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Phenotypic and genotypic screening of multi-drug resistant ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* from burn wound infection

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Key words: Burn Wound Infection, Bacterial Pathogens, Multi-drug Resistance, MAR Index, ESBL.

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Abstract

As burn cases are very frequent in Bangladesh, the aim of the present study was to screen the multidrug resistant extended-spectrum-β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* from burn wound infection. *E. coli* and *K. pneumoniae* were isolated on MacConkey agar and Eosin Methylene Blue (EMB) agar and identified by their colony characteristics and biochemical tests. Antimicrobial susceptibility of the isolates was performed by Kirby-Bauer disc diffusion method**.** After phenotypic ESBL confirmation, isolates were checked for the presence of ESBL genes by polymerase chain reaction (PCR). Among the isolates, *K. pneumoniae* (62.5%, n = 10) was highly prevalent one followed by *E. coli* (37.5%, n = 6). *E. coli* were sensitive to nitrofurantoin (66.67%) while *K. pneumoniae* were sensitive to amikacin (40%) and gentamicin (40%). Both of the isolates showed complete resistance (100%) to cefotaxime (30 µg), ceftazidime (30 µg) and cefoxitin (30 µg). MAR index was in the range of 0.69-1.0 for all the isolates except, one. All the isolates (100%, n = 16) were phenotypically positive for ESBL production. In PCR analysis, dominant ESBL class was found as bla_{TEM} (87.5%, n = 14) followed by bla_{SHV} (37.5%, n = 6). The co-existence of bla_{TEM} and bla_{SHV} in *K. pneumoniae* was also observed (50%, n = 5). However, none of the *E. coli* isolates harbored bla_{SHV} gene. The findings of the present study showed that all the isolates were multidrug resistant and ESBL positive, which might be a serious threat to the clinical management of burn wound infection.

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Introduction

Burns are a significant health issue in case of morbidity, long-term disability and mortality throughout the world; especially in developing countries like Bangladesh (Ekrami and Kalantar, 2007). Though treatment procedures are developing all over the world, wound colonization still is one of the major causes of infection in burn intensive care units (Santucci *et al.*, 2003). Thermally injured damaged skin may act as favorable site of infection by opportunistic nosocomial pathogens from hospital environment due to insufficient disinfection of hospital surfaces, instruments and rooms (Abreu *et al.*, 2013; Taneja *et al.*, 2013). As the burned tissue becomes avascularized, host defense system and systematically administered antibiotics fail to act against the pathogens (Church *et al.,* 2006). Additionally, burn patients are always at highest risk zone because of the burn injury itself, immune system depression (humoral and cellular), high cutaneous bacterial load, possibility of gastrointestinal bacterial translocation and other intensive diagnostic and therapeutic procedures (Bowen-Jones *et al.*, 1990; Jones *et al.*, 1990; Lari and Alaghehbandan, 2000).

The pathogens responsible for causing infection in burn wound vary depending on place and time (Pruitt *et al.*, 1998). Furthermore, multidrug resistance traits of these pathogens has become a global health issue, resulted in more complicated infections, prolonged hospital stay and increased mortality (WHO, 2014). The recent 'Review on Antimicrobial Resistance' reported that 10 million people will die per year worldwide due to superbug infections by 2050 (O'Neil, 2014). Though bacteria use various kinds of antibiotic resistance mechanisms, ESBLs are one of the main leading causes of beta-lactam antibiotic resistance among Gram-negative bacteria (Rawat and Nair, 2010). ESBLs are plasmid-encoded β-lactamases conferring resistance to penicillins and first, second and third generation cephalosporins e.g., cefotaxime, ceftriaxone, and ceftazidime (Rawat and Nair, 2010; Paterson and Bonomo, 2005). Several risk factors are responsible for the colonization of ESBL producer such as antibiotic administration, prolonged ICU stay, severe illness, catheterization, or instrumentation etc. (Chaudhary and Aggarwal, 2004). bla_{TEM}, bla_{SHV}, and bla_{CTX-M} are the major ESBL genes harbored in clinically important Gram-negative bacteria (Paterson and Bonomo, 2005; Bradford, 2001). These genes are commonly found in *Klebsiella pneumoniae* and *Escherichia coli,* but are also reported recently in other genera of Enterobacteriaceae family (Kaur and Aggarwal, 2013; Adeyankinnu *et al.*, 2014). bla_{TEM} is the most common plasmid-mediated β-lactamase of Gram-negative bacilli e.g., *E. coli*, whereas bla_{SHV} is produced mostly by *K. pneumoniae* (Livermore, 1995). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby, 1997). Appropriate detection of β-lactamases is highly required for antibiotic treatment, infection control purposes, and epidemiological investigations (Finch *et al.,* 2012).

Due to the geographical variation of microbial prevalence and ESBL genes distribution, current investigation was carried out to isolate *E. coli* and *K. pneumoniae* from burn wound site and to determine the antibiotic resistance pattern of the isolates by antibiotic susceptibility test and PCR analysis of common ESBL genes *viz.* bla_{TEM} and bla_{SHV} gene.

Materials and method

Study area

Samples were collected from the burn unit of a tertiary care hospital of Chittagong city. Thirty (30) burn wound patients admitted at in-door and out-door department of burn unit at any age and of both sexes were included in this study. Bacterial isolation was carried out at Department of Microbiology of University of Chittagong whereas bacterial characterization and antibiogram profiling was done at Department of Microbiology of Jagannath University, Bangladesh. PCR analysis was carried out at the Molecular Biotechnology Division of National Institute of Biotechnology, Dhaka, Bangladesh.

Sampling

Swab samples were collected aseptically by sterile

cotton swabs. At the time of swab collection, proper care was taken for avoiding contamination with other commensal microorganisms or other external sources. Surface swabs were collected from burn wound after the removal of dressings and cleaning of the remnant topical antimicrobial agents[5]. Swabs were moistened in 4 ml sterile normal saline and rubbed onto the burn wound surface. Then, the tubes were plugged carefully, labeled and transferred to the microbiological laboratory as early as possible for bacteriological isolation, identification and ESBL gene detection.

Microbiological analysis and biochemical characterization

Samples were collected twice from each patient; one was used for gram staining and another for culturing on selective media (Collee *et al.*, 2015). Each sample was cultured on MacConkey (MAC) agar and Eosine Methylene Blue (EMB) agar and incubated at 37°C for 24 hours. The particular microorganisms were isolated and identified according to standard protocols (Forbes *et al.*, 1998). Many biochemical tests were also performed to identify the isolated bacteria from the wound sample e.g., indole test, methyl-red (MR) test, Voges-Proskeur (VP) test, citrate utilization, catalase, oxidase test, motility etc (Cappuccino and Sherman, 1996).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of the isolates was performed by Kirby–Bauer disc diffusion method on Mueller–Hinton agar (Bauer *et al.*, 1966). The following antibiotics were used: amoxyclav (30µg), cefotaxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), colistin (10µg), cefoxitin (30µg), cephradine (30µg), ciprofloxacin (5µg), nalidixic acid (30µg), gentamicin (10µg), amikacin (30µg), streptomycin (10µg), co-trimoxazole (25µg), meropenem (10µg), azithromycin (30µg) and nitrofurantoin (300µg) (Oxoid; UK). The agar plates were uniformly swabbed with sterile cotton swabs and antibiotic discs were placed on the agar surface. After an overnight incubation of the inoculated MHA plate at 37°C, the zone of inhibition was measured and interpreted by comparing with the CLSI chart (CLSI, 2011). Control strains (*K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922) were used to monitor quality of antibiotic discs during antimicrobial susceptibility testing.

Phenotypic screening of ESBL producers

In primary ESBL identification test, the isolates were tested against ceftazidime (30 µg) and cefotaxime (30 µg) by Kirby-Bauer disc diffusion method. If the zone of inhibition was <22 mm for ceftazidime and <27 mm for cefotaxime, it was considered as ESBL producer (CLSI, 2017).

Genotypic screening of ESBL genes by PCR

Genomic DNA from the selected bacterial isolates were prepared by 'boiling method'. Briefly, the isolates were cultured on nutrient agar plate at 37°C for 24 hours. After incubation, three to five freshly cultured colonies were taken into a sterile centrifuge tube containing 100 µl of sterile-distilled water and vortexed properly. Then, the tubes were heated at 100 °C for 10 min in a heating block and centrifuged at 10,000 rpm for 10 min to collect the supernatant. The purity of the extracted DNA was checked by a nano-drop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and stored at -20°C until further use.

PCR detection of ESBL genes viz. *bla*$_{TEM}$ and *bla*$_{SHV}$ were carried out in a Veriti™ thermal cycler (Applied Biosystems, USA) using previously designed degenerate primer (Colom *et al.*, 2003). The primer sequence of *bla*$_{TEM}$ gene (product size - 516 bp) are TEM-C (5'-ATCAGCAATAAACCAGC-3') & TEM-H (5'-CCCCGAAGAACGTTTTC-3') whereas *bla*$_{SHV}$ gene (product size - 392 bp) are SHV-F (5'-AGGATTGACTGCCTTTTTG-3) & SHV-R (5'-ATTTGCTGATTTCGCTCG-3'). The PCR reaction was carried out in a total reaction volume of 25 µl containing 2×GoTaq® G2 Green Master Mix (Promega, USA), 10 pmol of each forward and reverse primer, 50 ng of template DNA and 9.5 µL of nuclease-free water. The PCR condition includes: initial denaturation at 95°C for 5 min, followed by 35

cycles consisting of denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 1 min and a final extension of 72°C for 10 min.

The PCR products along with 100 bp DNA size marker (Invitrogen, USA) were separated on 1% agarose gel in TAE buffer stained with ethidium bromide (0.5 mg/mL) and visualized under UV light in an Axygen™ Gel documentation system (Fischer Scientific, USA).

Multiple antibiotic resistance (MAR) index determination

The MAR index was calculated according to following formula-

$$\text{MAR INDEX} = \frac{\text{No of antibiotics against which organisms show resistance}}{\text{Total number of antibiotics used}}$$

MAR index values greater than 0.2 indicate high risk source of contamination where antibiotics are often used (Krumpernam, 1983).

Statistical analysis

Descriptive statistical analysis of the data was performed using Microsoft Excel 2013 for Windows.

Results and discussion

Superbug infection causes the most devastating effect to the burn wound patients. Geographical location, time and life styles significantly affect the prevalence of the infecting pathogens as well their antimicrobial susceptibility patterns. So, it is highly needed to continuously monitor the drug resistance traits of the bugs. The β-lactam ring hydrolytic enzyme, e.g, β-lactamase enzymes is one of the most common reasons of causing resistance to β-lactam antibiotics.

Failure in detection of these enzymes has led to therapeutic failure (Shiju *et al.*, 2010). Hence, this study aimed at the detection of ESBL producing MDR *E. coli* and *K. pneumoniae* from the burn wound infection.

Table 1. Morphological and biochemical characterization of the bacterial isolates.

Observation	*E. coli*	*K. pneumoniae*
Gram reaction	Negative	Negative
Cell Shape	Short rod	Long rod
Indole test	Positive	Negative
MR test	Positive	Positive
VP Test	Negative	Negative
Citrate Utilization	Negative	Positive
H_2S production	Negative	Negative
Motility test	Positive	Negative
Catalase test	Positive	Positive
Oxidase test	Negative	Negative

Table 2. Phenotypic and genotypic screening of ESBL producing *E. coli* and *K. pneumoniae*.

Organisms	Phenotypic Screening (% / n)	Genotypic Screening (% / n)		
		bla_{TEM}	bla_{SHV}	bla_{TEM} + bla_{SHV}
E. coli (n = 6)	100% (6)	66.67%(4)	ND	ND
K. pneumoniae (n = 10)	100% (10)	100% (10)	60% (6)	50% (5)

*ND= Not Detected.

Bacterial Prevalence in burn wound infection

E. coli produced dark colony with green metallic sheen on EMB agar whereas *K. pneumoniae* formed large pink and mucoid colony on MAC agar. The microscopic and biochemical test results were included in Table 1. In the present study, *K. pneumoniae* accounted for the highest percentage (62.5%) followed by *E. coli* (37.5%) (Fig. 1).

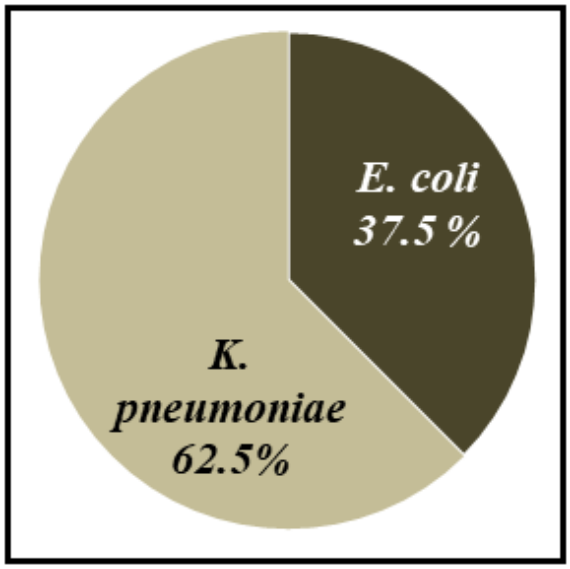

Fig. 1. Prevalence of *E. coli* and *K. pneumoniae* among burn wound infection cases.

Several studies claimed that *E. coli* and *K. pneumoniae* (also have been identified in the current study) are the most predominant pathogens associated with burn infection (Kavanagh, 1975; Chim *et al.*, 2007; Bayram *et al.*, 2013). A study of Bangladesh reported *Staphylococcus aureus* as the most prevalent bacteria (55.57%) followed by *E. coli* (23.7%) and *Pseudomonas* sp. (8.2%) (Roy *et al.*, 2017). Another Bangladeshi study reported maximum proliferation in burn wound with *P. aeruginosa* followed by *S. aureus* (80%) and *Klebsiella* sp. (50%) (Alam *et al.*, 2014).

Antimicrobial susceptibility pattern of bacterial isolates

Antimicrobial susceptibility of the isolates was determined by measuring the zone of inhibition on MHA agar plate according to CLSI guidelines (CLSI, 2011) as shown in Fig. 2.

Among 16 antibiotics, *E. coli* showed 100% resistance to cefotaxime, ceftriaxone, nalidixic acid, cephradine, ceftazidime, cefoxitin, ciprofloxacin and amoxyclav. Previously, 89.9% resistance was recorded against cephradine which was followed by co-trimoxazole (69.7%) and cefotaxime (64.2%) in a study of Bangladesh conducted in 2012 (Islam *et al.*, 2012).

Fig. 2. Antimicrobial susceptibility test by disc diffusion method.

According to Roy *et al.* (2017), the sensitivity towards cephradine, ceftriaxone, ciprofloxacin, azithromycin, cefotaxime, gentamicin and ceftazidime was 43.5%, 91.3%, 34.8%, 91.3%, 86.9%, 82.6% and 78.3% respectively. The increasing resistance towards these commonly prescribed antibiotics is limiting the scope of treatment. Highest sensitivity of *E. coli* was recorded against nitrofurantoin (66.67%). Similarly, it was 78.3% sensitivity in a previous study indicating its decreasing resistance traits day by day (Roy *et al.*, 2017) (Fig. 3).

In case of *K. pneumoniae* isolates, cefotaxime, ceftazidime, colistin and cefoxitin accounted the highest resistance (100%). This is in accordance with a Bangladeshi study of ESBL producing *Klebsiella* sp.

exhibiting 100% resistance against the antibiotics (Islam *et al.*, 2012). The highest sensitivity (40%) showed towards amikacin and gentamicin which is also decreasing as it was documented 77.77% and 55.55% for the respective antibiotics (Sultana *et al.*, 2015). Only 10% of *K. pneumoniae* was sensitive to nalidixic acid, cephradine and ceftriaxone. Previously, it was reported 45% and 34.9% for nalidixic acid and ceftriaxone respectively (Islam *et al.,* 2012) (Fig. 4).

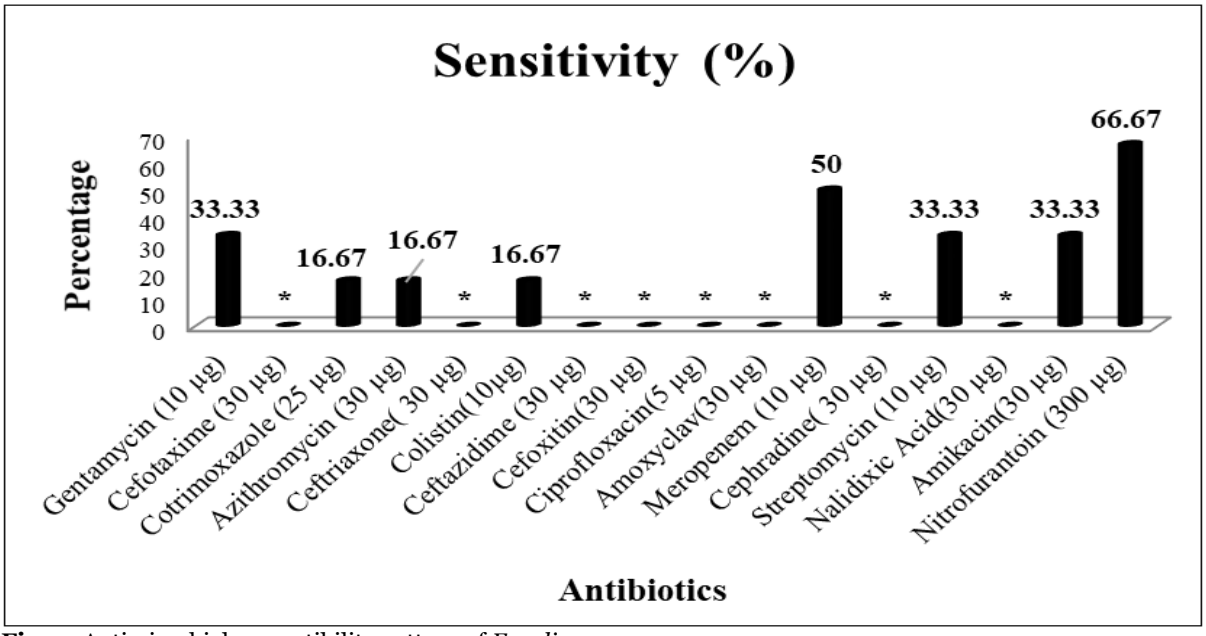

Fig. 3. Antimicrobial susceptibility pattern of *E. coli.*

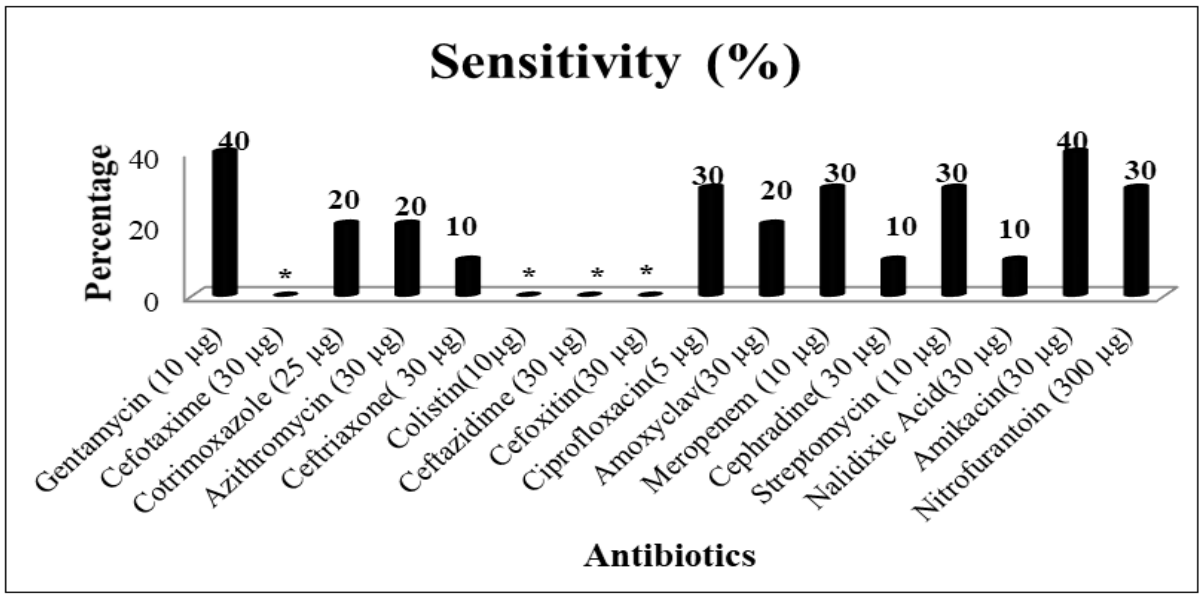

Fig. 4. Antimicrobial susceptibility pattern of *K. pneumoniae.*

Phenotypic and Genotypic detection of ESBL producers

All the isolates (n = 16) of *E. coli* and *K. pneumoniae* isolates showed positive results in initial screening of ESBL production as their inhibition zone was ≤22 mm for ceftazidime and ≤27 mm for cefotaxime. The isolates were then subjected to PCR for ESBL genes *viz.* bla_{TEM} and bla_{SHV} detection (Fig. 5 & 6). In both organisms, bla_{TEM} was found as the most prevalent ESBL gene (87.5%, n = 14) as shown in Table 2. The prevalence of bla_{TEM} was 66.67% (n = 4) and 100% (n = 10) for *E. coli* and *K. pneumoniae,* respectively.

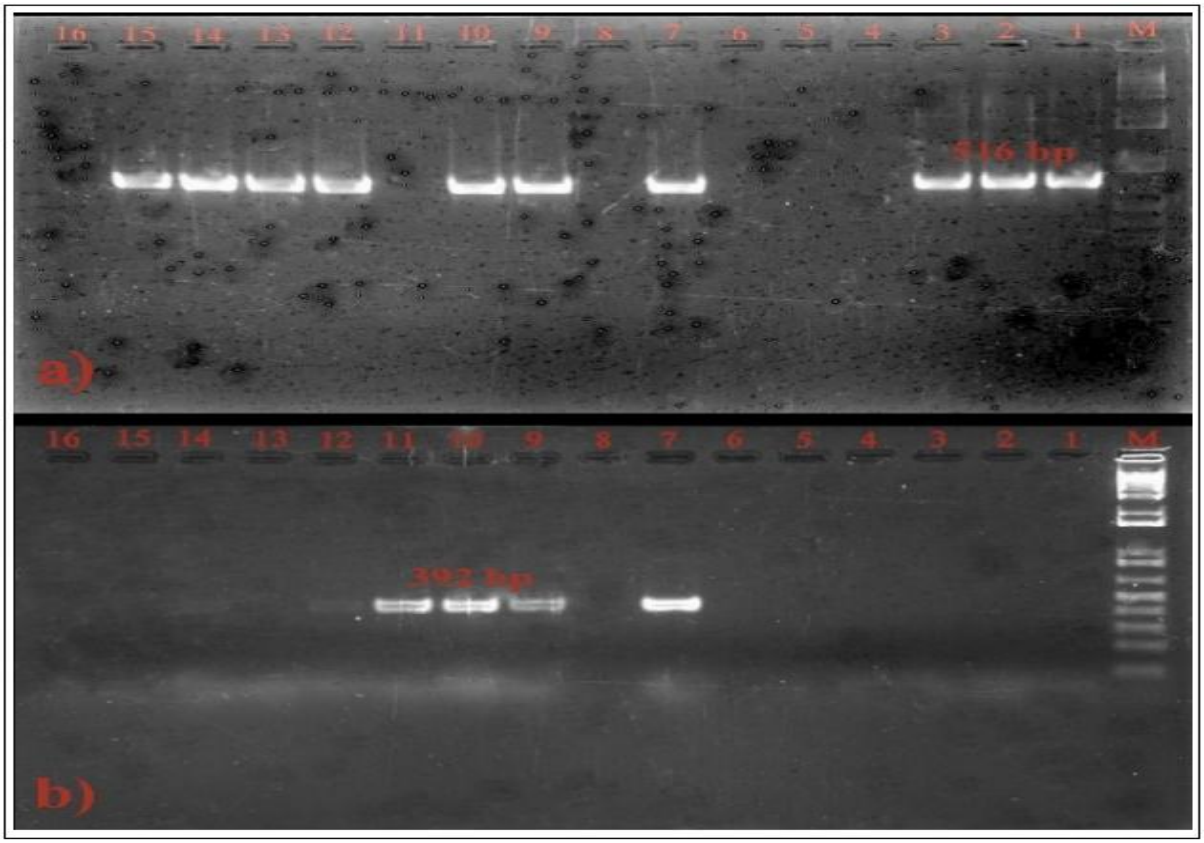

Fig. 5. 1% Agarose gel electrophoresis of PCR product of a) *bla*$_{TEM}$ and b) *bla*$_{SHV}$ genes of *Klebsiella pneumoniae* isolates. Lane M: 100 bp DNA Marker, Lane 1-16: *K. pneumoniae* isolates.

Several studies reported the predominance of *bla*$_{TEM}$ gene over *bla*$_{SHV}$ gene in ESBL producing *E. coli* than *K. pneumoniae* (Sultana *et al.*, 2016; Khaleque *et al.*, 2017). *bla*$_{SHV}$ gene was detected only in *K. pneumoniae* (60%, n = 6) while *E. coli* didn't possess the respective gene.

Another study carried out in Bangladesh reported the prevalence of *bla*$_{SHV}$ gene in *K. pneumoniae* rather than *E. coli* (Khan *et al.*, 2018). Co-existence of *bla*$_{TEM}$ and *bla*$_{SHV}$ (50%, n = 5) genes were also observed in *K. pneumoniae*. Simultaneous presence of both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were also reported in a previous study (Karim *et al.*, 2017).

MAR Indexing and its relation to ESBL gene prevalence

According to our findings, most isolates had MAR indices of >2.0, indicating overuse and irrational antibiotic use in the particular source/environment. However, the practical significance of such indexing in a developing country like Bangladesh may be very useful where sometimes all the antibiotics fail to give proper treatment.

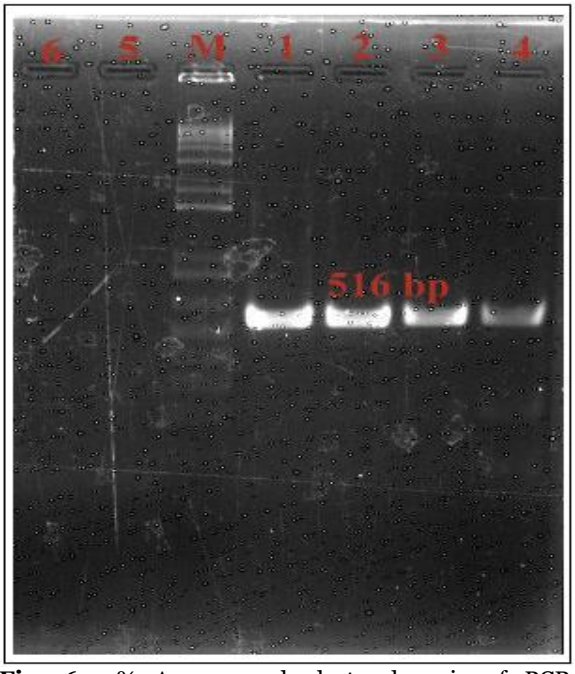

Fig. 6. 1% Agarose gel electrophoresis of PCR products of *bla*$_{TEM}$ gene of *Escherichia coli* isolates. Lane M: 100 bp DNA Marker, Lane 1-6: *E. coli* isolates.

In our study, MAR indices of all the *E. coli* and *K. pneumoniae* isolates were between 0.69-1.0 except 0.25 for one *K. pneumoniae* isolate. Two of *E. coli* (33.33%) and four of *K. pneumoniae* (40%) had 1.0 MAR index i.e., the isolates were resistant to all of the antibiotics used. The report is giving a strong indication to all of us that this ever-increasing drug resistance may restrict our treatment regime associated with burn wound infection in the near future (Fig. 7 and 8).

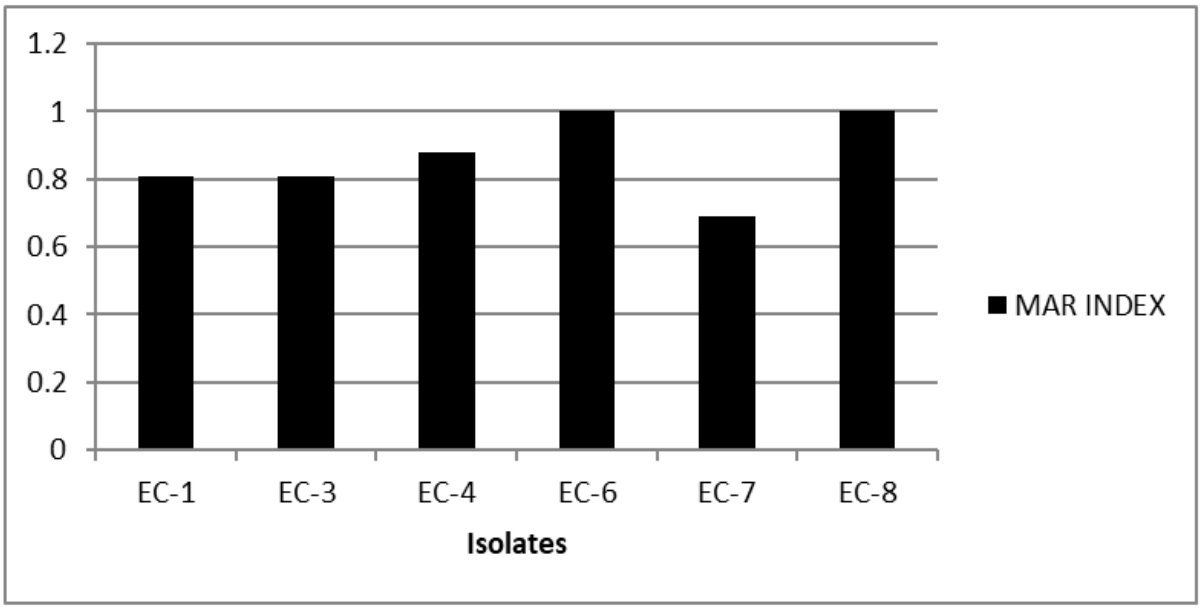

Fig. 7. Multiple Antibiotic Resistance (MAR) Index of *E. coli.*

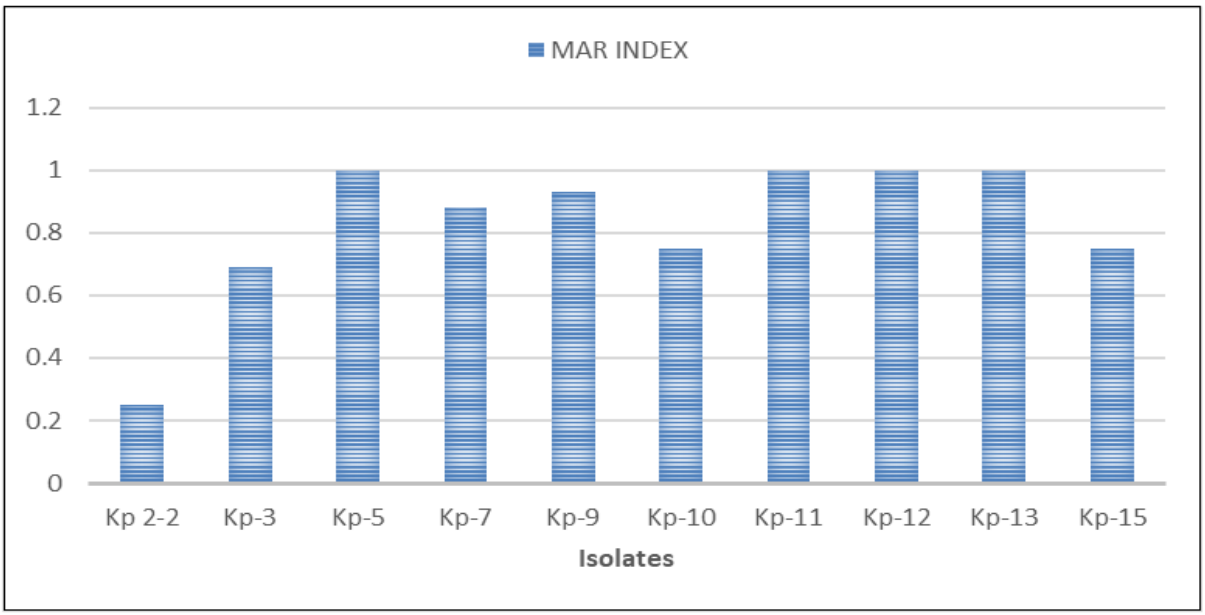

Fig. 8. Multiple Antibiotic Resistance (MAR) Index of *K. pneumoniae.*

The present study also correlates the presence of ESBL genes and MAR indexing of the multiple-drug resistant pathogens. It was observed that the organisms having highest MAR index possessed the ESBL genes, which means multi-drug resistant organisms were positive for ESBL genes. Among all the ESBL positive isolates, two of *E. coli* and three of *K. pneumoniae* isolates were resistant to all of the antibiotics used (MAR index=1.0).

Conclusion

In the present study, the most prevalent bacteria was

K. pneumoniae followed by *E. coli*. *K. pneumoniae* were sensitive to amikacin and gentamicin as well as *E. coli* showed sensitivity to nitrofurantoin. Among the two ESBL genes, bla_{TEM} gene was the most predominant gene, found in both *E. coli* and *K. pneumoniae* isolates. Co-existence of bla_{TEM} and bla_{SHV} was detected in *K. pneumoniae* but bla_{SHV} gene was not detected in *E. coli*.

The MAR indices of all the isolates were indicating their increasing drug resistance traits. Hence, public awareness campaign should be set up to spread knowledge about the fatal consequences of MDR and adequate hospital hygiene is essential for a sound management of burn cases.

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Conflict of interest

The authors declare that they have no conflict of interests.

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