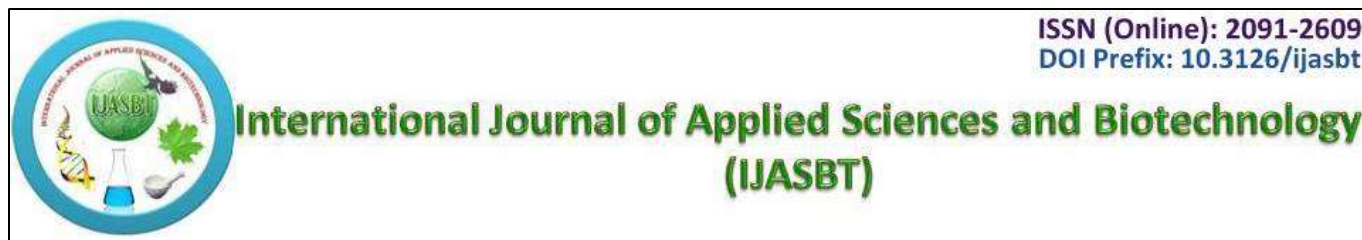

Research Article

Incidence of Multi Drug Resistance and Diversity of TEM-1 Beta Lactamase in Gram Negative Bacteria Isolated from Clinical and Environmental Sample

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Abstract

Occurrence and incidence of drug resistant bacteria are becoming very high and common with overuse of antibiotics. This study focused on the isolation of drug resistant strains from the clinical and environmental sample that produce extended spectrum β-lactamase (ESBL) and identification of TEM-1 genes in the plasmid and genomic DNA. 45 Clinical samples from the hospital and 7 environmental samples from the polluted water sources were collected. Among the collected samples, *E. coli* showed a higher incidence (36.1 %) followed by *Klebsiella* sp., *Staphylococcus* sp., *Streptococcus* sp., *Pseudomonas* sp., *Proteus* sp., and *Salmonella* sp. Antibiotic susceptibility of the isolates were investigated against 25 commercially available antibiotics. All the isolated strains showed MAR index value of more than 0.2. Among the 36 isolates, 7 Gram negative isolates (19.4 %) showed positive results for ESBL production in Double disk synergy test. The plasmid and genomic DNA were isolated and analyzed using PCR with specific primers for the presence of the TEM-1 gene (716 bp). A selected PCR products of the TEM-1 gene was sequenced and analyzed using BLAST.

Keywords: Multi drug resistance; β-lactamase, ESBL; TEM-1

Introduction

Drug resistant bacteria are emerging worldwide as a threat to common infections in community and hospital settings. The β-lactam antibiotics interfere with the biosynthesis of the peptidoglycan (Coyette *et al.,* 1994). Beta lactamase produced by several Gram negative and Gram positive bacteria are responsible for their resistance to β-lactam antibiotics such as penicillins, cephalosporins, cephamycins, etc. β-lactamases is one of the leading causes of resistance to β-lactam antibiotics in Gram negative bacteria (Sibhghatulla *et al.,* 2015). These enzymes inactivate the antibiotics by hydrolyzing the amide bond in the β-lactam ring.

β-lactamase can be chromosomal or plasmid encoded enzymes and produced in a constitutive or inducible

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manner. These enzymes secreted into the periplasmic space of Gram negative strains or into the outer membrane by their Gram positive bacteria. The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was reported in 1983 (Knothe *et al.,* 1983). The occurrence of mutation in these β-lactamase encoding genes results in the production of expanding the number of enzymes with a new substrate profile (Paterson and Bonomo, 2005).

In the past 15 years, the extensive and sometimes abusive clinical utilization of these drugs have been responsible for the development of an increasing number of resistant strains (Coyette *et al.*, 1994). In most cases, this be attributed to the production of new Extended Spectrum β-lactamase (ESBL) which are mediated by plasmids conferring resistance to cefotaxime, ceftazidime, and aztreonam. ESBLs belongs to the Class A β-lactamases that inactivates penicillin, oxyimino-cephalosporins, and monobactams (Mehrgan and Rahbar, 2008).

More than 150 different ESBLs have been described so far and the majority of these enzymes belongs to TEM and SHV families (Jacoby and Medeiros, 1991). Resistant organisms generally possess one of the two major enzyme types, the SHV enzymes (sulfahydryl variable) which originated from *Klebsiella* sp. and the TEM enzymes (isolated from a patient in Athens, Greece, named Temoneira) found in a variety of *Enterobacteriaceae* members.

These β-lactamases have been found in many genera of *Enterobacteriaceae* in particular *Klebsiella pneumoniae, Klebsiella oxytoca* and *E. coli* (Falagas and Karageorgopoulos, 2009). ESBL producing strains were isolated from abscesses, blood, catheter tips, lung, peritoneal fluid, sputum and throat swabs (Emery and Weymouth, 1997). Plasmid and transposon mediates the spread of TEM to other species of bacteria. There are now more than 90 TEM type β-lactamase and more than 25 SHV type enzymes. Both these groups of enzymes with a few point mutations at selected loci within the gene give rise to the extended spectrum phenotype. TEM-1 β -lactamase one of the leading cause of ampicillin resistance in 90% of *E. coli* (Livermore, 1995). These enzymes also found in non-*Enterobacteriaceae* gram-negative bacteria (Mugnier *et al.*, 1996).

The present investigation is focused to reveal the incidence of multi-drug resistant bacteria in clinical and environmental samples collected from Coimbatore, India and to study beta lactamase production and to analyze the diversity of TEM-1 using molecular markers.

Material and Methods

Collection of Samples

The clinical sample (n=45) and environmental samples (n=7) were collected for the isolation of *Enterobactericaea* members. Clinical samples (Pus, blood, urine, sputum, stool, and catheter) from the patient with chronic and acute illness was collected from the Kovai Medical Centre and Ganga hospitals, Coimbatore, India. The clinical isolates were collected based on the Gram's staining and patient's history maintained by the clinical laboratory. The isolates were collected in a sterile container which contain 3 mL of peptone water and transported immediately to the laboratory.

Polluted water (river and sewage water) was considered to be the best source for the isolation of enteric pathogens, thus polluted water samples were collected from the water sources around Coimbatore. The samples were stored at 4°C and processed within 24 h.

Processing of Samples

The water samples were filtered through a nitrocellulose membrane filter with a pore size 0.4 µm. After filtration, the membrane filter was inoculated into a sterile Luria Bertani broth to study the incidence of bacterial population (Mulamattathil *et al.,* 2014). The isolates from the clinical and environmental samples were inoculated to the selective agar medium specified by the HiMedia, India for the isolation of different bacterial genera. Morphologically different bacterial isolates were identified on the basis of a biochemical profile recommended by Bergey's manual of systemic bacteriology (1994). All the identified strains were stored in nutrient agar slants at 4°C.

Antibiotic Susceptibility

An antibiotic susceptibility test was carried out for the isolated strains by the disk diffusion method (Bauer *et al.,* 1996). Antibacterial susceptibility and resistance pattern were determined against commercially available 25 antibiotics. The diameter of the inhibition zones was measured and compared with the performance standard for antimicrobial disk susceptibility test CLSI 2013 and classified as resistant, intermediate and sensitive. The intermediate strains were also scored as resistant. Multiple Antibiotic Resistance Index (MAR Index) of the samples was calculated by the formula (Chitanand *et al.,* 2010).

MAR Index = y/nx.

Where, y= Total number of resistance scored; n = number of isolates; x = Total number of antibiotics tested.

Analysis of ESBLs Production

Production of diffusible ESBL enzymes onto the agar medium was determined by double disk synergy test (Kaur *et al*., 2013). ESBL detection test was based on the Kirby-Bauer disk diffusion test methodology. Disks containing 30 µg of aztreonam, ceftazidime, cefotaxime, and

cefpodoxime was placed 30 mm apart (center to center) around a disk containing amoxicillin (20 µg) and clavulanic acid (10 µg). Isolates exhibit an increase in zone diameter of 5 mm or greater around either of the clavulanate combined discs compared to that of the disc alone was considered as ESBL producer.

Isolation of Genomic DNA

10 mL of exponentially grown culture in Luria Bertani (LB) broth was harvested by centrifugation at 8000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended with 567 µL of TE buffer and 30 µL of 10% SDS and 3 µL of proteinase K (10 mg/mL) were added. The samples were properly mixed by vortexing and incubated for 1 h at 37 °C for complete lysis of cells. After incubation, 100 µL of 5 M NaCl and 80 µL of CTAB-NaCl was added and kept for incubation for 10 min at 65 °C in water bath. Equal volumes of chloroform-isoamyl alcohol (24:1) mixture was added and mixed well by inverting the tubes gently until the phases were completely mixed and centrifuged at 10,000 rpm for 10 min at 4 °C. The aqueous phase was collected in a fresh tube and equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, mixed and spun at 10,000 rpm for 10 min at 4°C. Again, upper clear aqueous phase was collected in fresh centrifuge and 2 µL RNase (10 mg/mL) was added followed by incubation at 37°C for 1 h. DNA was precipitated with 0.6 volumes of isopropanol and the precipitated DNA was centrifuged at 12,000 rpm for 5 in at 4°C. The DNA pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 2 min at 4°C. Finally, DNA was suspended in 50 µL of TE buffer and stored in ice cold conditions.

Isolation of Plasmid DNA

The plasmids of different strains were isolated from the over night incubated culture in Luria Bertani broth by the alkaline lysis method. 10 mL of culture was centrifuged at 6000 rpm for 10 min at 4°C; the pellet was collected and resuspension was made with 100 µL of ice cold alkaline solution I (50 mM Glucose, 25 mM Tris-Cl, and 10 mM EDTA) and vortexed. The bacterial cells were lysed with 200 µL of alkaline solution II (0.2 N NaOH and 1% SDS) and mixed gently. Then, the content was neutralized with 150 µL of alkaline solution III (5 M Potassium acetate and Glacial acetic acid) and stored on ice for 3-5 min. After incubation, the tube was centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was collected and equal volume phenol-chloroform was added and centrifuged for 2 min at 12000 rpm. The aqueous phase was collected, mixed with equal volume of ice cold absolute alcohol, vortexed and incubated for 2 min and centrifuged at 12000 rpm for 15 min at 4°C. The pellet was collected, washed with 70% ethanol and centrifuged at 12000 rpm for 2 min. The pellet was collected, resuspension was made with 50 µL of TE buffer, vortexed gently for few a seconds and stored.

PCR Assay of TEM-1 Gene

Unique primers were designed for the amplification of the TEM-1 gene. Primers were designed from the conserved regions and searched for their uniqueness and specificity to the respective gene by basic local alignment search tool (BLAST). A 716 bp fragment encoding TEM-1 was identified by using a primer set and the amplification was performed with forward primer, 5′-GACAGTTACCAATGCTTAATCA-3′ and reverse primer, 5′-TTGGGTGCACGAGTGGGTTA-3′ (Sigma Aldrich Chemicals Pvt. Ltd., Banglore).

The DNA amplification was performed using a thermal cycler (MJ Research, Model PTC 100 Watertown, Mass., USA). The PCR reaction was performed in 15 µL reaction volume containing 2 µL of template DNA (20 ng μL^{-1}), 1 µL (10 pmol) each of forwarding primer and reverse primer, 7.5 µl µL of PCR master mix (Fermentas, USA) and 4.5 µL of nuclease-free water. The reaction conditions for TEM-1 genes were as follows: initial denaturation at 94°C for 5 min, 30 cycles of amplification, each consisting of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min.

The PCR products (10 µL) were electrophoresed in 1.5% agarose gel (low EEO grade, HiMedia, India) for 2 h at 50V in the Tris-acetate-EDTA buffer (pH 8.3; 40 mM Tris, 20 mM acetate, 1 mM EDTA). The sizes of the amplified products were determined by comparison with 500 bp DNA ladder (Amersham Biosciences Corp., Piscataway, N.J.). Following electrophoresis, gels were stained with ethidium bromide and visualized under a UV (245 nm) trans-illuminator (BioRad, USA). The sizes of the amplified products were determined by comparison with the DNA ladder.

Sequencing and Analysis of TEM-1

Amplified products under optimum conditions with expected size were purified using purification kit (Qiagen, India). The amplified PCR products of TEM-1 and SHV-1 were sequenced using random dye termination sequencing method (ABI3130 Genetic Analyser, USA). The sequence was analyzed and aligned by using the basic local alignment tool (BLAST).

Result

Incidence of Bacterial Species

The incidence of bacterial genera in the sample was studied with main focus on Gram negative organisms belonging to the family *Enterobacteriaceae*. A total of 52 samples (7 environmental and 45 clinical samples) was analyzed and the bacterial genera onto their respective selective medium showed good growth. Identification of isolates were done following their morphology in Gram's staining, cultural characteristics, and biochemical properties, as per the Bergey's manual of systemic bacteriology (1994). Among

the collected samples, *E. coli* showed a higher incidence (36.1%) followed by *Klebsiella* sp., *Staphylococcus* sp., *Streptococcus* sp., *Pseudomonas* sp., *Proteus* sp., and *Salmonella* sp. (Fig 1).

Antibiotic Susceptibility

Among the 63 isolates, 36 isolates of Gram negative bacteria were selected randomly for the analysis of antibacterial susceptibility and resistance pattern against commercially available 25 antibiotics. The study showed the existence of multiple antibiotic resistant strains. It was observed that a significant number of strains showed resistance against all the antibiotics (Fig. 2).

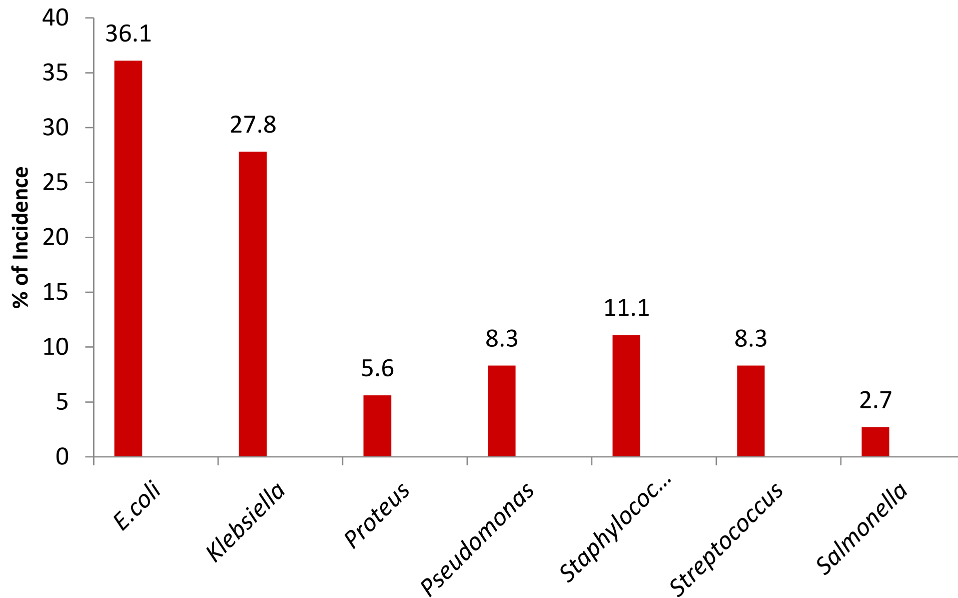

Fig. 1: Percentage incidence of bacterial isolates

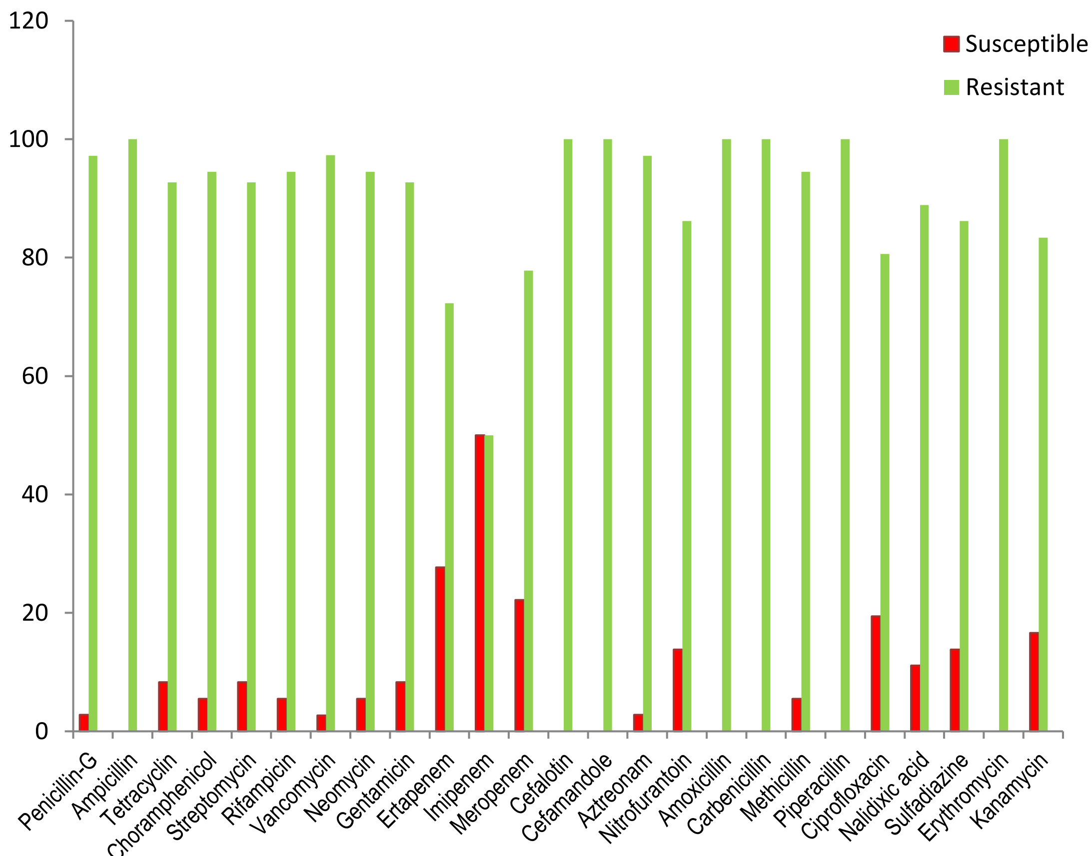

Fig. 2: Percentage of antibiotic resistance and susceptibility of bacterial species.

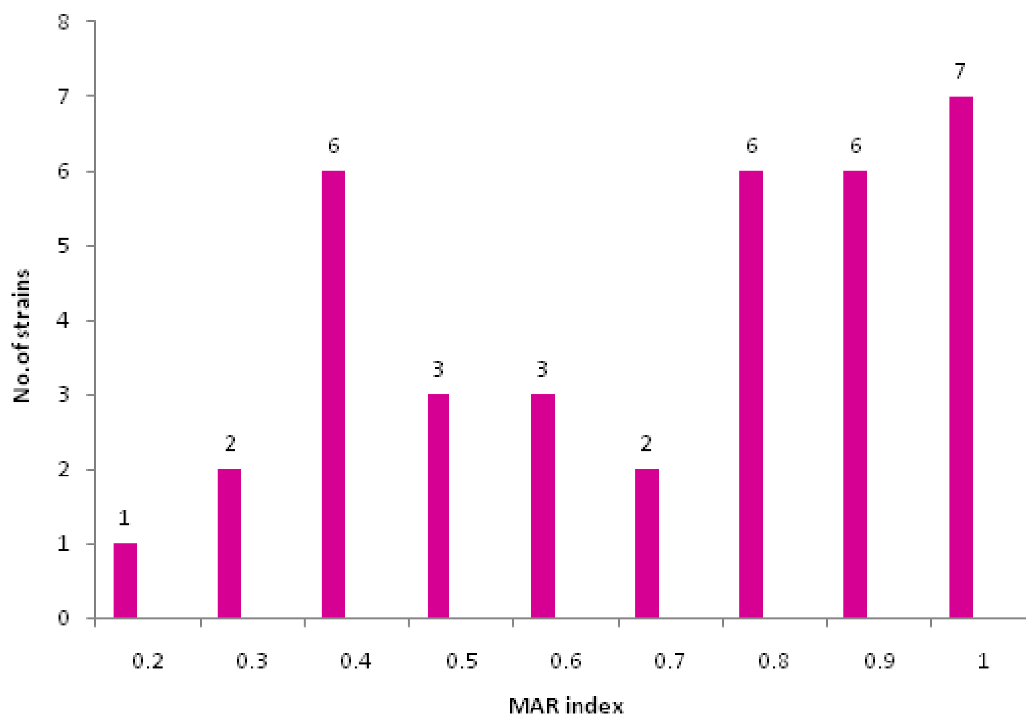

Fig. 3: MAR index of clinical and environment isolates

MAR index was calculated and it was also noticed that none of the strains possessed identical antibiotic resistance pattern. In the present study, it was observed that all the strains isolated from different clinical and environmental showed MAR index value of more than 0.2 that clearly indicates that all the strains might have originated from a high risk source of contamination. The MAR index value of the isolates was ranging from 0.2 to 1 (Fig.3).

Analysis of ESBL Production

All the 36 isolates were subjected to ESBL detection with double disk synergy test, where disks of ceftazidime, cephotaxime, aztreonam, and cefpodoxime was placed to center from a disk of amoxyclav. The plates were incubated at 37°C for 24 h and checked for the keyhole phenomenon. Among the 36 isolates, 7 isolates (19.4%) showed the keyhole phenomenon and 29 (80.5%) did not show the phenomenon.

PCR Assay of TEM-1 Gene

The TEM distribution was analyzed by PCR amplification by using specific primer by selecting 7 effective strains. The PCR product from the plasmid DNA of 5 strains produced bands corresponding to the expected size of TEM-1 was 716 bp. Among the 5 strains, 3 were *E. coli* and others were *Klebsiella* sp. (Fig. 4). The genomic DNA of 3 strains also showed the amplified product with 716 bp fragments, one among the three was *Pseudomonas* sp. and others were *E.coli* (Fig. 5).

Fig. 4: PCR Amplification of TEM-1 gene in plasmid DNA. Lane 1- 500 bp Ladder, lane 2- *Proteus* sp., lane 3 & 4 - *Klebsiella* sp. lane 5- *Pseudomonas* sp. and lane 6 to 8- *E.coli.*

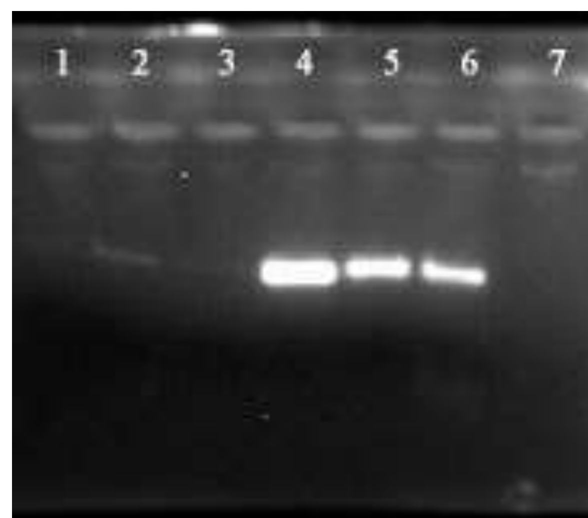

Fig. 4: PCR Amplification of TEM-1 gene in genomic DNA. Lane 1- *Proteus* sp., lane 2 & 3 - *Klebsiella* sp. lane 4- *Pseudomonas* sp. and lane 5 to 7- *E.coli.*

Table 1: Sequence analysis of TEM-1 using standard nucleotide BLAST

Isolate Number	Identity	(%) Similarity	Accession
E. coli isolate 1	*Escherichia coli* EV25	91%	NG_050251.1
E. coli isolate 5	Synthetic construct clone 85844BC03	96%	KY715697.1
Klebsiella isolate 2	*Klebsiella pneumoniae* strain AR 0120	89%	CP021835.1
E.coli isolate 3 *Klebsiella* isolate 5,		No significant similarity found	

Sequence Analysis of TEM-1

A selected PCR product of the TEM-1 gene was subjected to sequencing analysis. The resulted sequences were varied in their size with the maximum of 241 bp and a minimum of 157 bp. The partial sequence obtained was analyzed using bioinformatics tools, and the result was interpreted.

TEM-1 *E. coli* isolate 1, Partial sequence (F), 178bp, Plasmid DNA as template

GGTAAACCTAATCTTAGCTATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGAATTAA
AAAAACGATACGGGAGGGCTTACCATCTGGTTCC
AGTGCTGCAATGATACCCAATCATTGCTCTCTGTA
ATCTTTCAAGCAAAAGAATTGATTTCTTCTCTCAA
AAAA

TEM-1 *E. coli* isolate 2, Partial sequence (F), 186bp, Plasmid DNA as template

GTGAGTTTACGACATTTTTTCTCCCTCGCGCGCGC
GGAGTGTGAATACAACATTATTATATATTAGGGG
AAAAAAGGGGGGGTTTTTTTAGTTCATAATTTTTT
TAAAAAAGAGACCCTACTATTATTGTGTTGCTTTT
AGTGACACACATTGGAGCCCTTATTGCAAAAATT
ATTTTTAGGTTCG

TEM-1 *E. coli* isolate 5, Partial sequence (F), 211bp, Plasmid DNA as template

AGATGTCGCACTACGCGGTAGATCCTTGAGAGTT
TTCGCCCCGAATAACGTTTTCCTATGAAAAGAAA
ATGTGTTTAAAGGTGTCTTTATTTCCCTTTTAAAA
GGGGGCAGTAATAAATGTGTTGCTTATAGGGACA
GACATTCATCAAGTGTGACATTGGTGTCGGGCGT
TCAACTAAGTAGAAGCTTTTTGTTTATTTCCGCCC
CCACA

TEM-1 *Klebsiella* isolate 2, Partial sequence (F), 241bp, Plasmid DNA as template

TTTTAAAGTTAAACTTTCTCGCGATCTGTCTATTT
CGTTCATCCATAGTTGCCTGACTCCCCGTCGTGCC
TTATAACTACGATACGGGAGGGCTTACCATCTGG
CCCCAGTGCTGCATTGATACCGCTTGATCTATTCT
CACAGACTGCCGATTTATCAGAAACCATATTGGC
CTTCAGGAAGCCTGCTTGTTTATCTTAAAAGGGTC
TGTTTTTAAAACCTCACTTCTTGCATCCGTCGC

TEM-1 *Klebsiella* isolate 5, Partial sequence (F), 157bp, Plasmid DNA as template

GAGCTATTGCTTGTCCCTCCCACCGACGCGGACG
GTGTGAGAAGACACATGTGTATTCTGGGGAAAAA
AAAGAGGGGTCGTTTGTATGTCTTAGTGCGTTTTT
CTAGAGAGGGTCGGTGCAAATGTATGGGTGTTTC
GTGGTCGACGTGGTGGCGAC

Discussion

Over the last decade, several studies assessed the occurrence of ESBLs among *Enterobacteriaceae* recovered from hospital patients. In the present study, ESBL producers were found to be present in polluted water bodies also, which reveal the transfer of their resistance to other coliforms. The incidence of ESBL producing microorganisms was more prevalent in the ICU patients (Pena *et al.,* 1997). In contrast, a wider investigation revealed that the overall prevalence of ESBL producing *Enterobacteriacea* was 3.2% (De Champs *et al.,* 2000). These results were in agreement with Japanese data showing that ESBLs were expressed by less than 10% of isolates of *E. coli* and *Klebsiella* sp. (Lewis *et al.,* 1999).

MAR index is a tool that reveals the spread of resistant bacteria in a given population. A MAR index value greater than 0.2 implies that the strains of such bacteria originated from an environment where several antibiotics are used such as poultry, cattle, fishery and from human beings. MAR index value for all the isolates were greater than 0.2, indicating that all the bacteria isolates have been exposed to several antibiotics or it has originated from a high risk source of contamination. Resistance to the antibiotics among the isolates differs, revealing that the isolates were exposed to various antibiotics.

The confirmatory test for the identification of the ESBLs producers were done with the double disc synergy test. Sensitivity may be reduced when ESBL activity is very low, leading to wide zones of inhibition around the cephalosporin and aztreonam disks and it has been noted for *Proteus* (Paterson, 2005). A higher prevalence of ESBL

production was observed in *E. coli* and *K. Pneumoniae* (Aminzadeh *et al.,* 2008).

The prevalence of resistance gene in commensal *E.coli* is a useful indicator of antibiotic resistance in bacteria. The plasmids were isolated and analyzed for the presence of the TEM-1 genes. Among the 7, 5 isolates of the gram negative bacteria have shown the positive results which were isolated from the hospital environment sources. Three of the *E. coli* strains and two of the *Klebsiella we*re shown the positive result. This confirms the presence of the plasmid mediated TEM-1 genes in the isolates and indicates a high risk of transfer of these plasmids to other microorganisms.

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