CHAPTETR III

MATERIALS AND METHODS

1. Microorganisms

1.1 Clinical isolates

All 400 isolates was obtained from the 22,278 clinical specimens which were sent to the Division of Bacteriology, Department of Microbiology, Siriraj Hospital during the 6 - month period between August 2000 and January 2001. The clinical specimens included

a) Blood (9075 specimens)

b) Sputum from patients with respiratory tract infections(4757 specimens)

c) Urine from patients with urinary tract infections (8346 specimens)

1.2 Control strains

Positive ESBL producing *K. pneumoniae* included

K. pneumoniae ATCC 700603

K. pneumoniae ESBL low level control strain 1204*

K. pneumoniae ESBL high level control strain 1951*

Negative control included

K. pneumonaie ESBL non - producer control strain 911*

E. coli ATCC 35218

*Strain 1204 , 1951 and 911 were kindly provided by Mystic Laboratory , USA

2. Specimen

Feces were collected from 100 normal persons during the same period of time.

3. Isolation

Each of fecal sample was inoculated on blood agar and Mac Conkey agar , and incubated at 37 ° C for 24 hours. The colonies with typical characteristics of *K. pneumoniae* were selected for further identification. The characteristics included large, smooth, elevated, mucoid colony on blood agar while on MacConkey agar, it is large, smooth, lactose fermenter, elevated, mucoid colony.

4. Identification of *K. pneumoniae*

All the isolates obtained from the previous step were identified according to the methods described in Bergey's Manual of Systemic bacteriology. (12)

All 400 isolates from the clinical specimen were also included in this step for the confirmed identification.

The identification steps were as followed :

4.1 Gram staining

All the isolates were gram stained. *K. pneumoniae* is gram negative, straight rods,0.3 - 1.3 μm diameter and 0.6 - 6.0 μm in length, arranged singly, in pairs or short chains

4.2 Biochemical tests

The isolates were tested for the biochemical characteristics as shown:

Biochemical tests	Characteristics of *K. pneumoniae*
Indole	negative
Methyl red	negative
Voges- Proskauer test	positive
Citrate utilization	positive
Urease	positive
Motility test	negative
Lysine decarboxylase	positive
Argine decarboxylase	negative
Ornithine decarboxylase	negative
Lactose fermentation	positive
Sucrose fermentation	positive
Dextrose fermentation	positive
TSI	Acid butt/ Acid slant with gas

5. Detection of Extended spectrum β – lactamase (ESBL) producing *K. pneumoniae*

The ESBL producing *K. pneumoniae* were detected using the initial screen test and phenotypic confirmatory test as recommended in NCCLS 2000. (76) The methods were briefly described as followed:

5.1 Initial screen test (NCCLS, 2000)

1. Inoculum preparation

At least 3 to 5 well - isolated colonies from 18 - 24 hour agar plate of all *K. pneumoniae* isolates including the control strain *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, *K. pneumoniae* ESBL high level control strain 1951, *K. pneumoniae* ESBL low level control strain 1204, and *K. pneumoniae* ESBL non - producer control strain 911 were inoculated into the tubes containing normal saline. The suspension was adjusted to match the 0.5 McFarland turbidity standard.

2. Inoculation of test plates

(1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a steriled cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level . This step would remove the excess inoculum from the swab.

(2) The dried surface of a Mueller - Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, the plate was rotated approximately 60° each time to ensure an even distribution of inoculum. As the final step, the rim of the agar was swabbed.

(3) The plate lid was left a jar for 3 minutes to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

3. Application of disks to inoculated agar plate

The three antimicrobial disks (ceftazidime 30 μg, cefotaxime 30 μg, ceftriaxone 30 μg) was placed on each plate. Each disk must be pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 37° C for 24 hours, within 15 minutes after the disks were applied.

4. Reading plates and Interpreting results

After 24 hours of incubation, each plate was examined. The diameter of the zone of inhibition were measured. Zone were measured to the nearest whole millimeter, using a sliding caliper, which was held on the back of the inverti petri plate. The sizes of the zones of inhibition were interpreted by referring to the table of zone diameter standard of National Committee of Clinical Laboratory Standards (NCCLS, 2000) as shown in the Table 3.1.

Table 3.1 The inhibition zone of antimicrobial agents to interpreted suspicious ESBL producing organisms.

Antimicrobial agents	**Inhibition zone of suspicious ESBL producing organisms (mm.)**
Ceftazidime (30 μg)	≤ 22
Ceftriaxone (30 μg)	≤ 25
Cefotaxime (30 μg)	≤ 27

5.2 Confirmatory test of ESBL producing *K. pneumoniae*

The test has been performed in 2 ways. First, the combination disk method (76) were used and the other way; E - test (ESBL strip) as recommended by Cormican, 1996 and Zali, 2000, accordingly , was used.

5.2.1 Phenotypic confirmatory test (NCCLS 2000)

(The combination disk method)

1. The inoculum preparation and inoculation of test plates

The inoculum preparation and the inoculation of the test plates were done in the similar way as in the initial screen test method.

2. Application of disks to inoculated agar plate

The antimicrobial disks and combination disks (ceftazidime / ceftazidime + clavulanic acid, cefotaxime / cefotaxime + clavulanic acid) were placed on each plate. Each disk must be pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 37°C for 24 hours, within 15 minutes after the disks were applied.

3. Reading plates and Interpreting Results

After 24 hours of incubation, each plate was examined. The diameter of the zone of the complete inhibition were measured. Zone were measured to the nearest whole millimeter, using a sliding caliper, which was held on the back of the invert petri plate. The sizes of the zones of inhibition were interpreted by referring to the table of zone diameter standard of National Committee for Clinical Laboratory Standards (NCCLS, 2000). An organism was interpreted as the ESBL producer if there is an increase of $\geq$ 5 mm. in the inhibition zone of combination disk when compared to that of the cephalosporin disk.

5.2.2 E - test ESBL strip (Cormican, 1996 and Zali, 2000)

1. Inoculum preparation

At least 3 to 5 well – isolated colonies from 18 - 24 hour agar plate of all *K. pneumoniae* isolates including the control strain *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, *K. pneumoniae* ESBL high level control strain 1951,

K. pneumoniae ESBL low level control strain 1204, and *K. pneumoniae* ESBL non - producer control strain 911 were inoculated into the tubes containing saline suspension. The suspension was adjusted to match the 0.5 McFarland turbidity standard.

2. Inoculation of test plates

The steriled cotton swab was dipped in to the inoculum suspension. Excess fluid was removed by pressing the swab against the inside wall of the test tube. The entire Mueller - Hinton agar surface was swabbed three times and the plate was rotated approximately 60 degrees each time to ensure an even distribution of inoculum. Excess moisture was allowed to be absorbed for about 15 minutes so that surface was completely dry before applying E - test ESBL strips.

3. Application of E - test to inoculated agar plate

The E - test package was opened with a pair of forceps. The E - test strips contain either cefotaxime (CTX) and cefotaxime + clavulanic acid (CTXL) or cefazidime (CAZ) and ceftazidime + clavulanic acid (CAZL) as shown in the Fig 3.1. Both strips were placed on a dry clean Mueller - Hinton agar surface. The whole length of the strip had to be in the complete contact with the agar surface. If necessary, air pockets were removed by pressing gently on the strip with forceps, from the minimum concentration upwards. The plate were inverted and placed in an incubator at 37° for 24 hours.

4. Reading and Interpretation

When bacterial growth was visible, the MIC of CTX, CTXL, CAZ and CAZL could be obtained from the respective inhibition ellipsed intersect the strips. The presence of a phantom zone or ellipse deformation also indicated ESBL production due to synergy between CTX or CAZ and the clavulanic acid diffusing across from the CTXL or CAZL sections . The ratio of MIC of CTX / MIC of CTXL ≥ 8 or the ratio of MIC of CAZ/MIC of CAZL ≥ 8 confirmed extended spectrum β – lactamase producing isolates as shown in the Table 3.2

Table 3.2 Interpreted ESBL producing for E - test ESBL CTX /CTXL and CAZ / CAZL strips

ESBL	MIC(μg/ml) Ratio	MIC(μg/ml) Ratio
Positive	CTX ≥ 0.5 and CTX /CTXL ≥ 8	CAZ ≥ 1 and CAZ /CAZL ≥ 8
Negative	CTX < 0.5 or CTX /CTXL < 8	CAZ < 1 or CAZ /CAZL < 8
Non determinable	CTX >16 and CTXL > 1	CAZ > 32 and CAZL > 4

Note : CTX /CTL = Cefotaxime / Cefotaxime + clavulanic acid

CAZ / CZL = Ceftazidime / Ceftazidime + clavulanic acid

6. Antimicrobial susceptibility test

All the ESBL producing *K. pneumoniae* isolates were tested for antimicrobial susceptibility by 2 methods including disk diffusion method and MIC determination by E - test.

6.1 Disk diffusion methods

All the ESBL producing *K. pneumoniae* isolates were tested for antimicrobial susceptibility against 5 antimicrobial agents which have been commonly used in the treatment of infections due to *K. pneumoniae*. The antimicrobial diks were ciprofloxacin (30μg), gentamicin (10μg), amikacin (30μg), tobramycin (10μg), and trimethoprim - sulfamethoxazone (1.25 /23.75 μg). The test procedure was performed according to Kirby - Bauer Disk Diffusion method.(76,77)

After 24 hours of incubation, the diameter of the zone of complete inhibition were measured. The size of the zones of inhibition were interpreted by referring to table of zone diameter standard of National Committee of Clinical Laboratory Standards (NCCLS, 2000) as shown in the Table 3.3. The organisms were reported as either susceptible, intermediate susceptible, or resistant to the agents tested.

Table 3.3 Zone diameter interpretive standards antimicrobial agents of *Enterobacteriaceae* (NCCLS, 2000)

Zone diameter interpretive standards (mm.)			
Antimicrobial disk concentration	**Resistance**	**Intermediate**	**Susceptible**
Ciprofloxacin (30μg)	≤ 15	16-20	≥21
Gentamicin (10μg)	≤ 12	13-14	≥15
Amikacin (30μg)	≤ 14	15-16	≥17
Tobramycin (10μg)	≤ 12	13-14	≥15
Trimethoprim – sulfametthoxazone (1.25/23.75 μg)	≤ 10	11-15	≥16

μg = microgram

6.2 Minimal Inhibitory concentration (MIC) determination by E - test

E - test were performed in order to determine the MIC of the cepharosporins and imipenem against ESBL producing *K. pneumoniae* isolates. The drug tested included cefoxitin, cefuroxime, cefotaxime, ceftazidime, ceftriaxone, and imipenem. The test procedure was performed according to NCCLS, 2000.

1. Inoculum preparation and inoculation of test plates

The inoculum preparation and the inoculation of the test plates were done in the similar way as in the phenotypic confirmatory test (E - test ESBL strips).

2. Application of E - test to inoculated agar plate

The E - test package was opened with a pair of forceps, the strip was gripped at areas labeled CAZ, CTX, CRO, CXM, FOX, IPM and placed them on a dry clean surface . The strips were applied to the inoculated agar plate using forceps. The whole length of the strip had to be in the completely contact with the agar surface. If necessary, air pockets were removed by pressing gently on the strip with forceps. The plates were inverted and placed in an incubate at 37°C for 24 hours.

3. Reading and Interpretation

After 24 hours of incubation, read the MIC of CAZ, CTX, CRO, CXM, FOX, IPM at the end point of intersection between the inhibition ellipse edge and E - test strip. The MIC values were interpreted by referring to the table of MIC values standard of National Committee of Clinical Laboratory Standards (NCCLS, 2000) as shown in the Table 3.4. The organisms were reported as either susceptible, intermediate susceptible or resistant to the agents tested.

Table 3.4 **Minimal Inhibitory Concentration interpretive of antimicrobial agents of *Enterobacteriaceae* (NCCLS, 2000)**

Antimcrobial agents	Minimal inhibitory concentation interpretive		
	Resistance	Intermediate	Susceptible
Ceftazidime	≥ 32	16	≤ 8
Cefotaxime	≥ 64	16-32	≤ 8
Ceftriaxone	≥ 64	16-32	≤ 8
Cefuroxime	≥ 32	16	≤ 8
Cefoxitin	≥ 32	16	≤ 8
Imipenem	≥ 16	8	≤ 4

7. Analysis of restricted fragments of chromosomal DNA by Pulsed - Field Gel Electrophoresis (PFGE)

DNA extraction and PFGE were performed according to the method described by Wongwanich *et. al.*(2000).(78) Briefly as followed :

7.1 Culture plug preparation

A single colony of ESBL producing *K. pneumoniae* from 24 hours on MacConkey agar plate was inoculated into 5 ml nutrient broth. The culture media were incubated under aerobic condition for 24 hours at 37°C. Organisms were

harvested by centrifugation at 3,500 rpm at 4 °C for 10 min. The cells were then washed with 5 ml TES buffer and then centrifuged. The supernatant was then discarded. The washing step was repeated 3 times. Cells were resuspended in the TES buffer and adjusted the turbidity to McFarland No 4. This suspension was then mixed with an equal volume of 1.6 % low melting - point agarose and poured into each well of the disposable plug molds (BioRad, USA) . It was allowed to solidify in the refrigerator for 30 min.

7.2 DNA extraction

Plugs were pulled out and incubated in 3 ml fresh lysis solution I (10mM Tris HCl pH 7.6, 10mM EDTA, 1% SDS, 10mg/ml of lysozyme and 25mg/ml of Rnase) overnight at 37°C on gentle shaking water bath. The plug was then transferred into the new tube contained 3 ml of fresh lysis solution II (1 % Sarkosyl , 0.5M EDTA and 20 mg/ml of Protease) on the new tube, incubated on gentle shaking water bath 37°C overnight. The protease was inactivated by the treatment with 1.5 ml of phenylmethylsulfonyl fluoride two times, 1 hour each, at room temperature. The plugs were then washed with 3 ml. 0.5x TE buffer, at room temperature, 3 times and were kept in 0.5x TE buffer at 4°C.

7.3 Restriction enzyme digestion

One plug of each strain was placed on the plate and covered with 150 µl of 1x TE buffer. After that the plug was cut into half with sterile surgical blade and

were added into the microcentrifuged tube containing 146 µl of the restriction enzyme *Spe* I solution (water,10x restriction enzyme *Spe* I buffer , and 100 units of *Spe* I) The tube was then incubated overnight at 37 °C and then stored at 4 °C.

7.4 Gel preparation and gel running

The running agarose gel was prepared by dissolving 1 g of Ultrapure high – melting temperature agarose (1 % wt/vol, BioRad) in 100 ml of 0.5x TBE buffer, melted by microwave and then cool at 56°C. The plug was poured out into the sterile plate and was washed with 0.5x TE buffer. One half of the plug was placed in the microcentrifuged tube containing 1 ml of 0.5x TE buffer. The another half was placed onto the horizontal side of the comb. Buffer around the plug was absorbed with sterile tissue. The comb was then adjusted to the vertical line on the gel block . One percent running agarose gel was poured into the block and let gel to solidify for 30 min at room temperature. After the gel has hardened, the comb was removed. The gel was placed in the PFGE box containing 0.5x TBE buffer enough to cover the gel to a depth of about 1 mm or just until the tops of the wells are submered. CHEF DNA size standards Lambda ladder (BioRad ,USA) was used as the molecular standard markers. PFGE was performed at 200 v constant voltage by using a contour-clamped homogenous electric field apparatus (CHEF - DR II system) with an initial switch time of 10 sec and a final switch time of 20 sec for 22 hours.

7.5 Gel visualization

The gel was stained with 0.5 μg /ml of ethidium bromide for 30 min. After that it was rinsed and destained with deionized water for 30 min. The gel was then photograpghed under UV illumination. Result was interpreted by according to Tenover *et al.*(1995)(79)

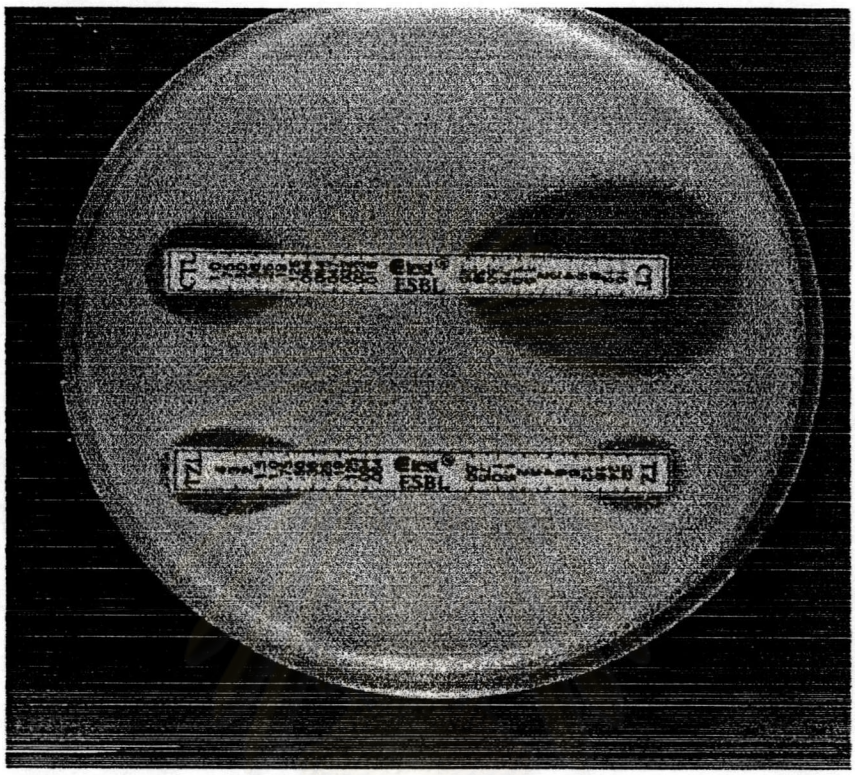

Figure 3.1 E - test ESBL strips (CTX/CTXL , CAZ/ CAZL)