



## Original Research Article

# Extended Spectrum $\beta$ -lactamase producing *Klebsiella pneumoniae* in Neonatal Units of Minya Governorate

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## ABSTRACT

Extended Spectrum  $\beta$ -lactamases (ESBLs) producing Gram-negative bacilli have become the major clinical concern worldwide. Therefore we have concerned our study with the aim to estimate the prevalence and possible types of ESBLs among, *Klebsiella pneumoniae* and other *Klebsiella* species clinical isolates. The study was carried out in the period from April 2014 to September 2014. Samples were collected from neonatal intensive care units of 3 hospitals in Minya governorate. Isolated colonies were confirmed by routine biochemical tests. Antimicrobial susceptibility disc diffusion method was used as a screening test for detecting ESBLs-producing isolates against cefotaxime, ceftazidime, cefotriaxone (3<sup>rd</sup> generation). A combined disk diffusion test and double disk synergy test were used as confirmatory methods for all suspected ESBLs-producing isolates. Detection of possible  $\beta$ -lactamases including ESBLs-types (TEM, SHV) was carried out for all ESBLs-producing strains by using molecular techniques. The present study showed that out of 20 blood samples from neonates, there were 2 positive samples (10%), from 100 health care workers, there were 3 positive samples (3%), from 50 environmental samples there was 1 positive sample (2%) and from 50 instrumental samples, there were 1 sample (2%). The P value was 0.32 which is not significant. All *K. pneumoniae* isolates (100%) were found to be susceptible to imipenem followed by amikacin 85.7% then ciprofloxacin 71.4% and cefotaxime 71.4%. Susceptibility were detected to streptomycin 28.5%, ceftazidime 57.1% and ceftriaxone 42.5. Resistances were detected to chloramphenicol 71.5% and rifampicin 71.5% as well. All *Klebsiella pneumoniae* isolates (100%) were resistant to ampicillin. The molecular analysis of ESBLs producing isolates of *Klebsiella pneumoniae* showed that all 7 isolates yielded positive results for both blaSHV and blaTEM (100%). Amplified PCR products for each of the blaTEM genes were sent for analysis by an automated DNA sequences system showed that 3 samples (health care worker, neonate and instrument) from one hospital had identical banding patterns.

## Keywords

ESBL,  
*Escherichia coli*,  
*Klebsiella pneumoniae*

## Introduction

*Klebsiella pneumoniae* is a member of Enterobacteriaceae family, non-motile, Gram-negative, oxidase negative, rod

shaped bacteria with a prominent polysaccharide based capsule. *Klebsiella* species are found everywhere in nature.

They are found in water, soil, plants, insects, animals and humans (Brisse S, 2006). Most of human infections with *Klebsiella* infections are caused by *Klebsiella pneumoniae*, followed by *Klebsiella oxytoca*. Infections are more common in very young, old people and immunosuppressed individuals such as diabetes mellitus and chronic pulmonary obstruction. Also the spread of infection is associated with contamination of invasive medical devices (Brisse S, 2004). It has been found that *Klebsiella pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections. Infections caused by drug resistant *Klebsiella pneumoniae* isolates, especially those produce extended spectrum  $\beta$ -lactamases (ESBLs) and multidrug resistant (MDR) are more difficult and expensive to be treated with worse treatment outcome (Magiorakos AP, 2012).

Resistance to third-generation cephalosporins in these isolates is largely due to the production of extended-spectrum  $\beta$ -lactamase (ESBL) enzymes, which hydrolyse oxyimino-cephalosporins that are inhibited by clavulanic acid (Bradford PA, 2001). The studies had shown that carbapenems possess the most consistent activity against extended spectrum  $\beta$ -lactamase (ESBL) producing *Klebsiella pneumoniae* bacteria. During the past decade, ESBL strains had frequently been implicated in neonatal intensive care units at tertiary care hospitals. TEM-1 is the most commonly-encountered  $\beta$ -lactamase in Gram-negative bacteria (Al-Jasser MA, 2006). The SHV-type ESBLs may be more frequently present in clinical isolates than any other type of ESBLs. SHV-5 and SHV-12 are among the most common (Paterson DL, 2003). Previous studies in the Middle East had revealed a high prevalence of ESBL-producing *Klebsiella pneumoniae*

isolates (Adler A, 2012). and extensive spread of those carrying CTX-m-15 was reported in Lebanon, Kuwait, and Egypt (Fam N, 2011). In Saudi Arabia, CTX-M-15 producing *Klebsiella pneumoniae* was responsible for a neonatal intensive care unit outbreak (Al-Agamy M, 2009). In the Arabian Gulf countries information on the types of ESBL is scanty (Sonnevend, A, 2006).

## Materials and methods

### Bacterial isolates

The study was carried out in the Microbiology and Immunology Department, Faculty of Medicine, Minya University, in the period from April 2014 to September 2014. Samples were collected from Neonatal Intensive Care Units of 3 hospitals in Minya governorate which are Minya University Hospital, Minya General Hospital and Minya Insurance Hospital. Seven *Klebsiella pneumoniae* isolates obtained from total number of 220 samples were collected as follow:

- 1) 20 blood samples were withdrawn from neonates who had evidence of sepsis after 48 hours of admission to the unit. Then 100 samples were taken from health care workers (nose, nasopharynx, nails, hands, clothes,.....etc) using sterile cotton swabs moistened by a sterile physiological saline solution.
- 2) 50 environmental samples using sterile cotton swabs moistened by a sterile physiological saline solution and used to collect samples from walls, floors, windows, beds, bedside tables, feeding bottles, scales, shelves, sinks and trays.
- 3) 50 instrumental samples using sterile cotton swabs from respiratory equipment,

suction tubes, phototherapy units, resuscitation equipment.

4) Isolation of *Klebsiella pneumonia* was done by culture on MacConkey's Agar and confirmed by biochemical reactions.

### Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by the disc-diffusion method on Mueller–Hinton agar, which was incubated at 35°C for 18 h. The results were interpreted according to the current guide lines of the Clinical Laboratory Standards Institute. The following antibiotics were tested: ampicillin, amoxicillin–clavulanate, streptomycin, amikacin, rifampicin, ciprofloxacin, chloramphenicol, imipenem, ceftriaxone, ceftazidime and cefotaxime. Detection of ESBLs production. By double disc approximation test. Synergy was determined between a disc of amoxicillin-clavulanate (20 µg/10 µg) (augmentin) and a 30-µg disc of each third-generation cephalosporin test (ceftriaxone, ceftazidime and cefotaxime) antibiotic discs placed at a distance of 20 mm from center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate.

### Molecular detection of ESBLs by PCR

All phenotypically confirmed ESBLs producing strains were subjected to plasmid DNA extraction, then amplification of the extracted DNA by PCR using *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> specific primers.

The enzyme used routinely was Taq PCR Master Mix 2X. The reaction mixture contained the following reagents: 12.5 µl Taq PCR Master Mix 2X, 1 µl of each primer mix as in Table 1, containing both forward and reverse final 10 µM for each, 2.5 µl from each DNA sample (1 µg) and 8 µl double distilled water. Water was added

instead of DNA for non template controls. PCR conditions for the SHV gene comprised an initial denaturation step for 5 min at 95°C, followed by 32 cycles of 94 °C for 1 min, 57° C for 1 min and 70° C for 1 min, with a final extension step at 72° C for 10 min. For TEM, the amplification cycle consisted of 5 min at 95° C, followed by 30 cycles of 94 °C for 30 s, 55° C for 1 min and 72° C for 1 min, with extension at 72° C for 10 min.

**Table.1** PCR primers used for detection of genes, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> (Dashti AA, 2010)

Gene	Primer Sequence
SHV- F*	ATGAGTATTCAACATTTCCG
SHV –R*	CCAATGCTTATTCAGTGAGG
TEM-F*	CTGGGAAACGGAAGTGAATG
TEM-R*	GGGGTATCCCGCAGATAAAT

\*F: Forward, R: Reverse

### DNA sequencing

Amplified PCR products for each of the *bla*<sub>TEM</sub> genes were sent for analysis by an automated DNA sequencing system, AB3500 DNA sequencer (Applied Biosystems). The resulting DNA sequences were compared to ascertain their genetic relatedness.

## Result and Discussion

### Patients and Bacterial isolates

Two hundred and twenty samples were collected from neonatal intensive care units of 3 hospitals in Minya governorate which are Minya University Hospital, Minya General Hospital and Minya insurance Hospital. Seven *Klebsiella pneumoniae* isolates obtained from 20 blood samples were withdrawn from neonates who had evidence of sepsis after 48 hours of admission to the unit, 100 nasal swaps from

health care workers, 50 environmental samples and 50 instrumental samples (using sterile cotton swabs moistened by a sterile physiological saline solution). *Klebsiella pneumoniae* strains grew as pink mucoid colonies on MacConkey's Agar as it is lactose-fermenting bacterium. Microscopically after staining with Gram stain *Klebsiella pneumoniae* is a Gram negative bacterium, rod-shaped surrounded by a large polysaccharide capsule and non motile. *Klebsiella pneumoniae* isolates were identified by the typical biochemical reactions (indole test (-ve), MR test (-ve), VP test (+ve), citrate test (+ve) and urease test (+ve) from left to right as shown in Figure 1.

The sources and characteristics of the *Klebsiella pneumoniae* isolates are presented as follows: 2 neonatal samples, 3 samples from health care workers, 1 sample from environment and 1 sample from instruments as shown in Table 2.

Our present study showed that out of 20 blood samples from neonates there were 2 positive samples (10%), from 100 health care workers there were 3 positive samples (3%), from 50 environmental samples, there were 1 positive sample (2%), from 50 instrumental samples, there was 1 sample (2%). P value was 0.32 which is not significant (Table 3, Figure 2).

Our study showed also that out of 7 male neonates there were 1 positive case (14.2 %) of *Klebsiella pneumoniae* and from 13 female neonates there were 1 positive case (7.6 %) and P value was 0.6 which is not significant (Table 4 and Figure 3).

The present study showed that out of 20 blood samples from neonates, there were 2 positive samples (10%), from 100 health care workers, there were 3 positive samples (3%), from 50 environment samples there

was 1 positive sample (2%), from 50 instrumental samples, there was 1 sample (2%). The P value were 0.32 which is not significant. All *Klebsiella pneumoniae* isolates (100%) were found to be susceptible to imipenem followed by amikacin 85.7% then ciprofloxacin 71.4% and cefotaxime 71.4 %. Susceptibility were detected to streptomycine 28.5 %, ceftazidime 57.1% and ceftriaxone 42.5. Resistances were detected to chloramphenicol 71.5% and rifampicin 71.5% as well (as shown in Table 5 and Figure 4). All *Klebsiella pneumoniae* isolates (100%) were resistant to ampicillin.

All samples of *Klebsiella pneumoniae* were confirmed to be ESBL producing by double disc approximation test/DDST (100%) (Figure 5).

The molecular analysis of ESBLs producing isolates of *Klebsiella pneumoniae* showed that all 7 isolates yielded positive results for both blaSHV and blaTEM (100%). The results of amplification of the products of the blaSHV (308 bp) and blaTEM (858 bp) genes are presented in Figures 6 and 7.

**DNA sequencing:** Amplified PCR products for each of the blaTEM genes were sent for analysis by an automated DNA sequencing system, AB3500 DNA sequencer (Applied Biosystems) as shown in Figures 8 and 9. The resulting DNA sequences were compared to ascertain their genetic relatedness. The results showed that 3 samples (health care worker, neonate and instrument) from one hospital had identical banding patterns indicating that a single clone of *Klebsiella pneumoniae* was involved.

The problem of gradually increasing resistance to antibiotics has threatened the entire world. Production of  $\beta$ -lactamase, which hydrolyses and inactivates  $\beta$ -lactam antibiotics, has been one of the most

important resistance mechanisms of many bacterial species, mainly in the family Enterobacteriace (Akcam FZ, 2004). Our study presented a warning event of ESBL production among all isolates of *Klebsiella pneumoniae*. Resistance to extended spectrum  $\beta$ -lactams among Gram negative pathogens is increasingly associated with ESBLs (Kimura S, 2007).

We identified a high percentage of *Klebsiella pneumoniae* infection with a frequency of (10%) among neonates and with (100%) ESBL producing. Other studies conducted in Egypt had reported that the percentage of *Klebsiella pneumoniae* infection was (7%) among infants and (67%) ESBL producing (Abdel-Hady H, 2008).

Another study from Egypt reported an outbreak in a neonatal intensive care unit in Cairo, Egypt, in which 80% of the isolates were *Klebsiella pneumoniae*, of which 58% were ESBL producers (Moore KL, 2005).

Our data demonstrated, 1 environmental sample was detected from 50 samples with percentage of 2%, one instrumental sample was detected as well from 50 samples with the same percentage 2 % and both samples were found to be ESBL producing (100%). Another study in Egypt revealed that there was 7.6% of environmental samples were positive to *Klebsiella pneumoniae* and 14.8% of these samples were ESBL producing (Ahmed SH, 2010). The difference between this study and ours study is in the number of samples as our study was done with 50 samples only while the other one was done with 475 environmental samples. In Kuwait, there was no any environmental samples was detected in a study done in a Kuwait hospital (Dashti AA, 2010). The percentage of *Klebsiella pneumoniae* in health care workers in this study was 3% which is relatively high comparing with similar studies performed in

Egypt, Mansuora 0.8% (Abdel-Hady H, 2008), however in Kuwait a study performed showing that 20% is the percentage of *Klebsiella pneumoniae* in health care workers (Dashti AA, 2010).

It is worth mentioning that one of the health care workers positive cases is diabetic, having low immunity of diabetic patients and their high vulnerability to infections. Information as regards ESBLs are very uncommon in our environment and as a result, most clinicians probably don't know when to test for ESBLs or any preventive measures to adopt that will help to control its spread. This level of ignorance could culminate to devastating consequences. As the presence of these enzymes significantly impacts the efficacy of  $\beta$ -lactam therapy, there is a need for clinical laboratories to accurately recognize ESBLs producers so as to better support therapy and provide accurate prevalence data (Bradford, PA, 2001).

A survey, carried out in 2001–2002 which covered medical centers in northern and southern European countries, Egypt, Lebanon, Saudi Arabia and South Africa, reported the highest incidence of extended spectrum  $\beta$ -lactamases (ESBLs)-producing isolates in Egypt (Bouchillon SK, 2004). We noticed that premature and low birth weight babies were more likely to acquire ESBL *Klebsiella pneumoniae* because of relative immaturity of their immune system. In addition, they are likely to be subjected to different interventional procedures.

Moreover, total parenteral nutrition and mechanical ventilation were significant predictors of ESBL *Klebsiella pneumoniae* infections as the 2 neonates with *Klebsiella pneumoniae* having respiratory distress syndrome. In contrast to our findings, previous studies have demonstrated that low birth weight and mechanical ventilation

(Boo NY, 2005) were not independent predictors of ESBL *Klebsiella pneumoniae* infection. We found also in our study *Klebsiella pneumoniae* isolates were susceptible to amikacin 85.7% then ciprofloxacin 71.4% and cefotaxime 71.4%. Susceptibility were detected to streptomycine 28.5 %, ceftazidime 57.1% and ceftriaxone 42.5.

Resistances were detected to chloramphenicol 71.5% and rifampicin 71.5% as well. This is in agreement with the findings of (Winokur PL, 2003; Jones RN, 2001; Jeong SH, 2004) in United States with resistance rates of 10% to amikacin. According to a study by Moubareck C, 2005, a higher resistance rates to amikacin of 17% were reported. In contrast to this study, it was reported that (Jain A, 2003) resistance to cefotaxime of more than 80.9% and up to 59.5% to ceftazidime. Rafay AM, 2007 observed 100% resistance to cephalosporin-ceftazidime, cefotaxime, and ceftriaxone (Duttaroy B, 2005) showed resistance of 75% to cefotaxime, 85% to ceftazidime and 60% to ceftriaxone.

Studies from Spain (Rodriguez-Bano J, 2004), Italy (Mugnaioli C, 2006), Greece (Pournaras S, 2004), UK (Woodford N, 2004) and Canada (Pitout JD, 2007), have shown an alarming trend of associated resistance to other classes of antimicrobial agents among ESBLs-producing organisms isolated from community sites. These surveys showed co-resistance to cotrimoxazole (up to 64%) gentamicin (up to 61%) and ciprofloxacin (up to 68%). Thus their limited use in the treatment of infections due to these pathogens is necessary. This co-resistance arises probably because these plasmid-mediated enzymes are transferable between bacterial species and are also capable of incorporating genetic material coding for resistance to other antibiotics.

Similar to our study, (Jain et al., 2003) showed that ESBL-producing organisms were resistant to ampicillin (Jain et al, 2003). DNA amplification by PCR for ESBL positive isolates, we considered that all 7 isolates contained the genes for TEM and SHV. Similarly, in Assiut the type of  $\beta$ -lactamases gene was determined among *Klebsiella pneumoniae* strains by using a polymerase chain reaction, which showed that SHV was the main type of  $\beta$ -lactamases, followed by TEM genes (Ahmed SA, 2009). Another study done in Egypt revealed that SHV genes are associated with an ESBL resistance phenotype in *Klebsiella pneumoniae* in Egypt in percentage of 98% which is near to in the previous investigation as well (Newire EA et al, 2013).

In a Turkish hospital, they found that TEM type ESBLs were observed in (9/15) *Klebsiella pneumoniae* isolates. SHV type ESBLs was found in (5/15) *Klebsiella pneumoniae* isolates with 6 isolates harboring both *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes (Bali EB, 2011). Another study was performed in Lebanon reported that 61% and 21% of ESBLs producers as *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> containing isolates, respectively (Chmelnitsky I, 2005) but it is lower than the results of a study has been done by (Hernández JR, 2005) in Spain (*bla*<sub>TEM</sub>: 77.64% and *bla*<sub>SHV</sub>: 37.64% respectively). On the basis of results of other studies, the frequency of SHV-type enzymes was higher than other genotypes of ESBLs (Shahcheraghi F, 2007) in Tehran (*bla*<sub>SHV</sub>:69.6 % and *bla*<sub>TEM</sub> 32.2 %). It is noteworthy that another study which was carried out in India 2007, isolates having both TEM and SHV were more common in contrast to TEM and SHV alone (Lal P, 2007).

In our research study DNA sequencing showed that 3 samples (health care worker,

neonate and instrument) from one hospital (Minya Insurance Hospital) had identical banding patterns indicating that a single clone of *Klebsiella pneumoniae* was involved. This suggests that the healthcare worker either acquired the bacteria from the neonate or may have been involved in its transmission at some point. This highlights the role of healthcare workers in horizontal transmission of outbreak strains. We found in our study that the instrumental sample also identical which confirms the serious role of the hospital environment and instruments in the transmission and spread of infections.

In conclusion, the results of this study revealed the transmission of a clone of ESBL-producing *K. pneumoniae* among neonates and healthcare workers and instruments in a Minya hospitals. Further investigation is required to determine the prevalence of such isolates in the community and hospital settings on a large scale. Accordingly, further studies must be done in other departments of Minya Insurance Hospital to report spread of *Klebsiella pneumoniae* isolates detected in this study

**Table.2** Characteristics of *Klebsiella pneumoniae* isolates

Sample	Source	Date	Age	Sex	Diagnosis
1	Blood	14/5/2014	6 days	Male	PT(32 weeks), RDS
2	Blood	22/8/2014	2 days	Female	PT(29 weeks), RDS
3	Nose	17/6/2014	42 years	Female	DM
4	Nose	14/5/2014	26 years	Female	-
5	Nose	22/8/2014	33 years	Female	-
6	Wall	14/5/2014	-	-	-
7	Incubator	17/6/2014	-	-	-

\*PT, Preterm birth; RDS, respiratory disease syndrome; DM, diabetes mellitus.

**Table.3** Percentage of distribution among samples

	Total number of samples	Number of positive cases	Percentage	P value
Neonates	20	2	10%	0.32
Health care workers	100	3	3%	
Environment	50	1	2%	
Instrument	50	1	2%	

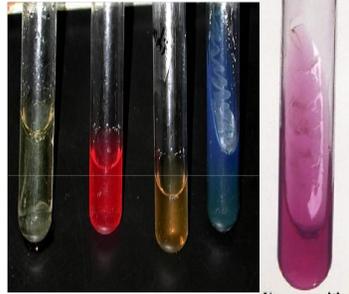
**Table.4** Sex distribution of *Klebsiella pneumoniae* among neonates

Antibiotic	Sensitive	Resistant	P value
Ampicillin	0	7 (100%)	0.17
Chloramphenicol	2 (28.5%)	5 (71.5 %)	

**Table.5** Antimicrobial susceptibility pattern of *Klebsiella pneumonia*

	Total number	(+ve) cases	Percentage	P value
Male	7	1	14.2 %	0.6
Female	13	1	7.6 %	

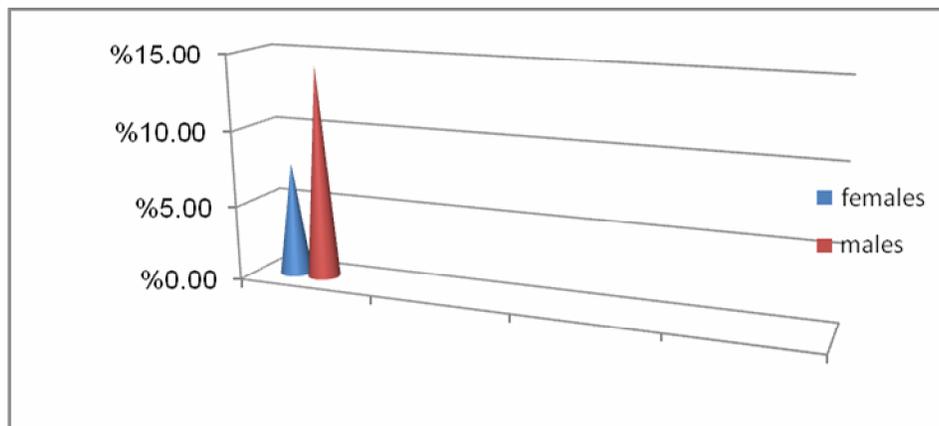
**Figure.1** Results of Biochemical reactions of *Klebsiella pneumonia*  
Indole VP MR citrate urease



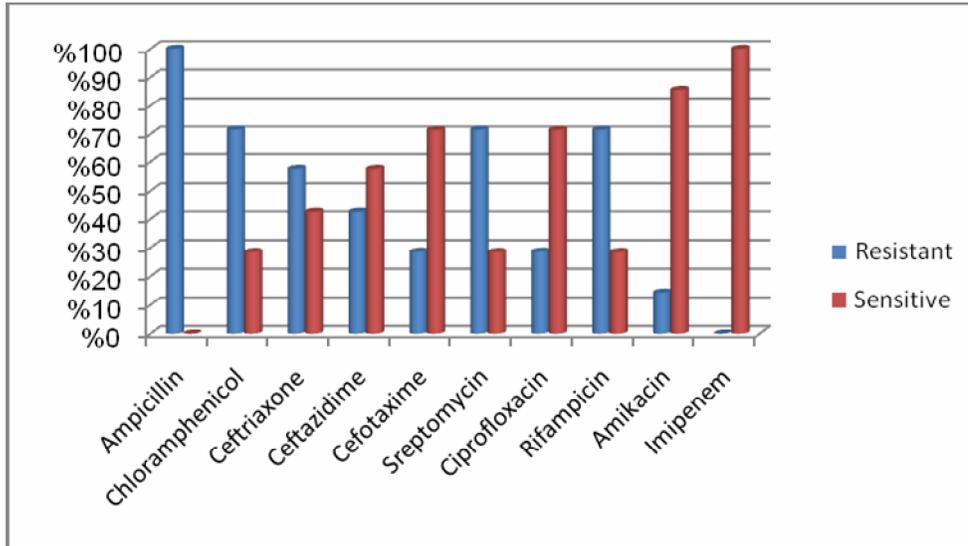
**Figure.2** Percentage of distribution among cases



**Figure.3** Sex distribution of *Klebsiella pneumoniae* among neonates



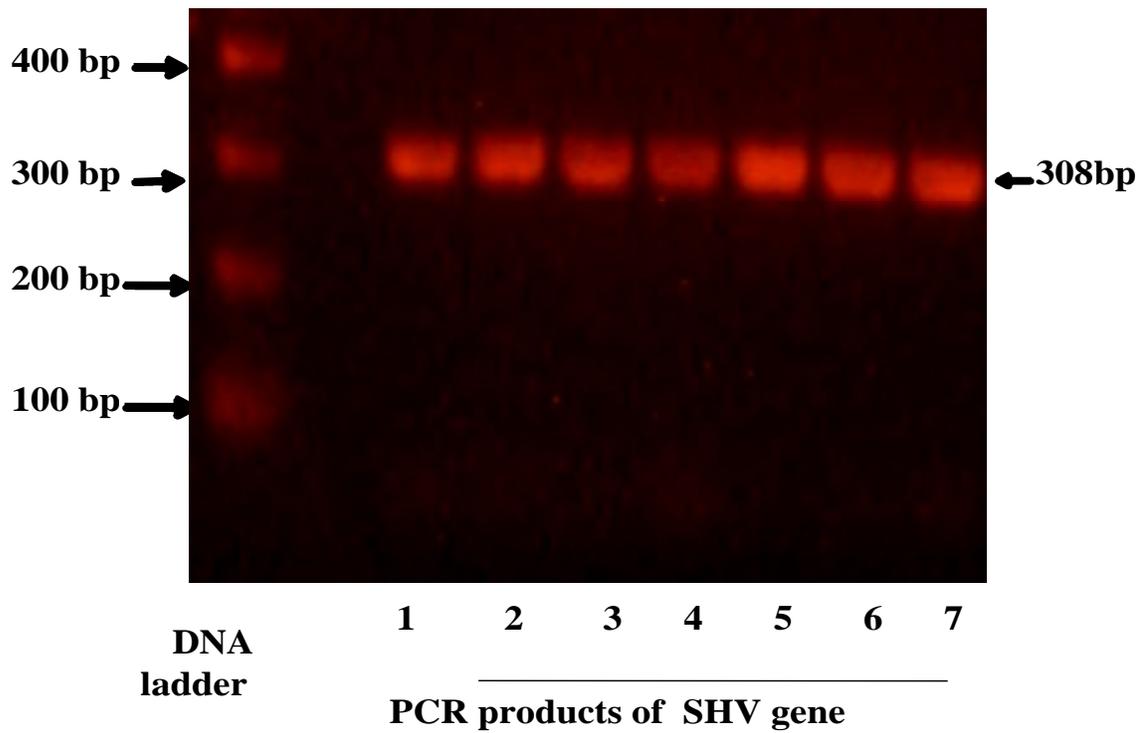
**Figure.4** Antimicrobial susceptibility pattern of *Klebsiella pneumoniae*



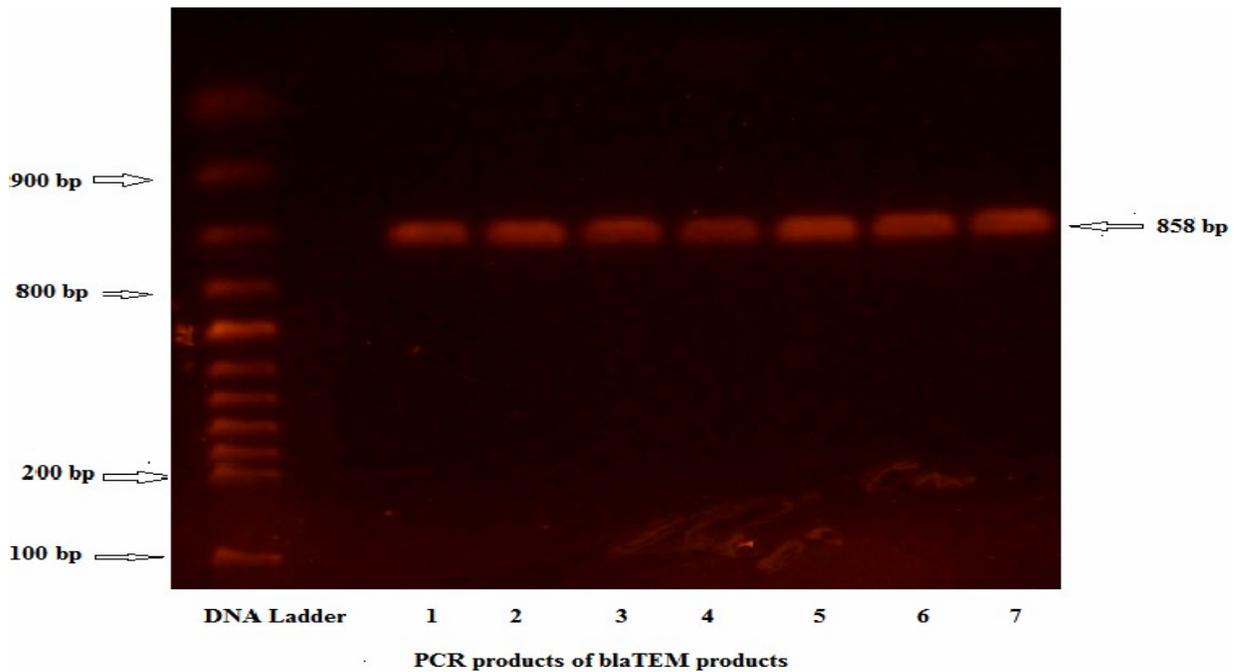
**Figure.5** Positive ESBL production test



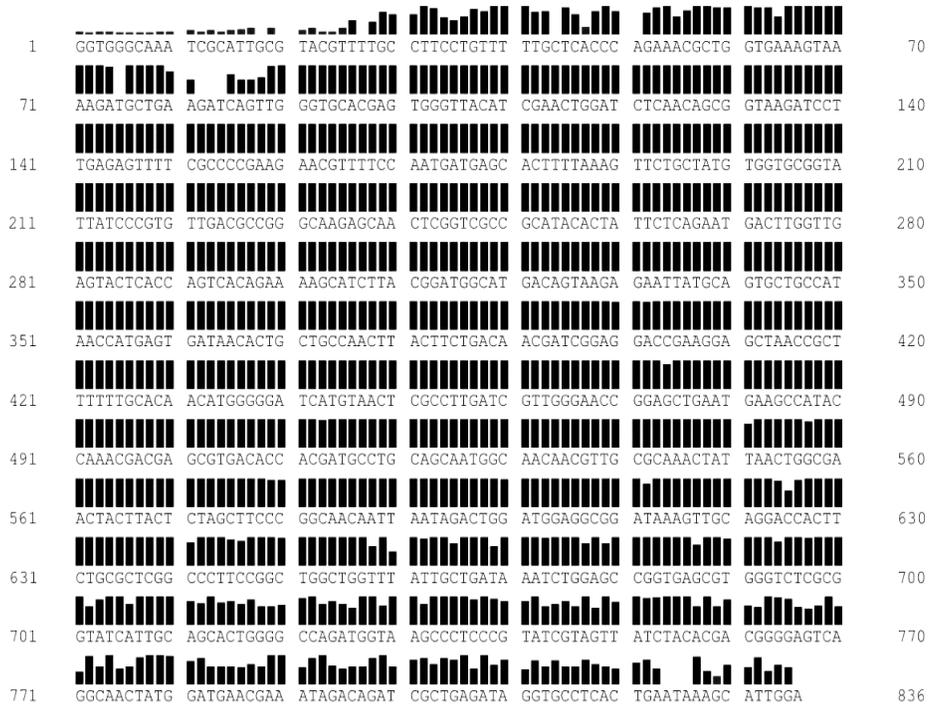
**Figure.6** Gel electrophoresis for PCRs performed In the study showing presence of blaSHV amplicon with a molecular size of 308 bp.



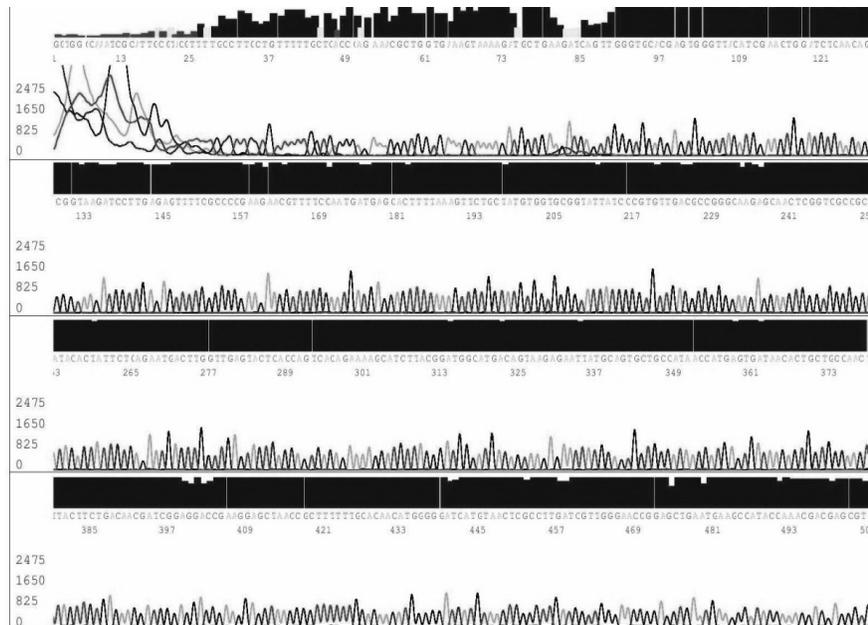
**Figure.7** Gel electrophoresis for PCRs performed in this study showing Presence of the blaTEM amplicon with a molecular size of 858 bp



**Figure.8** Sequence of bases of TEM gene obtained from DNA of healthcare worker, neonatal and instrumental samples



**Figure.9** Electropherogram data of TEM gene obtained from DNA of healthcare worker, neonatal and instrumental samples.



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