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Prevalence and Characterization of Extended Spectrum Beta-Lactamase Production in Clinical Isolates of *Klebsiella pneumonia*

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Abstract

Aim: This study was carried out to determine the presence of TEM and SHV genes in extended- spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae*. The study was also aimed to compare results of phenotypic confirmatory double disc diffusion test and genotypic methods.

Materials and Methods: A total of 679 strains of *Klebsiella pneumoniae* were selected for the study from June 2012-December 2013. Kirby – Bauer disk diffusion method was performed to determine the antibiotic resistance pattern. Screened for ESBL and confirmed by phenotypic confirmatory disc diffusion test (PCDDT). 100 randomly selected isolates were investigated for the presence of TEM and SHV genes via Polymerase chain reaction (PCR) using two different sets of primers. Multiplex PCR was also performed for the same.

Results: Phenotypic confirmatory test was able to detect ESBL production in 90.13% of *Klebsiella pneumoniae* isolates. Among the two ESBL genotypes, the most prevalent genotype was found to be TEM. Majority of ESBL producing isolates possess both ESBL genes.

Conclusion: Multiplex PCR can be used as a rapid method to identify common genes (TEM and SHV) responsible for extended spectrum beta lactamase production in *Klebsiella pneumoniae*. It will prove valuable for surveillance and for determining the line of treatment against drug resistant organisms, thus saving precious time and resources. PCDDT results correlated with genotypic method in all the tested strains.

Keywords: ESBL; *Klebsiella pneumonia*; TEM; SHV

Introduction

Microbes are remarkably adaptable and amazingly versatile. Through the course of evolution; they have developed sophisticated mechanisms for preserving genetic information and disseminating it efficiently in the interests of their survival. They recognize no boundaries. The resistance developed in one part of the country or indeed in the world can be disseminated readily [1]. Emergence of resistance to β-lactam antibiotics began even before the first β-lactam, penicillin, was developed. The first β-lactamase was identified in *Escherichia coli* prior to release of penicillin for use in medical practice [2]. ESBLs were first reported in *Klebsiella pneumoniae* in 1983, from Germany [3]. Production of beta lactamase is the most common mechanism of antibiotic resistance to beta lactam antibiotics. These are produced by aerobic Gram positive, Gram negative bacteria and also in anaerobes [4]. Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended- spectrum β-lactamases (ESBLs) [5].

These enzymes have been derived from TEM and SHV genes by mutations and have been well described in family *Enterobacteriaceae* especially *Klebsiella pneumoniae* [6]. *Klebsiella* species has rapidly become the most common ESBL producing organism, making it difficult to eradicate this organism from the high risk wards such as intensive care units [6].

Material and Methods

Bacterial isolates: The study was carried out in Microbiology Laboratory, Government Medical College Hospital, Aurangabad, during June 2012-Dec 2013. A total of 679 consecutive non duplicate clinical isolates of multi- drug resistant *Klebsiella pneumoniae* isolated from various clinical specimens were included in the study.

The specimens included Pus (371), ET secretion (81), Blood (75), Urine (49), Pleural fluid (32), Sputum (32), other fluids (39). All the samples were processed and isolates identified by standard methods [7-9].

To determine their multi-drug resistance, antimicrobial susceptibility testing was performed by Kirby-Bauer's disc diffusion method as per Clinical Laboratory Standards Institute-2012 (CLSI) recommendations. Antibiotic discs were procured from Hi-Media Laboratories, Mumbai (India).

All strains of *Klebsiella pneumoniae* found resistant or intermediate sensitive to the third generation cephalosporin

ns namely cephotaxime, or ceftazidime were tested for the production of ESBL by the following CLSI ESBL SCREENING TEST. Amongst the strains sensitive to these drugs, all were not excluded from tests for ESBL. CLSI has recommended that even if a strain falls in sensitive range (≥23 for Cephotoxime and ≥18 for Ceftazidime) some of them could still be ESBL producers. CLSI advises that those strains with zone of inhibition of ≤27 for Cephotoxime and ≤22 for Ceftazidime belong to this group.

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As a screening test, isolates showing inhibition zones ≤27 mm for Cefotaxime, ≤22 mm for Ceftazidime were selected as potential ESBL producers. Extended spectrum beta lactamase detection in *Klebsiella pneumoniae* isolates positive by screening test was confirmed by phenotypic confirmatory disk diffusion test (PCDDT). In brief it is as follows. Disk of cephotaxime and ceftazidime alone and those containing a combination of Clavulanic acid with these antibiotics were used as per CLSI guidelines. Following control strains were used for ESBL detection.

Positive control for ESBL *Klebsiella pneumoniae* ATCC: 700603.

Negative control for ESBL- *Escherichia coli* ATCC: 25922.

Interpretation: Organism was considered ESBL producer if there was more than 5 mm increase in zone diameter for Ceftazidime and Cefotaxime tested in combination with Clavulanic acid versus its zone when tested alone [10]. Some of the phenotypic tests for detection of ESBLs are: Double Disc Synergy test, three dimensional test, Inhibitor potentiated disc diffusion test, Disk approximation test, Vitek ESBL test and E test [11].

Molecular detection tests include: DNA probes (specific for gene family e.g., TEM or SHV.) PCR (Easy to perform, specific for gene family e.g. TEM or SHV) Oligotyping (Detects specific TEM variants) PCR-RFLP (Easy to perform, can detect specific nucleotide changes) PCR-SSCP (can distinguish between a number of SHV variants) LCR (can distinguish between a number of SHV variants) Nucleotide sequencing (The gold standard, can detect all variants) [5].

For detection of ESBL genes, PCR was performed using the following two different sets of primers. Set 1 had primers labeled as TEM and SHV. (Table 1) The strains under study were subjected to PCR separately using these primers. Set 2 had primers labeled bla_{TEM} and $bla_{SHV.}$ (Table 1). The strains under study were subjected to PCR separately using these primers also. In addition multiplex PCR was also performed with these two sets (TEM and SHV; bla_{TEM} and bla_{SHV}). Thus for set1 TEM, SHV and multiplex PCR was performed. Similarly for set 2 bla_{TEM}, bla_{SHV} and multiplex PCR was performed. All primers were synthesized by Merck-Bangalore, India.

The primers of set1used to amplify TEM gene correspond to the position 55 to 75 and 752 to 771 respectively, with 717 bp fragment size. Primers to amplify SHV gene correspond to position 509 to 526 and 962 to 979 respectively with a fragment size of 471 bp. The primers of set 2 are used to amplify 867 bp bla_{TEM} and 930 bp bla_{SHV} coding region in *Klebsiella pneumoniae.*

Preparation of DNA

Colony PCR of Clinical isolates were performed for screening the resistance genes using TEM, SHV, bla_{TEM} and bla_{SHV} primers. For colony PCR bacterial isolates were grown overnight on LB agar. A colony of each isolates were suspended in 100 µl double distilled water and boiled at 95^{0}C for 10 min. Following centrifugation at 10,000 rpm for 1 min the supernatant was used as the crude DNA.

Target	Primer sequence(5'-3')	Product size (bp)
SHV F	TCAGCGAAAAACACCTTG	471
SHV R	TCCCGCAGATAAATCACC	
TEM F	CTTCCTCCTGTTTTTGCTCACCCA	717
TEM R	TACGATACGGGAGGGCTTAC	
bla_{TEM} F	ATAAAATTCTTGAAGACGAAA	867
bla_{TEM} R	GACAGTTACCAATGCTTAATCA	
bla_{SHV} F	GGGTTATTCTTATTTGTCGC	930
bla_{SHV} R	TTAGCGTTGCCAGTGCTC	
F: Forward; R: Reverse		

Table 1: Primers used for detection with their expected fragment sizes1 [5], 2 [10].

Amplification of TEM and SHV genes (set 1) were performed in a 25 µl volume containing 3.5µl PCR master mix (Merck, Bangalore, India), 0.5 µl each forward and reverse primers. 5 µl template DNA preparation was added to the reaction mixture. PCR amplifications were carried out in a Peltier Thermal Cycler (PTC-200, MJ Research, USA).

The cycling conditions for amplification were as follows [6]. For SHV gene, initial denaturation at 94°C for 2 min and 30 cycles of 1 min at 94°C, 30 sec at 52°C and 45 sec at 72°C, followed by 5min at 72°C.

For TEM gene, cycling conditions were as follows [6]. Initial denaturation of 2 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, followed by 7 min at 72°C.

The resulting PCR products were analyzed by electrophoresis with 1.5% agarose gel in Tris-borate-EDTA buffer. The gels were stained with ethidium bromide and band observed were photographed on an UV trans illuminator. A molecular weight standard (100bp ladder-Merck, Bangalore) was included on each gel.

For set 2, reactions were performed in a Peltier Thermal Cycler (PTC-200, M J Research, USA), according to the protocol [11]. For both bla_{TEM} as well as bla_{SHV}, PCR profile was an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for one min, 52°C for one min and 72°C for 10 min. PCR products were analyzed by electrophoresis with 1.5 per cent agarose gel. After staining with ethidium bromide the gel was observed on trans illuminator and the image was recorded. In an earlier study [12] same cycling conditions were used for PCR when bla_{TEM} and bla_{SHV} were amplified separately. Hence we performed multiplex PCR using same primers (set 2) and followed same cycling conditions.

Results

Antibiotic resistance pattern was as follows: Gentamicin (79.23%), Amikacin (65.53%), Ciprofloxacin (68.92%), Cefotaxime (85.12%), Ceftazidime (83.21%), Cefipime (82.32%), and Meropenem (17.37%).

It was observed that many (≥80%) of our isolates of *Klebsiella pneumoniae* were resistant to third generation Cephalosporin's and other antibiotics, making them Multi Drug Resistant (MDR) strains. Most of these were isolated from in-patients, indicating probable Hospital Acquired Infection.(HAI)

Out of 679 strains of *Klebsiella pneumoniae,* 35 strains were sensitive to 3GC. All others i.e. 644 (94.84%) strains were subjected to PCDDT. Of these 644 strains, 612 strains showed increase in zone diameter of 5 mm to the combination of Cefotaxime + Clavulanic acid when compared to Cefotaxime alone. Same 612 strains gave similar results with Ceftazidime + Clavulanic acid when compared to Ceftazidime alone. All these 612 (90.13%) strains were reported as ESBL producers. (Figure 1) Of the 612 ESBL positive clinical isolates, 100 random isolates were subjected to genotypic characterization by PCR for presence of TEM and SHV gene (Table 2). With Primer Set: 1- All the hundred isolates (100%) showed presence of TEM gene, while forty isolates (40%) showed presence of SHV gene. Thus forty isolates contained both TEM and SHV genes (40%). Sixty isolates (60 %) had only TEM gene. No isolates showed SHV gene alone (Figure 2) With Primer Set: 2 Amplification with bla_{TEM} and bla_{SHV} seventy isolates (70%) showed presence of TEM gene with bla_{TEM} primers, while fifty isolates (50%)

showed presence of SHV gene with bla_{SHV} primers. Twenty isolates (20%) had both bla_{TEM} and bla_{SHV} gene (Figure 3 and Table 3)

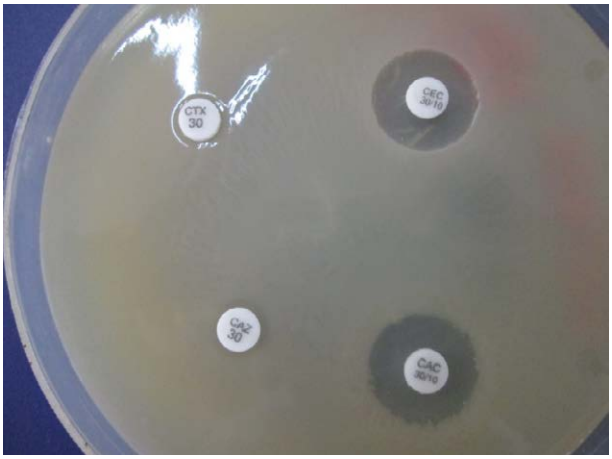


Figure 1: Phenotypic Confirmatory Disc Diffusion Test (PCDDT): Ceftazidime and Cefotaxime showing an increase in zone diameter of >5 mm with the addition of Clavulanic acid, indicative of ESBL production in a *Klebsiella pneumoniae* isolate.

	Multiplex TEM+SHV (Set 1)	Multiplex bla_{TEM} +bla_{SHV} (Set 2)
Number of Isolates having both markers	70%	30%
Number of Isolates having unique marker-TEM	30%	50%
Number of Isolates having unique marker -SHV	Nil	20%

Table 2: Showing the percentage of isolates amplified using multiplex PCR.

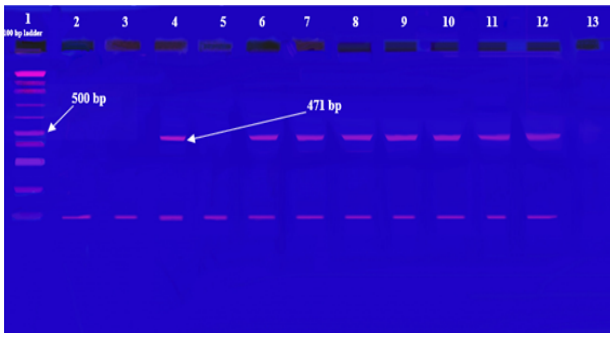
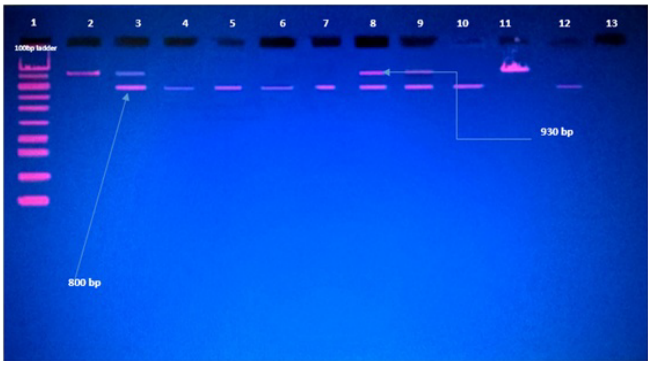


Figure 2: Multiplex PCR using TEM and SHV primer.



In the above figure lane1 is 100 bp ladder, lane 2-11 are samples, lane 12 positive control and lane 13 negative control.

Figure 3: Primer set 2-Multiplex amplification with bla_{TEM} and bla_{SHV}.

Discussion

Amongst GNBs, common organisms having ESBL production are *Klebsiella pneumoniae* and *E.coli*. Of these *Klebsiella pneumoniae species* has gained importance due to high proportion of strains showing ESBL production. Hence we concentrated our study only to *Klebsiella pneumoniae*.

Klebsiellae are opportunistic pathogens and can give rise to severe diseases such as septicaemia, pneumonia, UTI, and soft tissue infection. Typically, *Klebsiella* infections are nosocomial. The hospitalized, immune compromised patient with underlying diseases is the main target of these bacteria. Thus, *Klebsiella* infections may serve as a paradigm of hospital-acquired infections [13].

In the last 12 years ESBL have gone from being an interesting scientific observation to a reality of great medical importance. Initially restricted to the hospital acquired infections, they have also been isolated from infections in outpatients. Major outbreaks have been reported from all over the world thus making them emerging pathogens.

In some center's like Ujjain, Bijapur, Kanchipuram (some of the cities in India) the incidence of ESBLs is still very low [14-16]. But in metropolitan cities (of India) like New Delhi there is gradual increase in ESBL producers, from 80% in 2002, 91% in 2005, 97% in 2007 and 100% in 2010 [17-19].This probably relates to rampant and inadvertent use of third generation cephalosporin's). Over the counter availability could be another cause when patients resort to self-medication [20]. Two studies from Chennai in 2006show different rates of ESBLs [21,22]. Similarly two studies in 2012 from Davangere show different rates of

ESBL positive clinical isolates	TEM(100 bp)	SHV(471 bp)	Multiplex TEM+SHV	blaTEM(800 bp)	blaSHV(930bp)	Multiplex bla_{TEM} +bla_{SHV}
1	+				+	
2	+			+	+	+
3	+	+	+	+		
4	+			+		
5	+	+	+	+		
6	+	+	+	+		
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+		
10	+	+	+		+	
11	+	+	+	+		

Table 3: Showing PCR amplification of TEM and SHV genes among the random few ESBL positive clinical isolates.

ESBLs. It has been already reported that incidence of ESBLs differs not only across the country but from institute to institute [23,24].

The incidence of ESBLs in Manipal has actually decreased over the years. From 41% in 2007; it has decreased to 27.39% in 2009 [25,26]. They developed quick screening methods to assess the different mechanisms of ESBL production, so that the patients can be treated with appropriate antibiotics. Prevalence of ESBLs is reported to be high from Medicine ICU, Surgery wards and NICU/ Pediatric wards [17,27-30]. Our study correlates with these studies. The exact reason for this cannot be pointed out. But probably it relates to drug prescribing habits of these wards [31].

There was an increasing trend of rate of ESBLs in China. In was 33.3% in 2005, 41% in 2006 and 57% in 2007 [32]. It was further increased to 77.8% in 2009 [33]. ESBL production of *Klebsiella pneumoniae* from Thailand ranged from 44.4%-100% [34,35] and Korea 30% [36]. Another Asian Country Iran showed an increase in ESBL production by *Klebsiella pneumoniae* of 52.5% in 2008 [37] to 59.2% in 2009 [38], reaching 96% by 2010 [39]. A study from Nigeria recovered *Klebsiella pneumoniae* from patients suspected to have urinary tract infections; 35.3% were ESBL producers [40]. From Sharjah, UAE, found 40% of *Klebsiella* isolates as ESBL producers [41]. In a study from Cairo, Egypt ESBLs were detected in 55.3% of *Klebsiella pneumoniae* [42]. Riyadh, Saudi Arabia reported 55% of *Klebsiella pneumoniae* as ESBL producers [43]. From Pakistan urinary tract infections caused by ESBL producing *Klebsiella pneumoniae* was 71.7% [44]. Patients having infections caused by ESBL producing organisms are at increased risk of treatment failure with broad spectrum β-lactam antibiotics. Therefore, it is recommended that any organism confirmed for ESBL production be reported as resistant to all broad-spectrum β-lactam antibiotics, regardless of the susceptibility test result [10].

With the spread of ESBL producing strains in hospitals all over the world, it is necessary to know the prevalence of ESBL positive strains in a hospital so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms is much higher [17].

In the present study, it was observed that ESBL positive isolates exhibited high levels of multidrug resistance and the prevalence of ESBL producing *Klebsiella pneumoniae* was found to be high (90.13%). Similar results were achieved by other studies [6,18].

From India high prevalence of ESBL producing *Klebsiella* strains has been reported by various groups. Reported frequency of ESBL producing *Klebsiella* species in India ranged from 14-100% [16,19]. Prevalence of ESBL producing *Klebsiella* around the world varies between 3%-8% to 100% [35,45]. The easiest approach to genetic detection of β–lactamases is the amplification of the whole β–lactamase (bla) gene or its parts using PCR with oligonucleotide primers that are specific for the target gene. They can be helpful in providing explanations for unusual complex antibiograms, confirming specific resistance mechanisms

Out of the 679 isolates, majority of ESBL producers were from urine (97.87%) followed by pleural fluid (96.87%) and other fluids (94.73%). Isolates from urinary samples were found to be maximum. This could be due to injudicious use of antibiotics in patients suffering with recurrent urinary tract infections and persistent use of indwelling catheters in Intensive Care Unit patients. In the present study, all the ESBL producers were found resistant to 2 or more drugs, whereas multidrug resistance in non ESBL producers was comparatively less. Our study showed that ESBL production was high among uropathogens, and that the ESBL producers were mostly multi drug resistant. The situation is worsened due to increased multidrug resistance seen in ESBL producers [46].

Monitoring of ESBL production and antimicrobial susceptibility testing are necessary to avoid treatment failure in patients with UTI. ESBL producers are associated with increased morbidity and mortality. The majority of ESBL producing *K. pneumoniae* were resistant to the common antibiotics used in the treatment of urinary tract infections. The early detection and reporting of suitable antibiotics can reduce the treatment failure in ESBL UTI [44].

An increasing number of endemic and epidemic outbreaks in paediatric wards have been reported. Especially common are *Klebsiella* infections causing septicaemia and meningitis in newborns in neonatal intensive care units. Since more and more of these outbreaks have been caused by multidrug-resistant strains, *Klebsiella* neonatal infections are becoming a major concern of the paediatrician. Especially peculiar has been the repeated frequent isolation of multidrug-resistant *Klebsiella* isolates. The incidence of ESBL-producing strains among clinical *Klebsiella* isolates has been steadily increasing over the past several years. Currently, the available data suggest a further increase in the incidence of ESBL-producing isolates. As a result, the therapeutic options are becoming limited, so that in the near future there will be an urgent need for hospital infection control measures that counter the spread of ESBL-producing bacteria [13].

Carbepenems are often the last line of effective treatment available for infections caused by MDR *Enterobacteriaceae* [47]. In the present study 17.37% isolates were resistant to Meropenem, which correlates with another study [48]. Meropenem is a substrate for the multidrug efflux system. Overexpression of this efflux system raises the MIC of Meropenem and other substrate antibiotics, but not Imipenem. In the presence of a β–Lactamase or reduced permeability from down regulation of a critical outer membrane protein, frank resistance to Meropenem can occur [49]. The incidence of carbapenem resistance in *E.coli* and K. *Pneumoniae* isolates reveals the most serious threat to the clinician. Increasing resistance to carbepenems and the event of superbug in Indian hospitals and community has now spurred the government and regulatory agencies to impose strict statutory guidelines for rational use of antibiotics [50]. We have used two phenotypic methods for detection of ESBL production. One screen and other PCDDT. Out of the total 679 isolates, 644 strains were ESBL positive by screening test. When these 644 ESBL screen positive isolates were subjected to PCDDT, 612 were confirmed to be ESBL producers (90.13%). This means that the screening test gives some (4.96%) false positives or there may be other mechanisms of resistance to 3GC. Put together overall the TEM+SHV primers (Set 1) are more effective in revealing the presence of the resistance gene as compared to bla_{TEM} + bla_{SHV} primer (Set 2). We found that multiplex PCR was successful in amplifying both the resistance genes targeted in this study. As the primer combination is effectively covering 100% of the resistant isolates which were tested in the amplification study, multiplexing can thus be efficiently used for diagnosing the resistant strains. However in institutes where PCR facilities are not available, PCDDT appears to be a good phenotypic confirmatory test for detecting ESBL production. Studies from other parts of the world reported that SHV gene was common in *Klebsiella pneumoniae* isolates [6]. A study from Tanzania showed SHV to be more prevalent than TEM [51], similarly in another study from Germany TEM was present in 100% isolates and SHV in 80%. [52]. Whereas studies from China [33] Korea [36] and India [12,53] reported TEM to be more prevalent than SHV.

Considering these findings, present study and other Indian studies

referred to above, it appears that TEM is more prevalent in Asia. Genetic studies can be helpful in providing explanations for unusual complex antibiograms, confirming specific resistance mechanisms and epidemiological studies in a geographical area.

Conclusion

Reporting of ESBL producing isolates from clinical samples is useful for the clinicians to select appropriate antibiotics for the treatment of ESBL producing strains and to take proper precaution to prevent the spread of these resistant organisms to other patients.

The routine susceptibility test done by clinical laboratories fail to detect ESBL positive strains and can erroneously detect isolates sometimes to be sensitive to any of the third generation cephalosporin's, hence a special phenotypic confirmatory test is indispensable for detecting ESBLs. PCDDT is a good phenotypic confirmatory test.

Thus the high level of ESBLs among *Klebsiella pneumonia* isolates is alarming and warrants special attention from clinicians and Microbiologists. We as Microbiologists should readily identify these isolates, so that proper therapy can be instituted to avoid misuse or overuse of antibiotics.

Rapid diagnosis can be done by using the PCR amplification without the need for phenotypic characterization.

Multiplex PCR can be used as a rapid method to identify common genes (TEM and SHV) responsible for extended spectrum beta lactamase production in *Klebsiella pneumonia* isolated from different clinical samples. It will prove valuable for surveillance and for determining the line of treatment against drug resistant organisms, thus saving precious time and resources.

The TEM+SHV primers (Set: 1) were more effective in revealing the presence of the resistance gene as compared to bla_{TEM} + bla_{SHV} primer (Set: 2) combination.

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