Resistant Genes blaCTX-M, blaTEM and blaSHV encoding ESBL in surgical site infection causing *Escherichia coli and Klebsiella pneumoniae*- A report from a tertiary care hospital

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Abstract

Introduction: Surgical site Infections (SSI) rank third among hospital acquired infections worldwide. According to the WHO report of 2016, the global burden of SSI ranges from 2.5% to 41.5%. ESBL producing *E.coli* and *Klebsiella pneumoniae* are frequently being isolated from these infections owing to the high transmission density of plasmid-mediated ESBLs in hospital setting.

Aim: To survey for antimicrobial resistance encoding genes of ESBL (bla_{TEM}, bla_{CTX-M} and bla_{SHV}) among *Escherichia coli* and *Klebsiella pneumoniae* isolated from surgical site infections in a tertiary care hospital.

Materials and Methods: A cross sectional study was carried out over a period of one year in the department of Microbiology, SRM Medical College from March 2016-March 2017. A total of 136 pus swabs or pus aspirates were collected from post-operative male and female adult patients. Microbiological investigations of Gram staining, Culture and biochemical tests were performed with the samples. Antimicrobial susceptibility testing for screening and phenotypic detection of ESBL production was carried out according to Clinical Laboratory and Standards Institute guidelines, 2016. Conventional Polymerase chain Reaction (PCR) was performed using specific primers to amplify ESBL encoding genes among ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolates.

Result: Out of 136 samples tested 76 (55.88%) Gram negative bacteria were isolated. The most common causative agent of SSI was *Escherichia coli* 30 (39.47%), followed by *Klebsiella pneumonia 17* (22.37%). Phenotypic testing by combination disk method detected 40% of *E.coli and* 23.5% of *Klebsiella* to be ESBL producers. They were highly resistant to Ampicillin (86%) Cefepime (46.93%), Ceftazidime (62.75%) and Ciprofoloxacin (50.98%). bla_{TEM} was the predominant ESBL gene detected by Conventional PCR.

Conclusion: Early and accurate detection of ESBL is essential to reduce the spread of drug resistance among nosocomial bacteria.

Keywords: Surgical Site infections, *Escherichia coli*, *Klebsiella pneumoniae*, Extended Spectrum Beta lactamase, PCR, resistant genes, TEM.

Introduction

Surgical site infections were estimated to account for 31% of all Hospital acquired Infections (HAIs).[1] Centre for disease control and prevention (CDC) USA, defines a surgical site infection (SSI) as a form of hospital-acquired infection that occurs post operatively at the surgical site up to 30 days after an operative procedure, or up till 90 days for certain specified procedures and when an implant is left in place.[2] The risk of developing SSI varies inversely in proportion to the condition of the host, directly in proportion to the condition of the surgical site, directly in proportion to the virulence of the microorganism and also directly in proportion to the dose of bacterial contamination.[3] SSIs are a result of complex interaction between 1) patient related factors like obesity, extremes of age, cigarette smoking, systemic steroid use, malnutrition, compromised immune response, prolonged preoperative hospital stay; 2) operation related factors like duration of surgery, instrument sterilization, operation theatre environment; 3) microbial factors-like virulence of the microorganisms, toxin production and 4) perioperative antimicrobial prophylaxis-timing, choice and dosage.[4] Surgical Wound Classification by CDC, based on degree of contamination classifies post-operative wounds as clean wounds, clean-contaminated wounds, contaminated wounds and dirty wounds. Lower gastrointestinal tract surgeries like exploratory laparotomies that result in clean contaminated wounds are associated with a higher incidence of isolation of *E.coli* because the gastrointestinal tract is the natural habitat of Coliforms. Khairy et al in Saudi Arabia in 2014, reported *E.coli* and *Klebsiella* as the frequently isolated SSI pathogens.[2,5] Extended spectrum β-lactamases (ESBLs), Amp C type Beta-lactamase and carbapenemase production by the agency of plasmid transfer are common mechanisms of resistance in Multidrug resistant *Escherichia coli*, *Klebsiella pneumonia*e and other Enterobacteriaceae. Extended spectrum β-lactamases (ESBLs), a class A β-lactamases produced by these gram negative bacteria are a serious threat to the currently available antibiotic armory as it is a major resistance-mechanism among Gram negative bacteria leading to resistance to 3rd generation Cephalosporins in turn impeding treatment of infections caused by Enterobacteriaceae. ESBL producing bacteria

are not inhibited by penicillins and extended spectrum Cephalosporins and are inhibited by clavulunate, sulbactam or tazobactam.[10] Prevalence rates of ESBL producers vary widely. Idowu OJ et al in 2011 in Nigeria reported 35.5% of ESBL producing pathogens among orthopedic wound infections.[7] In a study in India by Rambabu et al, rate of ESBL production was 27.63% in 2015.[8] Mangayarkarasi et al had reported ESBL prevalence of 58.82% among urinary pathogens, in an earlier study in 2017 at our same institute.[9] ESBLs result from accumulation of point mutations in TEM-1 and SHV-1, that are the most common ESBL enzymes related to clinical isolates. Genes encoding TEM- and SHV- related ESBLs are commonly found on transferable plasmids. TEM derived its name from the patient temoneira in whom it was first isolated. SHV derived the name from Sulfhydryl variable active site. The β-lactamase enzyme, CTX-M was isolated from *Kluvyera species* and derived its name Cefotaximase. They are more active against Cefepime and Ceftriaxone than Ceftazidime, susceptible to β-lactamase inhibitors and are plasmid mediated. CTX-M is predominant in *E.coli and Klebsiella species.*[10,11] Lina et al in Bangladesh in 2014 in her study showed prevalence of $bla_{\text{CTX-M}}$ to be 100%, TEM gene to be 82.5% and SHV gene to be 47.5%. CTX-M is the predominant genotype identified among ESBL producers in studies in other parts of the world.[12] A study on ESBL genotypes by Patarachai et al in 2008 in Thailand, reported the prevalence of $bla_{\text{CTX-M,}}$ bla_{TEM} and bla_{SHV} genes to be 99.6%, 77.0% and 3.8% in E.coli and 99.2%, 71.7% and 87.4% in *Klebsiella pneumoniae respectively from various clinical samples.*[13] As there is limited data on SSI caused by ESBL producing gram negative bacteria, this study was undertaken to determine the incidence of ESBL (bla_{TEM}, $bla_{\text{CTX-M}}$ and $bla_{\text{SHV)}}$ genes among *Escherichia coli* and *Klebsiella pneumoniae* isolated from surgical site infections at our tertiary level hospital in the Southern region.

Materials and Methods

The present cross sectional study was conducted from March 2016-March 2017 in central laboratory of SRM Medical College Hospital, Kattankulathur, Tamil Nadu. The survey was taken from postoperative patients who underwent Clean and clean-contaminated surgeries. Ethical approval for the study was obtained from Institutional Ethical Committee, SRM Institute of Science and Technology. Ethical Committee clearance number: 928/IEC/2016. A total of 136 pus swabs or pus aspirates were collected. Microbiological investigations of gram staining, culture and biochemical tests were performed on the samples. Antimicrobial susceptibility testing was done for phenotypic detection of ESBL production. Conventional Polymerase chain Reaction (PCR) was carried out using specific primers to amplify ESBL encoding genes among phenotypically detected ESBL producers.

Sample Collection: A total of 136 wound swabs and pus aspirates were collected from post-operative male and female hospitalized adult patients in post-operative units of Surgery, Orthopedics, ENT and Gynecology with clinical diagnosis of SSI. The samples were collected at the time of dressing change. After the surface cleaning of wound with antiseptic solution, wound swabs or pus aspirates were collected from depths of the wound under aseptic precautions. The swabs were immediately transported to the laboratory for further microbiological processing. Relevant clinical history of patients were noted from case records.

Bacterial Identification: Direct microscopy and Gram staining from pus samples was done. Aerobic bacterial culture using basic agar media was performed and after 24 hours of incubation of inoculated media at 37deg C, the colony morphology noted. The preliminary identification by motility, gram reaction, catalase and oxidase tests were done. Presumptively motile, catalase positive, Nitrate reducing, Oxidase negative and glucose fermenting gram negative bacilli were enrolled into Enterobacteriaceae. Further confirmation of each organism up to species level was done using biochemical tests which included Indole test, Methyl red test. Vogues Proskauer test, citrate utilization test, urease test, triple sugar iron test and o-nitrophenyl beta galactosidase**.**

Phenotypic Detection of ESBL: Antibiotic susceptibility testing was performed on Mueller Hinton agar to screen for ESBL producers using antibiotic panel for gram negative bacteria. The antibiotic disks tested included Ampicillin (10µg), Ciprofoxacin (5µg), Amikacin (30µg), Ceftazidime (30µg), Ceftazidime/Clavulanic Acid (30/10µg), Cotrimoxazole (1.25/23.75µg), Cefotaxime (30µg), Gentamycin (10µg), Piperacillin/Tazobactam (100/10µg), Imipenem (10µg) and Meropenem (10µg), Tigecycline (15µg) and Colistin (10µg) were used. Phenotypic confirmation of ESBL by combination disk method (Fig. 1) was included in the routine susceptibility testing. The test was performed as per CLSI guidelines, 2017 [CLSI guidelines, 2017 [CLSI guidelines; M100, 26th edition, Performance standards for antimicrobial Susceptibility Testing, 2016.]. An isolate was considered an ESBL producer when the difference between zone inhibition diameters of Ceftazidime/Clavulanic acid (30µg/10µg) and Ceftazidime (30µg) alone was >5mm. Genotyping for detection of ESBL genes was done by Multiplex-Polymerase Chain Reaction (PCR).Standard strains used for quality control of the procedure were *Escherichia coli* (ATCC 25922).

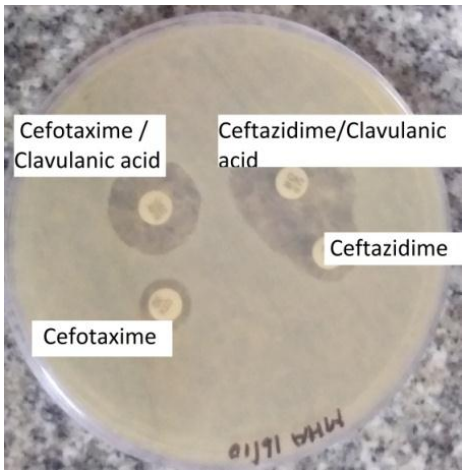


Fig. 1: Phenotypic ESBL detection by combination disk method

DNA Extraction: [HELINI biomolecules Purefast DNA extraction kit based]

As per the manufacturer's instructions, DNA isolation was done by centrifuging one ml of overnight culture of test bacterium at 6000rpm for 5min suspending pellets in 0.2ml Phosphate Buffered Saline (PBS). To the PBS suspension 180µl of lysozyme digestion buffer and 20µl of lysozyme were added and mixture incubated at 37^0C for 15 minutes. This was followed by addition 400µl of binding buffer and 20µl of Proteinase K to the mixture and incubation at 56ºC for 15min.This would inactivate all the nucleases. The sample was transferred to the PureFast spin column and centrifuged for 1min.This step enabled binding of nucleic acids to glass fibers in pure fast purification tube. The flow-through was discarded. 500µl Wash buffer-1 was added to the PureFast spin column followed by Centrifuge. After discarding the flow-through 500µl of Wash buffer-2 was added to the spin column and Centrifuged again for 30-60 seconds at 4500rpm.The flow-through was discarded again and column centrifuged for an additional 1 min. The contents of PureFast spin column was transferred into a fresh 1.5 ml micro-centrifuge tube and 100µl of Elution Buffer added. After centrifuging column for 2 min it was discarded and the purified DNA extracted in collection tube. Extracted DNA was stored at -20°C until PCR amplification.

PCR Amplification: PCR was carried out in 20µlreaction volumes for each sample, that constituted 10µl of HELINI PCR Master mix [containing 2µl of Taq DNA polymerase, in 10X Taq Reaction buffer, 2mM MgCl,1µl of 10mM dNTPs] (HELINI Biomolecules, Chennai, India), 5µl of each gene Primer. This mixture is added to5µl each of purified bacterial DNA. Primers used for the 3 genes are mentioned in Table 1.

PCR amplification of bacterial DNA was performed in Master Cycler [Thermal cycler, eppendorf].Cycling conditions are mentioned in Table 2.

Agarose Gel Electrophoresis: The products of PCR were added to the wells of prepared 2% agarose gel and subjected to electrophoresis at 50v. The positive bands were observed in UV transiluminator and Gel documentation was done using gel documentation system. (Fig. 2-4)

Table 1: Primers used for amplification of bla TEM, bla SHV, bla CTX-M genes in a master cycler-[Obtained from Helini biomolecules, Chennai]

Gene Detected	Primer	Amplicon size(bp)
bla CTX-M	F-ACGTGGCGATGAATAAGCTG R-AACCCAGGAAGCAGGCAGT	296bp
bla TEM	F-GATAACACTGCGGCCAACTT R-CTGCAACTTTATCCGCCTCC	250bp
bla SHV	F-CGCCGCCATTACCCATGACGCGAT R-ACCCGATCGTCCACCATGCCACT	275bp

Table 2: PCR Cycling Conditions

PCR step	Temperature	Duration	No. of Cycles
Initial Denaturation	95ºC	5 mins	1 cycle
Denaturation	94 ºC	30 secs	35 cycles
Extension	58 ºC	30 secs	
Annealing	72 ºC	30 secs	
Final Extension	72 ºC	5 mins	1 cycle

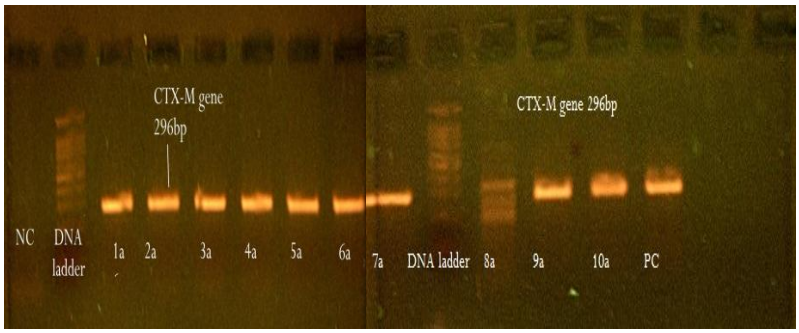
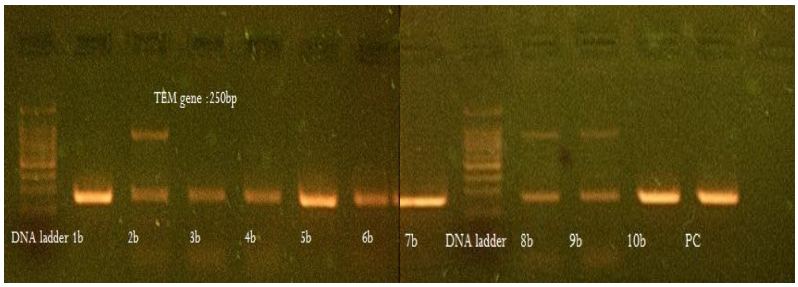


Fig. 2: Agarose Gel Pictures of confirmatory PCR for CTX-M gene in ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*
LANE-{NC (Negative control), DNA ladder,1a, 2a, 3a, 4a, 5a, 6a, DNA ladder, 8a, 9a, 10a, PC (Positive control)-CTX-M gene] Amplicon size: 296bp



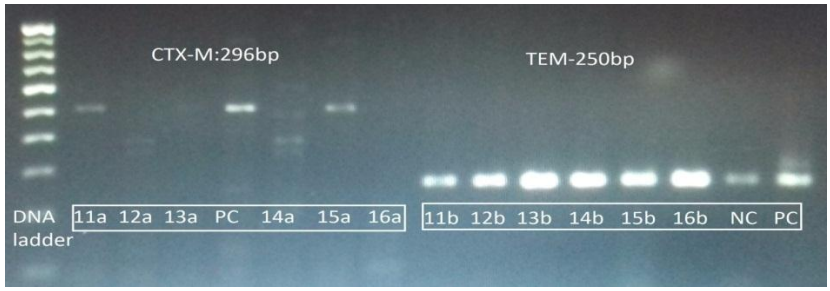


Fig. 3: Agarose Gel Pictures of confirmatory PCR for CTX-M and TEM genes in ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*
LANE-[DNA ladder, 1b, 2b, 3b, 4b, 5b, 6b, 7b, DNA ladder, 8b, 9b, 10b, PC-TEM GENE] LANE-[DNA ladder, CTX-M [1a, 2a, 3a, PC,4a, 5a, 6a], TEM [1b, 2b, 3b, 4b, 5b, 6b, NC, PC] amplicon size: 250bp

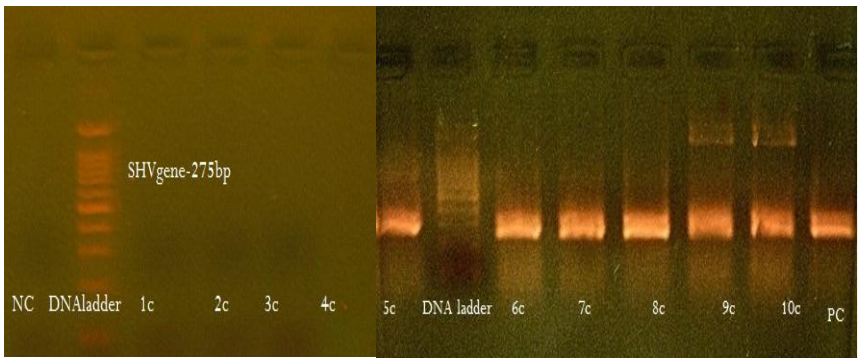


Fig. 4: Agarose Gel pictures of confirmatory PCR for SHV gene in ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*
LANE-[NC (Negative control), DNA ladder,1c,2c,3c, 4c, 5c, DNA ladder, 6c, 7c, 8c, 9c, 10c, PC-SHV gene]
LANE-[DNA ladder, NC (Negative control), PC (Positive control), 11c, 12c, 13c, blank, 14c,15c,16c-SHV GENE
Amplicon size:275bp

Results

A total number of 136 clinically diagnosed SSI patients were studied from whom 108 pus swabs, 1 tissue and 27 pus aspirates were collected. 87 samples were positive by culture, of which 77 were monomicrobial and 10 samples yielded polymicrobial growth.

Gender distribution of patients showed that Surgical Site Infection rate was higher in men (65.55%) than in women (33.33%). Maximum number of patients with culture positive SSIs belonged to the older age group of 41-60 years. The most common surgeries performed were hernioplasties followed by gastrointestinal surgeries, open reduction of Fractures and appendectomies.

Thirty seven (27.21%) out of 136 patients were Diabetics and culture positive SSI was identified in 22 (25.30%) diabetic patients. Hypertension, Malignancy and Hepatitis B infection were seen commonly in the 40-60 years age group.

According to the Center for Disease Control and Prevention, wound classification system, 92 (67.64%), of the pus swabs collected were classified as clean wounds, 43 (31.62%) categorized as clean contaminated wounds and 1 (0.74%) as contaminated wound. Of these 54.8% of Clean, 25.6% of clean-contaminated and 0.65% of contaminated wounds were confirmed microbiologically.

Bacteriological profile of SSI pathogens: 76 (55.88%) isolates out of 136 samples were gram negative bacilli that were predominant, and the remaining 21(15.44%) isolates included some gram positive cocci. Out of the total 76 gram negative bacterial isolates, the commonly isolated aerobic bacterium was *Escherichia coli 30* (39.47%), followed by *Klebsiella pneumoniae 17* (22.37%), *Pseudomonas species* 11 (14.47%), *Acinetobacter* 12 (15.79%), *Proteus vulgaris 4* (3.50%), *Citrobacter* species 1 (1.31%) and Non fermenting GNB 1 (1.31%).

Of the multidrug resistant Gram negative bacteria, 16 (21.05%) were ESBL producers. Out of 136 pus samples, ESBL production was seen in 12 (40%) isolates of *Escherichia coli* and 4 (23.5%) isolates of *Klebsiella pneumoniae*, while *Proteus vulgaris* and *Pseudomonas species* showed no ESBL production. ESBL producing *E.coli* and *Klebsiella pneumoniae* showed highest resistance to Ampicillin (86%), Cefepime (46.93%), Ceftazidime (62.75%) and Ciprofloxacin (50.98%). The molecular characterization of phenotypically confirmed ESBL producers revealed that $_{bla}$TEM gene was present in all 16 (100%) isolates while $_{bla}$CTX-M was seen in 12(62.5%) and $_{bla}$SHV gene was seen in only 6 (37.5%) of ESBL producing isolates.

Table 3: Gender Distribution of Study Population

Gender	Number (%) of bacterial cultures positive	Number (%) of bacterial cultures negative
Males	55 (65.47%)	41 (78.8%)
Females	29 (34.52%)	11 (21.15%)
Total (136)	84	52

Table 4: Bacteriological profile of SSI causing bacterial isolates

Bacterial Pathogens	No. of Bacterial Isolates (N=97)	ESBL Producing Bacteria
Escherichia coli	30 (31%)	12 (40%)
Klebsiella pneumoniae	17 (18%)	4 (23.5%)
Acinetobacter	12 (12.37%)	0
Pseudomonas	11(11.34%)	0
Staphylococcus aureus	9 (9.57%)	0
CONS	8 (8.24%)	0
Proteus vulgaris	4 (4.12%)	0
Enterococci	4 (4.12%)	0
Citrobacter	1 (1.03%)	0
Non-fermenting GNB	1 (1.03%)	0

Table 5: Gender wise distribution of Culture positive *Escherichia coli* and *Klebsiella pneumoniae*

Gender	Number (%) of *Escherichia coli* isolated	Number (%) of *Klebsiella pneumoniae* isolated
Males	20 (68.96%)	12 (70.59%)
Females	10 (31.03%)	5 (29.41%)
Total (136)	30	17

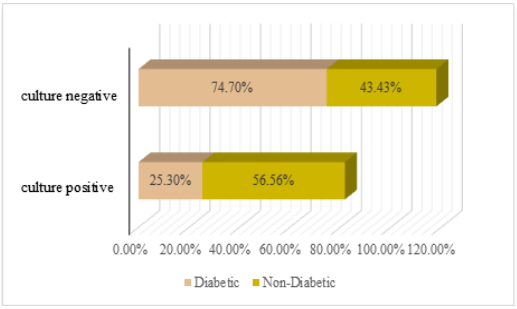


Fig. 5: Distribution of culture positivity in Diabetic and non-diabetics

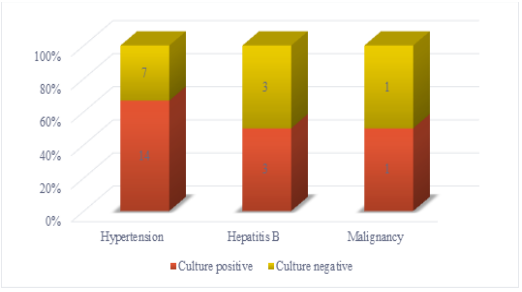


Fig. 6: Distribution of pus culture positivity in patients with hypertension, hepatitis B and malignancy

Table 6: Genotypic detection of TEM, SHV and CTX-M genes in *Escherichia coli* and *Klebsiella* spp isolated from surgical site infections

ISOLATE	Number (%) of ESBL detected by phenotypic confirmation method	CTX-M (%)	TEM (%)	SHV (%)	CTX-M, TEM	CTX-M, TEM, SHV	NON-ESBL producer
Escherichia coli	12 (40%)	10 (83.33%)	12 (100%)	5 (41.7%)	3 (83.33%)	7 (58.33%)	18 (60%)
Klebsiella pneumoniae	4 (23.5%)	2 (50%)	4 (100%)	1 (25%)	2 (50%)	1 (25%)	13 (35.71%)
Total(47)	16	12	16	6	5	8	31

Discussion

Post-operative hospital acquired infections are a serious health concern among post-surgical patients that lead to increased mortality, morbidity, length of hospital stay and fiscal burden on patients. Surgical site Infections (SSI) are the second most common hospital acquired infections, worldwide. According to WHO report of 2016, the global burden of SSI ranges from 2.5% to 41.5%. The prevalence of SSI in India, based on various studies ranged between 5 and 24 %.

Of the 136 pus swabs analyzed in this study, majority were obtained from men. This shows that men were at higher risk for surgical site infections, possibly due to differences in propensity for bacterial colonization of skin or other anatomical differences like thicker hair growth and shaving in men that could interfere with wound dressing adherence, causing higher risk of infection in them.

When assessing Diabetes as a risk factor for SSI, it was found that the majority of the culture positive cases were non-diabetics. This could be due to the proper perioperative glycemic control, that in turn reduced SSI risk among diabetic patients.

Bacteriological profile of SSI isolates in this study revealed that, Gram negative bacilli accounted for 55.88% (76/136) while Gram positive cocci accounted for 15.44% (21/136) out of the total samples studied. Shahane et al's study in 2012 in Pune also reported a higher rate of isolation of GNB.[16] *E.coli* and *Klebsiella* were the predominant SSI pathogens similar to report by Idowu. OJ et al in 2011 in Nigeria.[7] Majority of samples showed monomicrobial growth of aerobic bacteria, similar to the study by Negi et al in 2015 in Uttararkhand.[16, 17]

Owing to the high selection pressure posed by antibiotic treatment during hospital stay, ESBL producing Enterobacteriaceae are increasing in prevalence in our setting. This could be due to the high transmission density of plasmid mediated ESBLs among nosocomial bacteria.

The most common bacterial pathogens associated with SSI were *E.coli* (39.47%) and *Klebsiella pneumoniae* (22.37%) in the current study. This could be explained by the fact that there is a high risk of contamination of the surgical wound by endogenous

flora especially during gastrointestinal surgeries. As *E.coli* forms a part of the normal intestinal flora of humans, contamination of the surgical site with this organism is common. The rate of isolation of *E.coli* was comparable to other studies by Khairy et al in Saudi and Samir Anand et al in India in 2016 which were 35% and 57.1% respectively.[5, 14] *E.coli* was the predominant Gram Negative Bacteria showing Multidrug resistance among all SSI isolates.

ESBL was the predominant multidrug resistant pattern exhibited by Gram Negative Bacteria. The prevalence rate of ESBL producing GNB isolated from SSI in this study was 16/76 (21.05%) which was lower compared to report by Idowu OJ et al in 2011.[7] ESBL prevalence in the current study was higher when compared to studies by Lina et al in 2014 who reported an ESBL prevalence of 11.45% from urinary tract infection and surgical wound infection isolates.[12]

Escherichia coli and *Klebsiella pneumoniae* were resistant to Ampicillin, Ceftazidime and Ciprofloxacin. The reason for this high multidrug resistance was probably due to selective pressure resulting from extensive and incorrect use of these antibiotics during preoperative hospital stay and administration of total parenteral nutrition that was continued post-surgery. This highlights the need for implementation of a proper antibiotic policy in our hospital setting that could be strictly followed by surgeons to ensure judicious administration of perioperative antimicrobial prophylaxis. Both *E.coli* and *Klebsiella* were sensitive to Colistin. This might be due to the fact that these antibiotics are seldom utilized in the hospital due to their high cost and therefore very rarely misused.

The molecular characterization of phenotypically confirmed ESBL producing *E.coli* and *Klebsiella* spp.in the present study showed a prevalence of CTX-M gene to be 10 (66.67%). All isolates were positive for TEM gene 15 (100%) and 5 (33.33%) for SHV gene. In our study, the TEM gene predominated SHV and CTX-M among the ESBL encoding genes. These results disagreed with Lina et al in their study showing prevalence of $bla_{\text{CTX-M}}$ to be 100%, TEM gene to be82.5% and SHV gene to be 47.5%.None of the isolates carried CTX-M and SHV genes together In addition none of the isolates had CTX-M or SHV genes alone. The prevalence and type of ESBL genes may vary from one geographic region to another.[12] In an earlier study by Mangayarkarasi et al in 2017 on urinary pathogens, in this same institute CTX-M was the predominant gene demonstrated.[9] CTX-M is the predominant genotype identified among ESBL producers in other studies unlike in our study. The findings of the current study show that among pathogenic GNB causing SSI, in our setting, TEM-related ESBLs are still predominant.

TEM gene was observed in all genotypically positive isolates in our study, prompting the possibility of TEM being an appropriate candidate for molecular testing of ESBL among multidrug resistant isolates as suggested by Trupti et al in 2017 who had made similar observations of TEM predominance in her study in Madhya Pradesh.[15] Nucleic acid amplification tests are suggested as the method of choice for detection of ESBL producing strains of Enterobacteriaceae. Though genotypic tests are expensive and require advanced equipment with expertise, they are more sensitive as they can also detect hidden ESBL encoding genes. Sequencing of the ESBL genes identified in this study could not be carried out due to cost constraints; in future this will be considered.

Conclusion

E.coli and *Klebsiella spp*., the commonly isolated pathogens from surgical site infections in the current study showed highest resistance to Ciprofloxacin (33.33%) and ceftazidime (47%) respectively. ESBL production rate among gram negative bacteria was 21.05%. TEM gene was present in all genotypically confirmed ESBL isolates. This study concludes that ESBL detection by phenotypic testing accompanied with molecular amplification of genes will help provide the clinician accurate sensitivity pattern of drug resistant nosocomial pathogens thus preventing antibiotic misuse. Through pharmacological interventions directed towards effective antibiotic stewardship and judicious administration of surgical antimicrobial prophylaxis surgical site infections can be prevented effectively.

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