Prevalence of Extended Spectrum β-Lactamases and Molecular Screening of *klebsiella pneumoniae* in the West Bank, Palestine

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Abstract

Klebsiella are Gram negative, non-motile, rod-shaped, lactose-fermenting, facultative anaerobic bacteria and encapsulated with a polysaccharide capsule. It causes opportunistic and nosocomial infections. Klebsiella produce plasmid-mediated extended-spectrum beta lactamases (ESBLs) which gives the bacteria the ability to resist beta-lactam antibiotics.

The prevalence of ESBLs among clinical isolates of Klebsiella pneumonia was determined. In addition, the specific molecular characterization of CTX-M genes were also determined.

Sixty seven urine, pus, and wound swabs were collected from patients of gynecology, surgery, medicine, Intensive Care Unit (ICU), and orthopedics from different parts of the West Bank of Palestine, mainly Ramallah, Hebron, and Jerusalem. Antibiotic sensitivity were done by combination disk method and double-disk synergy test. ESBL genes were detected by Polymerase Chain Reaction (PCR) and multiple PCR.

Results showed that 48 samples were positive for CTX-M universal gene. Sixty four samples were positive for SHV gene, and 51 samples were positive for TEM gene. Forty two samples were positive for CTX-M1 gene, 38 samples were positive for CTX-M9 gene. Seven samples from 42 samples were positive for CTX-M1 only, 3 samples from 38 samples were positive for CTX-M9 only, and 35 samples were positive for both genes (CTX-M1 and CTX-M9).

Our results were close to several studies around the world mainly in Egypt and Israel. In conclusion, bacteria are becoming more complex and highly spread in hospitals and the community in Palestine.

I. INTRODUCTION

Klebsiellae is a member of the Enterobacteriaceae family. It causes opportunistic and nosocomial infections. They are Gram negative bacteria, non-motile, rod-shaped, encapsulated, lactose fermenting, facultative anaerobic, with major polysaccharide capsule which covers the entire cell surface providing protection against most host defense mechanisms. Klebsiella pneumonniae displays two types of antigens on the surface of the cell, lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen) [1], both contributing to pathogenicity. Klebsiella produce plasmid-mediated extended-spectrum β-lactamases (ESBLs) which give bacteria the ability to resist β-lactam antibiotics. Acquired resistance to β-lactams is mainly mediated by extended spectrum β-lactamases (ESBLs) that confer bacterial resistance to all β-lactams except carbapenems and cephamycins, which are inhibited by other β-lactamase inhibitors such as clavulanic acid. Klebsiellae genus contains many species, the most common isolated species of this genus is Klebsiella pneumoniae. This species is encapsulated with polysaccharides capsule which protect the bacteria from the immune system and antimicrobial agents [2].

Klebsiella pneumoniae causes destructive changes to human lungs leading to inflammation and hemorrhage with cell death, that sometimes produce itching, bloody and mucoid sputum. Klebsiella pneumoniae is a major cause of hospital acquired infections, (nosocomial infections) (50% mortality in ICU patients). Klebsiella infections occur in immune-compromised patients, such as diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary disease (COPD), glucocoticoid therapy, renal failure,..etc. [2].
Klebsiella pneumoniae is the second cause of urinary tract infection after Escherichia coli. Symptoms caused by this organism include high fever, chills, flu-like symptoms and productive cough in lung infections. The mortality rate is high, and patients should seek medical care as soon as the symptoms appear [3].

Klebsiella spp. are ubiquitous in nature. In humans they may colonize the skin, pharynx, or gastrointestinal tract. Klebsiella may be considered as normal flora in colon, and intestinal tract and the biliary tract. Extensive use of broad-spectrum antibiotics in hospitals led to the appearance of multidrug-resistant strains that produce extended spectrum beta-lactamases (ESBLs). These organisms are highly virulent. Most outbreaks are due to single

clone or single gene. Humans are the primary reservoir for K. pneumoniae. Carriage rates of Klebsiella pneumoniae in the community range from 5% to 38% in stool, and 1% - 6% in nasopharynx. Klebsiella spp are rarely isolated from the skin. It was reported that the colonization rate in hospitalized patients is 77% in stool, and 19% in pharynx [4].

ESBLs are enzymes produced by some bacteria and are responsible for their resistance to β-lactam antibiotics like penicillin, cephamycins, and cephalosporines. The lactamase enzyme breaks the β-lactam ring open, inactivating the molecule's antibacterial activity.

II. MATERIALS AND METHODS

The following materials are needed to conduct the experiment related to this project:

a. Mackonky agar.
b. Trypticase Soya Agar TSA.
c. Analytical protocol index 20 E (API 20E) Or Enterotest tube (Hy LAB).
d. Muller Hinton agar.
e. Antibiotics such as augmentin, cephotaxime, ceforoxime, ceftriaxone, ceftazidime, meropenem, imipenem, amikacin, aztreonam.
f. Primers.
g. Master mix .
h. Agarose.

Screening of ESBL-Producing Strains Klebsiella pneumoniae:

Clinical and Laboratory Standard Institute (CLSI) has developed screening tests for identifying the ESBLs–producing *Klebsiella* species. Strains showing zone of inhibition of ≤ 22 mm for ceftazidime, ≤ 25 mm for ceftriaxone, and ≤ 27 mm for cefotaxime were ESBLs confirmatory tests. Double disk Synergy Test (DDST) was done by placing one disk containing clavulanic acid (20/10µg) in the center of the plate. A disk of (30µg) cefotaxime, and ceftazidime (30 µg) were placed on either side of augmentin disk with center to center distance of 20 mm to centrally placed disc. The plate is incubated at 37˚C overnight. ESBLs production was interpreted as the 3rd –generation cephalosporins disc inhibition was increased towards the Augmentin disk, or bacterial growth was inhibited where the two antibiotics were diffused together.

Phynotypic Confirmatory Disc Diffusion Test (PCDDT):

Third generation cephalosporins, ceftazidime (30 µg) disc and ceftazidime + clavulinic acid (30µg + 10 µg) disc were placed 25mm apart. An increase of ≥ 5 mm in zone of inhibition for ceftazidime + clavulinc acid compared to ceftazidime was confirmed as ESBLs producers [6].

Research Procedures :

Samples, were cultured on a special media called MacConkey Agar. *Klebsiella pneumoniae* was confirmed using biochemical tests such as Indole , Methyl Red, Voges Proskauer, Citrate , Triple Sugar Iron, and Urease . DDST was used to identify ESBL – producing *Klebsiella pneumoniae* . DNA extraction was performed then amplified by PCR, and then gel electrophoresis was used in order to identify the genes of ESBLs

Microbial methods:

Antimicrobial susceptibility test done on the 67 sample using 0.5 McFarland and cultured on Muller Hinton agar. Each sample was tested for susceptibility for Aztreonam (ATM) , Cefpodoxime (CPD), Cefotaxime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), Amikacine (AK), Imepenime (IPM), Meropenim (MEM), and CAZ/ Clavulanate combination, and CTX/Clavulanate combination. The susceptibility was assessed using break points shown in Table (1).

Combination disk method:

This test compares the zone of inhibition of CAZ antibiotic disc alone and zone of inhibition of CAZ with clavulanate disc, and CTX antibiotic alone and zone of inhibition of CTX with clavulanate disc. Bacteria was considered ESBL positive if zone of combination ≥ 5mm than zone of the inhibition of cephalosporin antibiotics alone [5].

Double –disk synergy test:

This test performed by placing disks of third generation cephalosporin (CRO, CTX) at distances of 30 mm (center to center) from a disk augmentine. Test was considered positive if synergy shape was seen [5].

Molecular Method:

Most common method to detect the presence of β-lactamase is Polymerase Chain Reaction (PCR) with oligonucleotide primers that are specific for a β-lactamase gene. In this study we used PCR for ESBL detection and confirmation.

PCR Amplification:

DNA extraction was done sub-culturing isolated colonies on TSA overnight. 10-15 colonies of each sample were suspended in 200 µl of 1× Tris-EDTA buffer in a 2 ml Eppendorf tube. The mixture was placed at 95ºC for 10 minutes. Remove mixture and place in freezer at -20ºC for 5 minutes. Thawing and freezing were repeated 3 times. The tubes then centrifuged at 13,000 rpm, and the supernatant containing DNA was separated and stored in the freezer.

Single PCR Amplification for SHV, TEM and CTX-M:-

A single PCR amplification from genomic DNA was performed on each isolate for the presence of genes encoding SHV, TEM, CTX-M β-lactamases. The primers used in amplifications are listed in Table (2)

SHV and TEM amplification: The amplification was then performed in PCR thermocycler (c1000, Thermocycler, Biorad) using Eppendorf tubes. Five µl of sample DNA used as template in 25µl reaction volume. The complex mix for TEM and SHV amplification consisted of the following components: 12.5 µl Go Tag Green Master Mix , 2X (Promega), 1µl forward primer (0.1µM), 1 µl reverse primer (0.1µl), and 5.5 µl nuclease free water.

The amplification conditions were : an initial denaturation step at 95 ºC for 6 minutes , 35 cycle at 94 ºC for 30 seconds, annealing for 30 seconds at primer specific temperature (Table 2) and extension at 72 ºC for 2 minutes . This was followed by a final extention step at 72 ºC for 10 minutes.

CTX-M Amplification : Mixture in the CTX-M Amplification consist of : 12.5 µl Go Tag Green Master Mix , 2X (Promega), 0.5 µl forward primer (0.1µM), 0.5 µl reverse primer (0.1µl), and 6.5 µl nuclease free water.

The PCR conditions were : an initial denaturation step at 95 ºC , for 6 minutes , 30 cycle of 94 ºC for 30 seconds, annealing for 30 seconds at 58 ºC , and extension at 72 ºC for 50 seconds. This was followed by a final extension step at 72 ºC for 6 minutes.

Detection of CTX-M subgroups by Multiplex PCR:-

Multiplex PCR amplification from genomic DNA was performed on 47 samples of CTX-M producing *Klebsiella Pneumoniae* for the presence of genes encoding CTX-M groups. The primers used in the amplifications are listed in Table 3.

The Amplification mixture was composed of the following :12.5 µl Go Tag Green Master Mix , 2X (Promega), 0.5 µl of each CTX-M group forward primers (0.2µM), 0.5 µl of each CTX-M reverse primers (0.2µM), 5µl DNA template, and 3.5 µl nuclease free water in a final volume of 25 µl.

The PCR conditions were : an initial denaturation step at 95 ºC , for 6 minutes , 30 cycle of 94 ºC for 30 seconds, annealing for 30 seconds at 57 ºC , and extension at 72 ºC for 50 seconds. This was followed by a final extension step at 72 ºC for 6 minutes.

A negative (distilled water instead of template) and positive controls were used in each PCR run. PCR products (5µl) were run in an electrophoresis containing 1X TAE buffer using a 1% agarose gel stained with ethidium bromide . Gels were visualized on a UV transilluminator and photographed using GEL-DOC system (Biorad, USA).

III. RESULTS

A. Antimicrobial Susceptibility

Forty nine samples of *Klebsiella pneumoniae* were resistant to Ceftazidime (CAZ) and cefotaxime (CTX), but when combined with clavulanic acid, the zone of combination increased (≥5mm), Also synergic shape was shown when placing disks of third generation cephalosporin (CRO, CTX) , at distance of 30 mm (center to center) from a disk containing Amoxicilline – clavulanate, and cefotaxime –clavulanate. These two characteristic were used to identify ESBL. Also These 49 sample were resistant to third generation cephalosporin (CRO, CTX, CPD, ATM).

Carbapenems, Meropenem (MEM), Imipenem (IPM), and Amikacin are all effective for treatment of ESBLs in *Klebsiella pneumoniae. (*see Appendix A and Appendix B).

B. PCR Result

48 samples from 67 were positive for CTX-M universal gene (Figure 1). Another 64 samples from 67 were positive for SHV gene (Figure 2), and 51 samples were positive for TEM gene (Figure 3). 42 samples from 67 were positive for CTX-M-1 gene by multiplex PCR (Figure 4), 38 samples were positive for CTX-M- 9 gene group, 7 samples from 42 were positive for CTX-M-1 only, 3 samples from 38 were positive for CTX-M-9 only, and 35 samples were positive for both genes (CTX-M-1, CTX-M-9).

IV. DISCUSION

Today antibiotics have been used extensively and newer antibiotics are continuously being added for treatment of various infections. An extensive use of ß-lactam antibiotics in hospital and community has created major problems leading to increased morbidity, mortality and health care costs. Proper use of antibiotics is very important for various reasons. Development of bacterial resistance against newer antibiotics makes the main focus of research.

In this study, a total of 67 *Klebsiella pneumoniae* strains were isolated from various clinical specimens, in which the majority of the organisms were isolated from urine, wound swab and sputum cultures. Aztreonam, amoxyclave, third generation cephalosporins, ceftriaxone and cefotaxime were found 85-90% resistant, this is in agreement with other studies [8]. Aminoglycosides (Amikacin) have a good activity against ESBLs in *Klebsiella pneumoniae*, 91.5% isolates were susceptiple to Amikacin .

Carbapenems are the drug of choice for many infections caused by ESBLs in *Klebsiella pneumonia.* Our results showed that imipenem was 98% sensitive, and meropenem was 92.5% sensitive. These findings were similar to study [9]. Amikacine

was the second choice after imipenem and meropenem. So these drug resistant-organisms have limited theraputic options and necessitated the increased use of carbapenems, which cause new beta- lactamases to be developed by *K . pneumoniae* carbapenamase (KPC) which is resistant to carbapenems and has been spread worldwide [10]. Very limited options to treat carbapenems resistant strains and colistin may be the drug of choice [11].

In the present study ESBLs in *Klebsiella pneumoniae* prevalence was 74.6%, which was very close r to study done by Yasmin 2012 [12].

The high occurrence of ESBLs in *klebseilla pneumoniae spp is* of great concern since infections caused by this bacterium were very common. Resistance of the organism may be due to the presence of capsule that gives some level of protection to the cells. The presence of multidrug resistance efflux pumps , spreading easily, pathogenicity and efficiency at acquiring and disseminating resistance plasmid make the organism highly infectious [13]

.

Two combinations with clavulanic acid (CAZ/CAZC) and (CTX/CTXC) were used, and found that *Klebsiella pneumoniae* showed maximum ESBL production in CAZ/CAZC combination which correlate with other studies [14]. Ceftazidime plus clavulanic acid (CAZ/CAZC) was the best single disk diffusion test recommended [15].

In this study, TEM, SHV and CTX-M genes were found in 76.1%, 95.5% and 71.6% from phenotypically confirmed ESBLs producers respectively. CTX-M presence may be increased due to wide use of third generation cephalosporins, especially ceftriaxone and it is more resistant to cefotaxime

In this study among the 48 CTX-M genes present 47(98%) were cefotaxime and ceftriaxone resistant. TEM and CTX-M combine 37/48, and TEM, SHV and CTX-M were 35/48. SHV was detected as a single gene in 4 samples. CTX-M-1 and CTX-M-9 were 91.6% (44/48) and 83.3% (40/48) respectively .

V. CONCLUSION AND RECOMMENDATIONS

It can be concluded that Extended Spectrum B-lactamases are gradually increasing in Palestine with co - resistance to some other classes of antibiotics which is very alarming. There was limited number of drug sensitivity for this bacterium. The drug of choice is imipenem and meropenem, followed by Amikacin in injectable form. But most probably if irrational use is not stopped, infection with ESBLs will increase, resulting in high morbidity and mortality. This study shows that *K. Pneumoniae* is essential for the prompt recognition of antimicrobial resistant organism, as it is more resistant than *E. coli* .
Infection control practitioners and clinicians need the clinical laboratory to rapidly identify and characterize different types of resistant bacteria specially ESBLs efficiently to minimize the spread of these bacteria and help select more appropriate antibiotics. It is very dangerous for laboratory practitioner that some amounts of ESBLs are present in third generation cephalosporins sensitive bacteria. So ESBLs must be detected by double disc diffusion test.

The epidemiology ESBL- producing bacteria are becoming more complex,and highly spread in hospitals and the community.

Further studies are required to investigate Multi Drug Resistant (MDR) bacteria and ESBL from other parts of Palestine using more isolates studies of molecular epidemiology of these resistant genes and can also be used for comparison with genes already isolated from other parts of the world.

REFERENCES

[1] Lal P, Kapil A, Das BK, Sood S (2007) Occurrence of TEM & SHV gene in extended spectrum β-lactamases (ESBLs) producing Klebsiella spp. isolated from a tertiary care hospital. Indian Journal of Medical Research, 125 2: 173–178.

[2] Paterson DL, Bonomo RA (2005) Extended spectrum β-lactamases : a clinical update. Clinical Microbiology Reviews 18 4: 657- 686.

[3] Pitout JD, Laupland KB (2008) Extended spectrum β-lactamase– producing Enterobacteriaceae: an emerging public health concern, The Lancet Infectious Diseases 8 3 159–166.

[4] Gupta V (2007) An update on newer β-lactamases. Indian Journal of Medical Research 126 5: 417–427.

[5] Clinical Laboratory Standards Institute CLSI (2006) Performance standards for antimicrobial susceptibility testing in Proceedings of the 16th International Supplement (M100-S16). National Committee for Wayne, Pa, USA.

[6] Shukla I, Tiwari R, Agrawal M (2004) Prevalence of extended spectrum β-lactamase producing *Klebsiella pneumoniae* in a tertiary care hospital. Indian Journal of Medical Microbiology 22 2: 87–91.

[7] Emery CL, Weymouth LA (1997) Detection and clinical significance of extended-spectrum β-lactamases in a tertiary care medical center. Journal of Clinical Microbiology, 35 8: 2061–2067.

[8] Clinical Laboratory Standards Institute (2010) M100-520 VOL-30 Performance Standards For Antimicrobial Susceptibility Testing , Twentieth Informational Supplement (M100-520 VOL30) .

[9] Sasirekha B, Manasa R, Ramya P, Sneha R (2010) Antimicrobial sensitivity Pattern β-lactamase in E.coll and *Klebseilla pneumoniae* isolated in Tertiary Care Hospital. Journal Medical Science 3 4:265-271.

[10] Haque R, Sal M, MA (2010) Detection of ESBL producing nosocomial gram negative bacteria from a tertiary hospital in Bangladesh. PK J MD SCI 26 0 4:887-891.

[11] Rhe YJ, Park, YK, Lee MY, Peck KR, KK (2010) KPC-Producing Extreeme Drug – Resistant *Klebseilla Pneumoniae* Isolated From Patients with Diabetes Mellitus and Chronic Renal Failure On Haemodialysis in south Korea. Antimicrobial Agents and Chemotherapy 54: 2278-2279.

[12] Taslima, Y (2012) Prevalence of ESBL among E. coli and Klebsiella spp. In a tertiary care hospital and molecular detection of important ESBL producing genes by Multiple PCR. Department of Microbiology and Immunology Mymensingh Medical College. Mymensingh.

[13] Amaya E, Caceres M, Fang H, Ramirez AT, Palmagren AC, Nord CE, Weintraub A (2009) Extended spectrum beta- lactamase producing *Klebseilla Pneumoniae* in Neonatal intensive care unit in leon, Nicaragwa. International Journal of Antimicrobial Agents 33:386-387.

[14] Gruteke P, Goessens W, Gils JV, Peerbooms P, Toom NL, Bekum AV, Verbrug H (2003) Microbiology, 41 3:1161-1166.

[15] Rahman MM, Haque JA, Hossain MA, Sultana R, Islam F, Islam AHMS (2004) Prevelance of extended spectrum beta lactamase–producing *E.coli* and *Klebseilla pneumoniae* in an urban hospital in Dhaka Bangladesh. International journal of antimicrobial agents 24 5: 508-510.

Table 1 : The Breakpoint For Antibiotics Used In The Study [7].

Antibiotic	*R*	*I*	*S*
AMC (20/10)	*≤13mm*	*14-17mm*	*≥18mm*
CTX (30)	*≤22mm*	*23-25mm*	*≥26mm*
CRO (30)	*≤19mm*	*20-22mm*	*≥23mm*
CPD(30)	*≤17mm*	*18-20mm*	*≥21mm*
ATM(30)	*≤17mm*	*18-20mm*	*≥21mm*
AK(30)	*≤14mm*	*15-16mm*	*≥17mm*
IPM(10)	*≤13mm*	*14-15mm*	*≥16mm*
MEM(10)	*≤13mm*	*14-15mm*	*≥16mm*
CAZ(30)	*≤17mm*	*18-20mm*	*≥21mm*

R:Resistant , S:Sensetive, I:intermediate

Table 2 : The Primers Used In The Amplification Of The Tem,Shv, And Ctx- M Genes

Name	*Sequnce*	*Annealing temperature*	*Product Size(kb)*
TEM-F *TEM-R*	*5`-CGCCGCATACACTATTCTCAGAATGA-3`* *5`-ACGCTCACCGGCTCCAGATTTAT-3`*	*54.5˚C*	*444*
SHV-F *SHV-F*	*5`-ATGCGTTATATTCGCCTGTG-3`* *5`-TGCTTTGTTATTCGGGCCAA-3`*	*50˚C*	*747*
CTX-M UNV-F *CTX-M-UNV-R*	*5`-ATGTGCAGYACCAGTAARGTKATGGC-3`* *5`-TGGGTRAARTARGTSACCAGAAYCAGCGG-3`*	*58˚C*	*593*

`K:G or T, R :A or G,S :G or C, and Y is C or T

Table 3 : The Primers Used In The Amplification Of Ctx-M Groups.

Name	*Sequnce*	*Annealing temperature*	*Product size(kb)*
CTX-M-1 F *CTX-M-1 R*	*AAAAATCACTGCGCCAGTTC* *AGCTTATTCATCGCCACGTT*	*57*	*415*
CTX-M-2 F *CTX-M-2 R*	*CGACGCTACCCCTGCTATT* *CCAGCGTCAGATTTTTCAGG*	*57*	*552*
CTX-M-9 F *CTX-M-9 R*	*CAAAGAGAGTGCAACGGATG* *ATTGGAAAGCGTTCATCACC*	*57*	*205*
CTX-M-8/25 F *CTX-M-8/25 R*	*CTTTGCCATGTGCAGCACC* *GCTCAGTACGATCGAGCC*	*57*	*305*

F : forward, R: Reverse

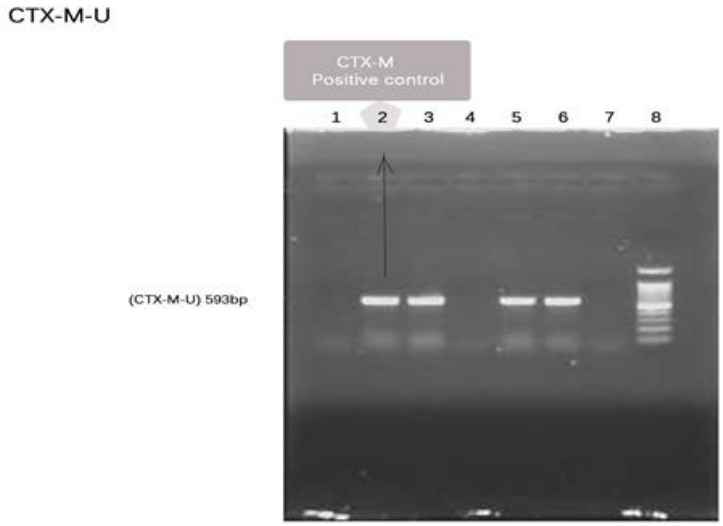


Fig. 1: CTX-M Universal Gene

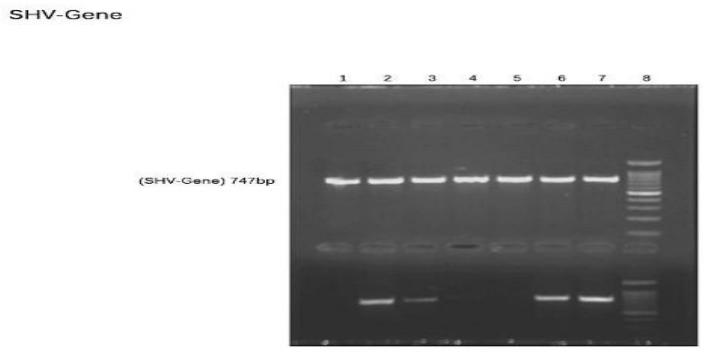


Fig. 2: Positive Samples For SHV Gene.

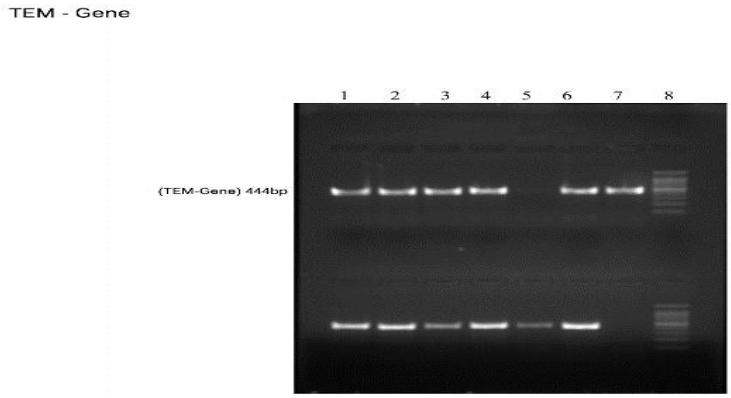


Fig. 3: Positive samples for TEM gene.

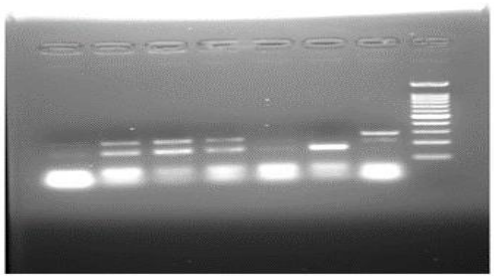
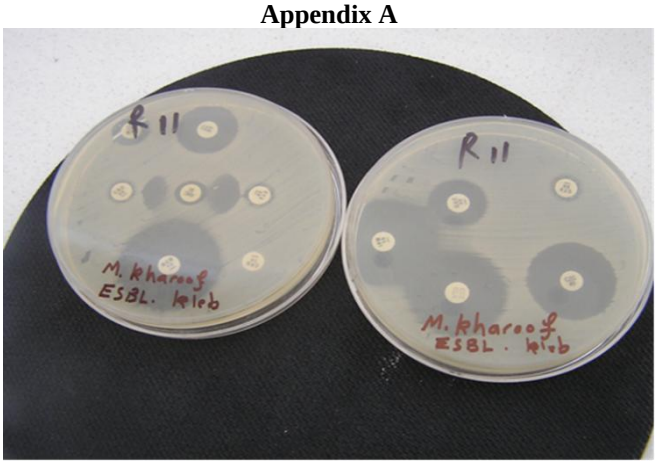
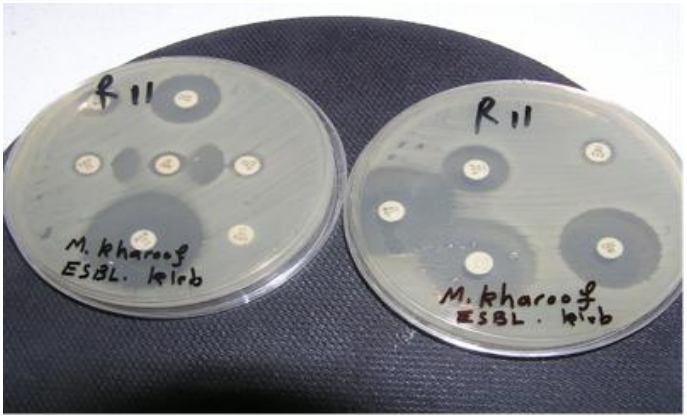


Fig. 4: Positive samples of Multiplex PCR for CTX-M-1 gene.

Appendix A





NO SAMPLE	AMC	CTX	CRO	CPD	ATM	AK	IPM	MEM	CAC	CTC	CAZ
M1	15I	1R	R	R	12R	2R	25S	3R	26	26	16R
M2	15I	2R	2R	1R	16R	21S	27S	3R	25	27	17R
M3	14I	1R	1R	1R	15R	21S	27S	27S	21	25	16R
M4	11R	R	R	1R	1R	18S	25S	27S	21	24	13R
M5	13R	14R		2R		2R	27S	30S	26	28	26S
M6	9R	9R	9R		15R	17S	26S	25S	21	25	16R
M7	9R	R	9R	R	16R	18S	25S	27S	25	25	18I
M8	22S	35S	26S		32S	22S	34S	34S	34	36	32S
M9	12R	26S	25S	21S	22S	17S	20S	26S	23	27	22S
M10	R	R	R	R	R	24S	11R	9R	9	R	8R
M11	R	R	R	R	R	R	9RR	8R	7	R	R
M12	R	R	R	R	R	10R	10S	10R	7	R	R
M13	15I	R	R	R	15R	21S	27S	30S	24	26	16R
M14	11R	32S	31S	35S	38S	21S	32S	31S	31	31	29S
M15	15I	28S	24S	34S	22S	21S	26S	30S	27	31	28S
M16	20S	32S	32S	27S	32S	21S		30S	28	28	27S
M17	R	18R	19R	R	26S	24S	26S	35S	30	26	25S
M18	18	R	R	R	9R	22S		31S	24	28	12R
M20	12R	8R	R	R	17R	21S	27S	28S	24	28	18I
R1	17I	30S	30S	28S	32S	21S	28S	30S	28	27	28S
R2	21S	32S	32S	28S	32S	22S	30S	28S	30	32	27S
R3	20S	32S	32S	28S	30S	21S	31S	32S	29	35	29S
R4	18S	32S	30S	30S	32S	21S	35S	32S	30	33	30S
R5	9R	9R	9R	R		18S	28S	31S	24	28	16R
R6	8R	10R	R	R	12R	18S		28S	22	25	15R
R7	8R	9R	9R	R	12R	18S	26S	30S	23	26	17R
R8	8R	8R	8R	R	12R	18S	28S	28S	25	27	27S
R9	7R	8R	8R	R	12R	18S	30S	29S	25	26	16R
R10	8R	8R	8R	R	12R	20S	32S	32S	27	30	17R
R11	10R	9R	9R	R	12R	20S	30S	30S	25	27	17R
R12	10R	9R	9R	R	12R	19S	26S	32S	24	27	15R
R13	20S	30	28	29S	30S	21S	30S	32S	30	30	28S
R14	8R	8R	8R	R	11R	19S	28S	28S	26	27	17R
R15	10R	10R	9R	R	12R	20S	30S	30S	26	28	16R
R16	20S	30	28	27S	29S	21S	30S	34S	28	30	28S
R17	9R	9R	9R	R	12R	21S	30S	32S	27	29	17R
R18	9R	9R	9R	R	15R	19S	30S	30S	25	28	20I
R19	10R	R	R	R	12R	20S	31S	31S	28	30	17R
R20	11R	9R	9R	R	12R	20S	32S	31S	25	29	17R
R21	9R	9R	9R	R	12R	20S	31S	32S	26	29	17R
R22	11R	11R	11R	R	16R	22S	32S	32S	27	29	18I
R22/2	9R	11R	10R	R	16R	21S	31S	31S	26	27	18I
R23	9R	9R	9R	R	12R	21S	31S	33S	27	28	17R
R24	11R	11R	11R	R	15R	21S	30S	30S	25	29	19I
R25	10R	10R	10R	R	15R	20S	31S	31S	25	29	18I
R26	10R	10R	10R	R	16R	20S	26S	31S	27	28	17R
R27	10R	10R	10R	R	12R	17S	27S	28S	24	27	14R
R28	8R	8R	8R	R	12R	R	32S	30S	23	26	17R
R62	10R	9R	R	R	11R	17S	27S	28S	25	27	13R
R64	10R	10R	10R	R	14R	14R	25S	30S	27	27	17R
R66	11R	16R	16R	R	9R	16I	30S	30S	32	29	12R
R67	15I	16R	17R	9R	22S	21S		30S	27	27	26S
R81	8R	8R	8R	R	15R	19S	28S	29S	25	28	17R
A	10R	10R	10R	R	17R	20S	30S	29S	26	27	26S
B	18S	31S	30S	26	31S	21S	28S	29S	28	32	28S

C	18S	30S	30S	27S	31S	21S	31S	30S	28	31	28S
D	13R	9R	9R	R	12R	21S	31S	30S	28	32	17R
E	14I	12R	11R	R	13R	21S	30S	33S	27	30	16R
F	12R	12R	11R	R	14R	20S	30S	30S	26	30	18I
G	12R	13R	12R	R	19S	20S	30S	30S	26	31	20I
H	11R	11R	11R	R	14R	21S	30S	32S	26	30	16R
I	10R	11R	11R	R	14R	20S	30S	32S	25	30	17R
J	12R	13R	11R	R	14R	20S	13R	34S	28	30	16R
K											
L	22S	16R	14R	R	17R	22S	30S	32S	28	32	20I
M	21S	30S	30S	27S	30S		30S	30S	27	30	25S
N		12R					31S	31S	26	30	15R
O		11R					28S	30S	25	27	15R