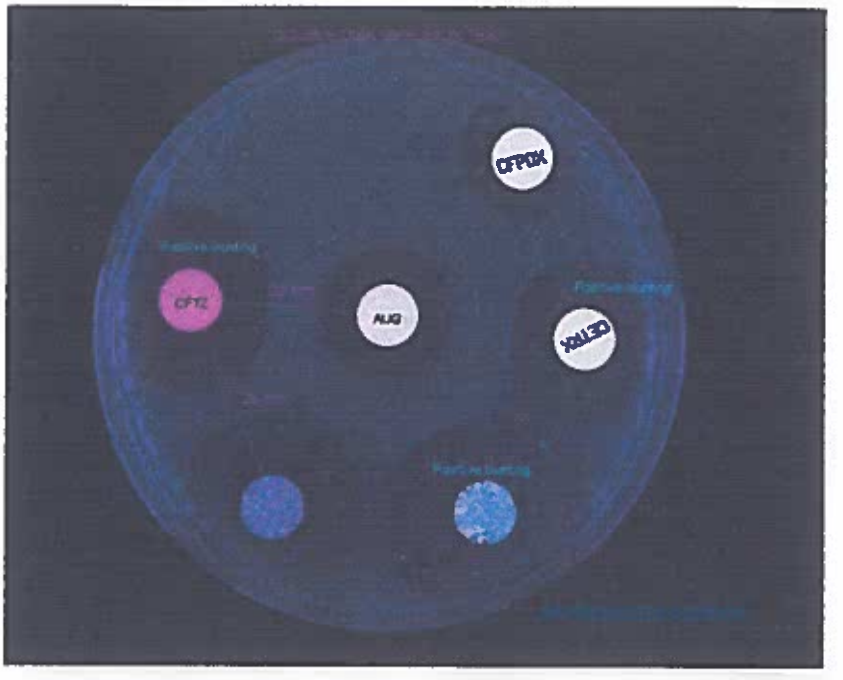


Figure 1 a

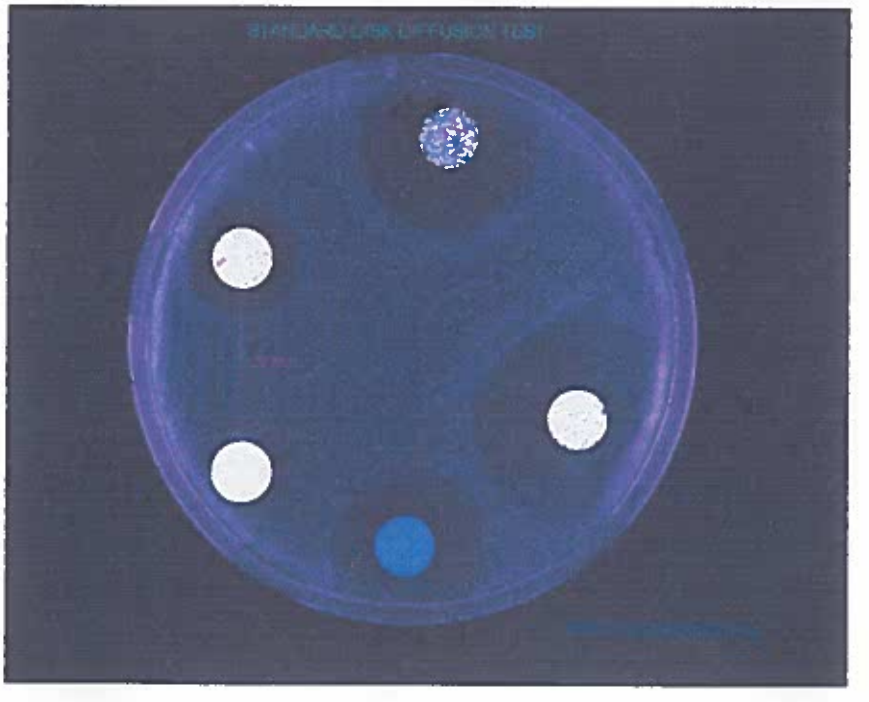
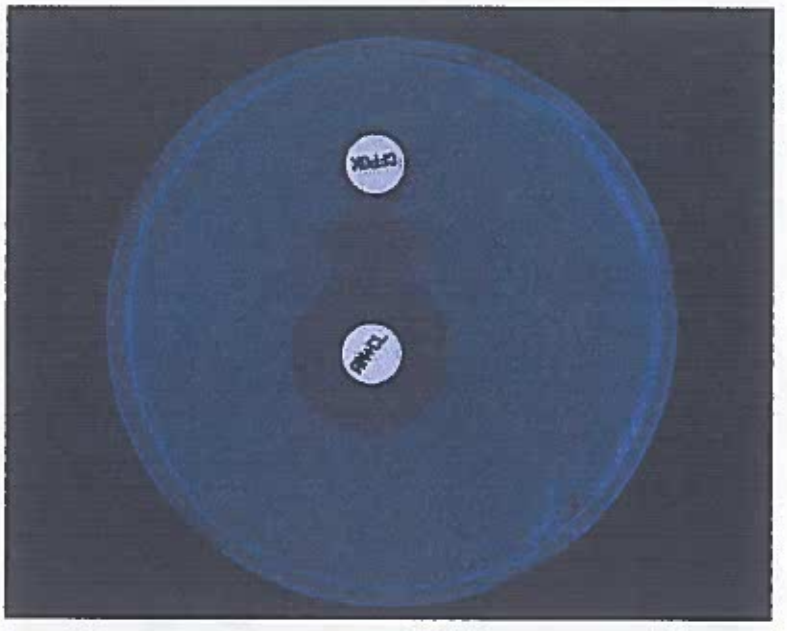


Figure 1 b

Figure 2 **(Double disk synergy test)**



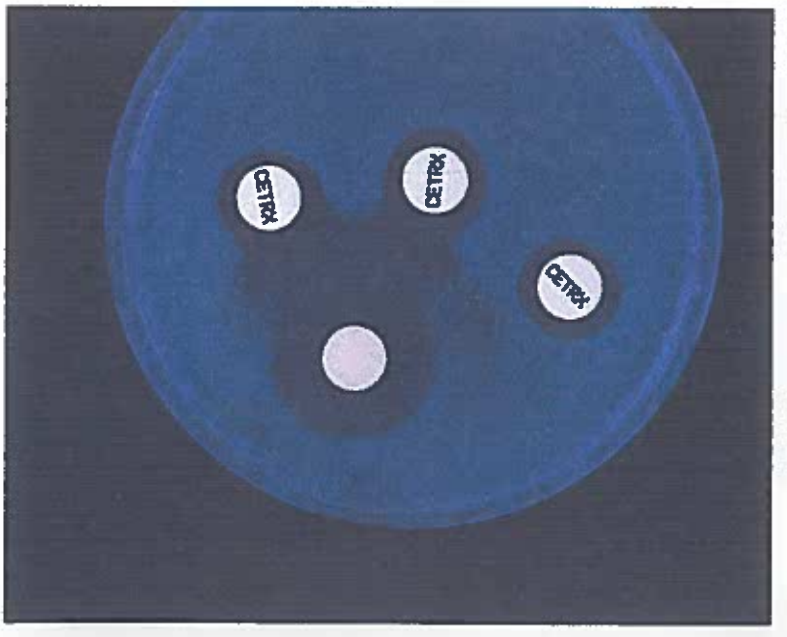


Figure 3 **(Positive results for ESBL E-test)**

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