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Invitro Activity of Tigecycline against Multidrug-Resistant Enterobacteriaceae from Blood Stream Infection at a Tertiary Care Hospital of Nepal

Ajay Yadav,[1] Basudha Khanal,[2] Amit Kumar Patel,[3] Alina Karna[4]

[1]*Department of Microbiology, Madan Bhandari Academy of Health Sciences, Hetauda, Nepal;*
[2]*Department of Microbiology, BP Koirala Institute of Health Sciences, Dharan, Nepal;*
[3]*Department of Pathology, National Medical College and Teaching Hospital, Birgunj, Nepal;*
[4]*Department of Pathology, Institute of Medicine, Kathmandu, Nepal;*

ABSTRACT

Introduction: Any bacterial isolates showing resistant to three or more than three different types of antimicrobial groups are termed as multidrug resistant. Such superbugs have shown significant challenge not only to microbiologist but also to clinicians. Multidrug resistant like extended-spectrum and metallo-beta lactamase are being encountered as the causative agents of blood stream infection. Accurate diagnosis of blood stream infection, timely isolation and identification of the causative agents and determination of their antimicrobial susceptibility are crucial, as effective management depends on the selection and timely administration of the most appropriate antimicrobial agent.

Objectives: This study is aimed to find out the efficacy of tigecycline against multidrug resistance enterobacteriaceae.

Methods: A descriptive cross-sectional study was conducted in the Department of Microbiology, BP Koirala Institute of Health Sciences, from 1st September 2014 to 31st August 2015. Confirmation for ESBL was done as recommended by Clinical and Laboratory Standard Institute (CLSI) and MBL production was detected by double disk synergy test. Antibiotic sensitivity test against tigecycline was done by Kirby-Bauer disk diffusion method.

Results: 98.96% of multidrug resistant enterobacteriaceae were sensitive against tigecycline. None of the isolates were resistant against tigecycline and only 1.04% of the isolates showed intermediate susceptibility. A total of 64 (33.4%) isolates were found to be multidrug resistant.

Conclusion: Tigecycline is found to have excellent invitro activity against MDR enterobacteriaceae from blood stream infection.

Keywords: Enterobacteriaceae; Extended-spectrum beta lactamase; metallo-beta lactamase; multidrug resistant; tigecycline.

INTRODUCTION

Blood stream infection (BSI) is potentially a life-threatening disease as it can cause serious secondary infections. BSI due to multidrug-resistant (MDR) organisms has been associated with multiple poor outcomes like duration of hospital stay, health care cost and a high morbidity and mortality rate.[1–3]

Correspondence
Ajay Yadav
ajay.yadav@mbahs.edu.np

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Over recent years, problem of multidrug resistant Enterobacteriaceae has increased dramatically and occupies the third most leading cause of BSI in most of the settings.[4] The escalating burden of multidrug resistance in Enterobacteriaceae is largely due to production of beta lactamase and subset of beta lactamase that are enzymes that bind, deactivate the different types of beta lactam antibiotics and confer broad resistance against them.[5]

Tigecycline is a broad-spectrum of antibiotic that binds 30S ribosomal subunit of bacteria and thereby blocking the interaction of amino acyl tRNA with A site of the ribosome.[6] It is also not associated with cross-resistance to other antibiotics, conferring

another advantage in its activity against several Extended Spectrum Beta-Lactamase (ESBL) and carbapenemase producing Enterobacteriaceae.[6] Tigecycline has become an alternative to face the challenges of many MDR organisms.[7]

A prospective study demonstrates that tigecycline along with standard dose imipenem showed good clinical efficacy on patients with bacteremia.[8] Therefore, we aimed to find out the efficacy of tigecycline against multidrug-resistant enterobacteriaceae.

METHODS

A descriptive cross-sectional study was conducted in the Department of Microbiology, BP Koirala Institute of Health Sciences, from 1st September 2014 to 31st August 2015. Ethical approval was obtained from Institutional Review Committee (IRC), BP Koirala Institute of Health Sciences, Dharan, Nepal. All methods in this study was carried out in the hospital premises of BPKIHS in accordance with guidelines provided by "BPKIHS- code no IRC/424/014".

A total of 11,264 blood specimens were submitted to Department of Microbiology for culture. Only Enterobacteriaceae isolates were included in this study, isolates other than Enterobacteriaceae falls under exclusion criteria. Convenient sampling technique was used for this study. Approximately 3 ml blood was collected in Brain Heart Infusion Broth. Cultures were processed in BD 9050 system (Becton and Dickinson, New York, USA). These broths were aerobically incubated at 35°C for 5 days and observed for the growth of microorganisms. Any sign of growth was followed by sub-culture on MacConkey's agar and Blood agar plates (Hi Media, Mumbai, India). Identification, characterization and sensitivity of the isolates were analyzed as per standard microbiological procedures.[9] Identification of isolates was performed following standard microbiological techniques.[10]

Antimicrobial susceptibility test of all the isolates was performed on Mueller Hinton agar (MHA) (Hi Media, Mumbai, India) by the standard disk diffusion technique of Kirby-Bauer method and interpreted as per CLSI recommendations.[11]

Antibiotics used were several beta-lactam and tigecycline (TGC) (15µg). Beta-lactam antibiotics used were Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Ceftriaxone (CTR) (30µg), Cefotaxime with clavulanic acid (CAC) (30/10µg), Ceftazidime with clavulanic acid (30/10µg), Ertapenem (ETP) (10µg), Cefoxitin (CX) (30µg), Cefepime (CPM) (30µg), Aztreonam (AO) (30µg), Imipenem (IPM) (10 µg). After 18-24 hours of incubation, each plate was examined for the zone of inhibition. Interpretation of antibiotic susceptibility test results was made as per the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI).[12] Isolates were further tested by performing sensitivity test against tigecycline by Kirby-Bauer disk diffusion method. As for interpretation of susceptibility pattern of tigecycline, isolates showing zone of inhibition (ZOI) ≥ 18 mm were interpreted as sensitive, 15 to 17 mm interpreted as intermediate susceptible and ≤ 15 mm interpreted as resistant. Most of the studies have done E-test or agar dilution test for calculation of minimum inhibitory concentration (MIC) of tigecycline, but in this study Kirby-Bauer disk diffusion method is used as this method is cost effective for community perspective as compared to MIC.[13,14] *Escherichia coli* ATCC 25922 was used as the control organisms for the antibiotic sensitivity test. Isolates showing resistant to at least one antibiotic in three or more antimicrobial classes were confirmed as multidrug-resistant (MDR) phenotype.[9,15]

Detection of ESBL

ESBL screening was done as per CLSI recommendations. Isolate showing zone of inhibition (ZOI) of ≤ 27 mm for cefotaxime (CTX) (30 µg) and ≤ 22 mm for ceftazidime (CAZ) (30 µg) was taken for ESBL confirmation.[12]

Detection of Carbapenemase

The isolates showing resistant to ertapenem (ETP) (10 µg) (ZOI ≤ 22 mm) and sensitive to imipenem (IPM) (10 µg) (ZOI ≥ 23 mm) were considered as carbapenemase producers. Besides this, rest of the antimicrobials should be resistant for the isolates to be carbapenemase producers.[16]

Metallo β-lactamase (MBL)

All carbapenemase producers were tested for double disk synergy test and were confirmed by Modified Hodge Test (MHT).

Double disk synergy test

Double disk synergy test was done by using anhydrous ethylene diamine tetra acetic acid (EDTA) disk of concentration 1.5 mg/disk (0.5 mol.) and imipenem (IPM) (10 µg). EDTA-IPM disks were kept at a distance of 10 mm apart (edge to edge). Enhanced ZOI showing synergistic effect between the two disks was considered as MBL producers.[17,18]

Modified Hodge Test (MHT)

ATCC *Escherichia coli* was inoculated on MHA plate. IPM disk was placed at the center of MHA plate with the help of sterile forcep. Isolated strain was inoculated perpendicular to the IPM disk. Following incubation, appearance of a clover leaf at the streaking line of the isolated strain was confirmed as MBL producers.[16]

Data and statistical analysis

The data generated during the study period were analyzed by using SPSS version 16.0 and were analyzed according to frequency distribution and percentage.

Consent to participate/consent to publish

Informed consent form was not included as this study deals with the bacterial isolates that belong to Enterobacteriaceae family only.

RESULTS

During the study period, a total of 11,264 blood samples were collected from the patients subjected to Blood Stream Infections. Of these isolates, 192 (1.70%) were Enterobacteriaceae comprising of *Escherichia coli* 95 (49.48%), *Klebsiella pneumoniae* 45 (23.44%), *Enterobacter aerogenes* 27 (14.06%), *Citrobacter freundii* 6 (3.13%), *Citrobacter koseri* 6 (3.13%), *Proteus vulgaris* 3 (1.56%), *Salmonella* Typhi 3 (1.56%), *Enterobacter cloacae* 2 (1.04%), *Proteus mirabilis* 2 (1.04%), *Klebsiella oxytoca* 1 (0.52%), *Morganella morganii* 1 (0.52%) and *Salmonella* Paratyphi A 1 (0.52%). (Figure 1)

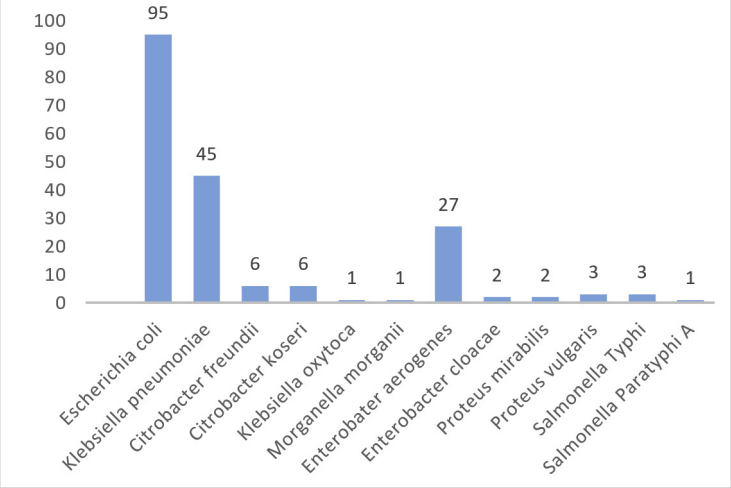

Figure 1: Frequency of Enterobacteriaceae isolates (n=192)

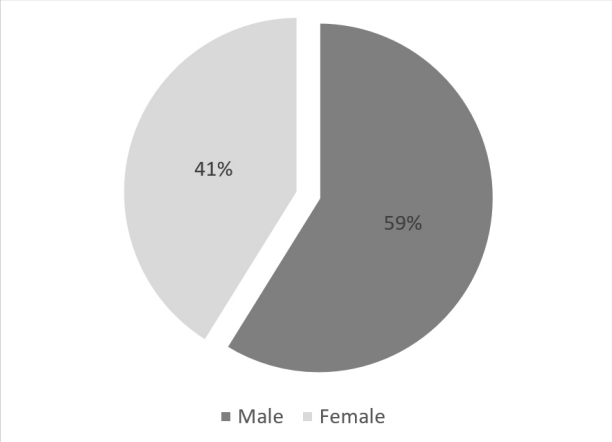

Figure 2: Sex wise distribution

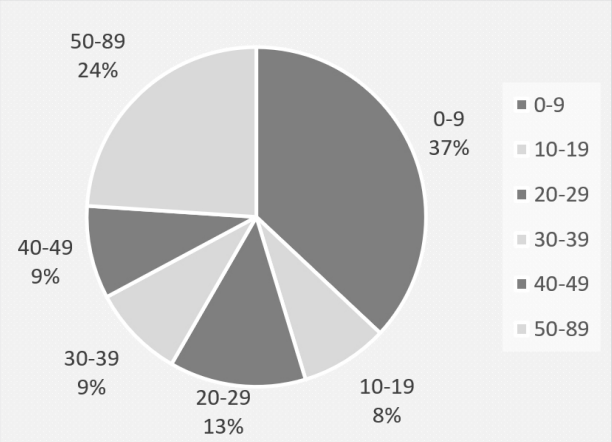

Figure 3: Age wise distribution (n=192)

Sex wise distribution of studied patients (n=192)

Of the total Enterobacteriaceae isolates, 113 (59%) were obtained from male patients and 79 (41%) from female patients. *Figure 2.*

Age wise distribution (n=192)

Enterobacteriaceae was isolated from blood in all aged groups. Among them, majority were from age group <10 years (71; 37%), followed by age groups 50-89 years (46; 24%) and age groups 20-29 years (25; 13%). The number of Enterobacteriaceae in other age groups were almost similar, i.e. 30- 39 years (17; 9%), 40-49 years (17; 9%) and 10-19 years (16; 8%). There were 15 neonates, 19 infants and 30 children (<12 Years). Figure 3

Frequency of **β**-lactamases producers

Results of different types of β-lactamases produced by Enterobacteriaceae are given in figure 4. ESBL was the most common β-lactamase 94 (49%), followed by carbapenemase 51 (26.5%) and MBL 22 (11.5%).

Antibiotic sensitivity pattern of Enterobacteriaceae

Most of the isolates were resistant to third generation cephalosporins, cefotaxime (141; 73.40%), ceftazidime (139; 72.40%) and ceftriaxone (105; 54.70%). Similar sensitivity pattern for cefepime (113; 58.80%) was observed compared to third generation cephalosporins. Most of the isolates of *E. aerogenes* (17; 63%) and *C. freundii* (4; 67%) were sensitive to cefepime. All three isolates of *S.* Typhi were sensitive to cefepime. Regarding monobactam, 77 (40%) isolates were found sensitive and the number of resistant isolates were equal to the sensitive isolates. Among carbapenems, 22 (11.5%) isolates were resistant to imipenem and 51 (26.5%) isolates were found resistant to ertapenem. Most of the isolates (164; 85.4%) were sensitive to imipenem.

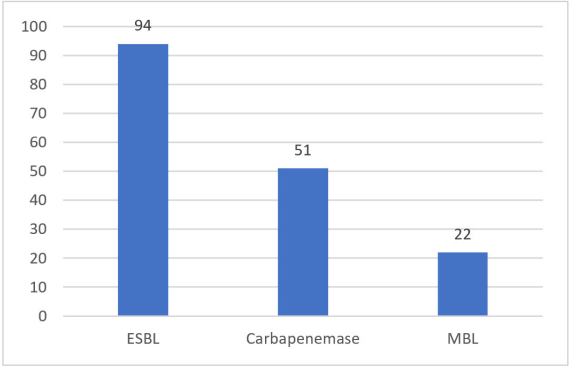

Figure 4: β-lactamase producers (n=192)

Table 1: Antibiotic sensitivity pattern of the Enterobacteriaceae

Antibiotic used	Sensitive (%)	Resistance (%)	Intermediate (%)
Cefotaxime	25.50	73.40	1.10
Ceftazidime	23.96	72.40	3.64
Ceftriaxone	42.70	54.70	2.60
Cefotaxime-clavulanic acid	71.90	28.10	0.00
Ceftazidime-clavulanic acid	68.30	31.70	0.00
Cefoxitin	41.70	41.70	16.6
Cefepime	39.60	58.9	1.50
Aztreonam	40.10	56.25	3.65
Ertapenem	66.63	26.56	7.81
Imipenem	85.40	11.50	3.10
Tigecycline	98.96	0.00	1.04

Almost 99% of the isolates were found sensitive to tigecycline (zone of inhibition ≥18 mm). Only two of the isolates (*K. pneumoniae*) were found to have intermediate susceptibility (zone of inhibition ≤16 mm) to tigecycline. (Table 1)

MDR Enterobacteriaceae

Among 192 Enterobacteriaceae, 64 (33.3%) isolates were found to be multidrug resistant. *Escherichia coli* (26) was found to be the leading one among the MDR isolates, followed by *Klebsiella pneumoniae* (21), *Enterobacter aerogenes* (8), *Citrobacter koseri* (3), *Citrobacter cloacae* (2) and one each of *Citrobacter freundii, Klebsiella oxytoca, Proteus vulgaris,* and *Salmonella* Paratythi A. (Figure 5)

DISCUSSION

In our study, the frequency of Enterobacteriaceae from BSI was found to be 1.7% (192) out of total blood culture positive 17.3% (1948). It is similar to the study conducted by Abdallah *et al* in 2015 in Egyptian patients and in Nepal by Joshy M Easow *et al* in 2010,[19,20] where the number of Enterobacteriaceae isolated found to be 94 and 96 respectively, which was less as compared to our study.

β-lactamase production remains the most important mediator of β-lactam resistance in enterobacteriaceae.[21] In the present study, among 49% ESBL producers, 64% were *Escherichia coli* followed by *K. pneumoniae* 20%. Similar type

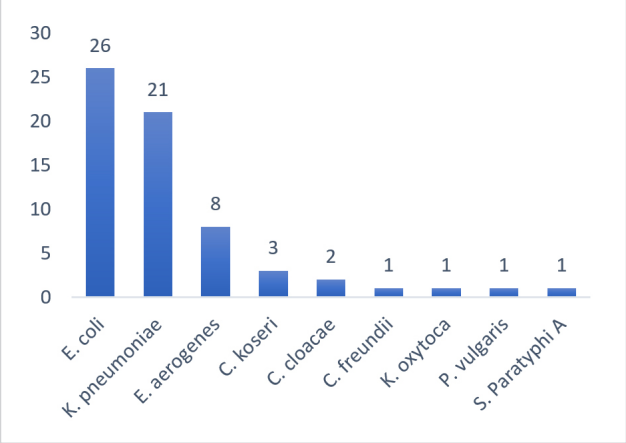

Figure 5: MDR Enterobacteriaceae (n=64)

of study conducted by Shrestha *et al* at BPKIHS in the year 2007 where the prevalence of ESBL among the clinical isolates of pyogenic infections were - *E. coli* (53%), *K. pneumoniae* (14.8%), *P. mirabilis* (12.9%), *Enterobacter* species (5.5%) and *Citrobacter* species (5.5%).[22] Emergence of ESBL is probably due to widespread use of third-generation cephalosporins which is believed to be the major cause of mutations in TEM and SHV enzymes.[21]

In this study, Carbapenemase producers was found to be 51 (26.5%). In screening MBL, 22 (11.5%) isolates were imipenem resistant, whereas 51 (26.5%) were resistant to ertapenem. MBL producing *Klebsiella* species in the present study was found to be higher in number than that shown by Shrestha *et al.*[23] In a study conducted by Vinod Kumar *et al*., 20% resistance to imipenem and 17% MBL production was reported. Similarly, Kamble *et al*., reported 20% MBL production in their study.[24]

Antimicrobial susceptibility profile of Enterobacteriaceae showed a high degree of resistance to the antimicrobials. Resistance against cefotaxime and ceftazidime were highest (72–74%) as compared to the resistance pattern against antimicrobials. In BSIs, third-generation cephalosporins have been extensively used as a first-line antibiotic, as a result of which they are rendered useless. Our isolates showed least resistance for imipenem (11.5%) and ertapenem (26.5%). The rate of resistance to the various drugs was in concordance with other studies.[21,24,25] In our study, 64 (33.3%) isolates were MDR. Various authors have reported high percentage of MDR in their study.[21,25,26]

Among the isolates, 190 (99%) were sensitive to tigecycline. Two *K. pneumoniae* were found to have intermediate susceptibility to tigecycline. In a study conducted by Mohanty and Mahapatra, 6.7% of the isolates (all *K. pneumoniae*) and seven *K. pneumoniae* (14.5%) was found resistant to tigecycline, which is different from our study.[6] Present study showed good activity of tigecycline (99%) against the isolates. Only two isolates were found to have intermediate susceptibility to tigecycline. Similar results were documented by Sader *et al.*[27] Tigecycline was very active and appears to be an excellent option for treatment of infections caused by these multidrug-resistant Enterobacteriaceae.[27,28]followed by VIM-1 (14 Clinical efficacy of tigecycline in BSI has not yet been established. In vitro, evaluation of its efficacy in ESBL and MBL producing isolates in septicemia have been reported by Roy *et al*. in two different studies.[29,30]

Limitations: We are unable to perform minimum inhibitory concentration (MIC) for tigecycline as E-strip for tigecycline is expensive and also fund was not available for this study. It would not be helpful for the community people for diagnostic purpose as it is not cost effective. So, Kirby-Bauer disk diffusion test is done for determination of sensitivity pattern of tigecycline.

CONCLUSIONS

Tigecycline has showed excellent effect against MDR Enterobacteriaceae. The limited availability of suitable alternate therapeutic armamentarium necessitates the use of tigecycline with a critical and urgent need to continuously monitor the emergence and spread of resistance. Present study has documented the increasing antimicrobial resistance among isolates from BSI which is a matter of concern for clinicians and microbiologists. This reflects the need for early detection and prevention of further spread of resistance to other bacteria.

Conflict of Interest: None

NJHS

REFERENCES

1. Edmond MB, Ober JF, Dawson JD, Weinbaum DL, Wenzel RP. Vancomycin-resistant enterococcal bacteremia: natural history and attributable mortality. Clin Infect Dis. 1996;23(6):1234–9.
2. Bearman GML, Wenzel RP. Bacteremias: a leading cause of death. Arch Med Res. 2005 Jan;36(6):646–59.
3. Wang J, Pan Y, Shen J, Xu Y. The efficacy and safety of tigecycline for the treatment of bloodstream infections: A systematic review and meta-analysis. Ann Clin Microbiol Antimicrob. 2017;16(1):1–10.
4. Demirturk ND and Demirdal T. Causative agents of nosocomial bloodstream infections and their antimicrobial susceptibility patterns: Southeast Asian J Trop Med Public Health. 2013 Nov;44(6):1036-42.
5. Ruppé É, Woerther PL, Barbier F. Mechanisms of antimicrobial resistance in Gram-negative bacilli. Ann Intensive Care. 2015;5(1).
6. Mohanty S, Mahapatra A. In vitro activity of tigecycline against multidrug-resistant Enterobacteriaceae isolates from skin and soft tissue infections. Ann Med Surg. 2021;62(1):228–30.
7. Muralidharan G, Micalizzi M, Speth J, Raible D, Troy S. Pharmacokinetics of tigecycline after single and multiple doses in healthy subjects. Antimicrob Agents Chemother. 2005;49(1):220–9.
8. Jean S, Hsieh T, Hsu C, Lee W, Bai K, Lam C. ScienceDirect Comparison of the clinical efficacy between tigecycline plus extended-infusion imipenem and sulbactam plus imipenem against ventilator-associated pneumonia with pneumonic extensively drug-resistant Acinetobacter baumannii bacteremia , and co. J Microbiol Immunol Infect. 2016;49(6):924–33.
9. Parajuli NP, Acharya SP, Mishra SK, Parajuli K, Rijal BP, Pokhrel BM. High burden of antimicrobial resistance among gram negative bacteria causing healthcare associated infections in a critical care unit of Nepal. Antimicrob Resist Infect Control. 2017;6(1):1–9.
10. Garcia LS. Clinical Microbiology Procedures handbook. 2nd ed. Washington, DC: ASM press; 2007.109-118.
11. Clinical and Laboratory Standard Institute. Performance standard for antimicrobial susceptibility test. 9th ed. CLSI document M100-S24. 2014
12. Clinical and Laboratory Standards Institute. Wayne, PA: Clinical and Laboratory Standards Institute; 2014. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 24 th Informational Supplement. CLSI document M100-S24. 2014.
13. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. 2016;(December 2009):1–23.
14. Antimicrobial Susceptibility Systems HiCrome ™ Mueller Hinton Agar. 2010; 24-30.
15. Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268–81.
16. Anderson KF, Lonsway DR, Rasheed JK, Biddle J, Jensen B, McDougal LK, et al. Evaluation of methods to identify the Klebsiella pneumoniae carbapenemase in Enterobacteriaceae. J Clin Microbiol. 2007 Aug;45(8):2723–5.
17. Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem-susceptible metallo-β-lactamase- producing gram-negative bacilli in the clinical laboratory. J Clin Microbiol. 2006;44(9):3139–44.
18. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol. 2003 Oct ;41(10):4623–9.
19. Abdallah HM, Wintermans BB, Reuland EA, Koek A, al Naiemi N, Ammar AM, et al. Extended-Spectrum β-Lactamase- and Carbapenemase-Producing Enterobacteriaceae Isolated from Egyptian Patients with Suspected Blood Stream Infection. PLoS One. 2015 Jan;10(5):e0128120.
20. Joshy M Easow, Noyal M Joseph, Banodita A Dhungel, Bipin Chapagain PS. Blood Stream Infections among febrile patients attending a Teaching Hospital in Western Region of Nepal . Australasian Medical Journal AMJ 2010, 3, 10, 633-637. 2010.
21. Modi Dhara J, Patel Bhaumik V, Patel Mitesh H, Bhatt Seema S, Sood Nidhi K V, M M. A study of extended spectrum β-lactamase (esbl) and ampc β-lactamase producing klebsiella pneumoniae in neonatal intensive care unit at tertiary care hospital, ahmedabad. Natl J Community Med. 2012.
22. Shrestha S, Amatya R, Dutta R. Prevalence of extended spectrum beta lactamase (ESBL) production in gram negative isolates from pyogenic infection in tertiary care hospital of eastern Nepal. Nepal Med Coll J. 2011 Sep;13(3):186–9.
23. Mishra SK, Shrestha R, Rijal BP, Pokhrel BM. The bad, the ugly and the demon: a tale of extensively drug-resistant, extended-spectrum-beta-lactamase- and metallo-beta-lactamase-producing superbugs associated with nosocomial pneumonia. Asian Pacific J Trop Dis. 2015 Jan;5(1):71–6.
24. Gajul S V, Mohite ST, Mangalgi SS, Wavare SM, Kakade S V. Klebsiella Pneumoniae in Septicemic Neonates with Special Reference to Extended Spectrum β-lactamase, AmpC, Metallo β-lactamase Production and Multiple Drug Resistance in Tertiary Care Hospital. J Lab Physicians. 2015 Jan;7(1):32–7.
25. Chandel DS, Johnson JA, Chaudhry R, Sharma N, Shinkre N, Parida S, et al. Extended-spectrum beta-lactamase-producing Gram-negative bacteria causing neonatal sepsis in India in rural and urban settings. J Med Microbiol. 2011 Apr;60(Pt 4):500–7.
26. VinodKumar C S , Kalappanavar NK , Umakanth Patil BKG. Change in spectrum of microbial aetiology in relation to gestational age and birth weight and emergence of ESBL in tertiary neonatal intensive car. Int J Biol Med Res. 2011;2(3):727–34.
27. Castanheira M, Sader HS, Deshpande LM, Fritsche TR, Jones RN. Antimicrobial activities of tigecycline and other broad-spectrum antimicrobials tested against serine carbapenemase- and metallo-beta-lactamase-producing Enterobacteriaceae: report from the SENTRY Antimicrobial Surveillance Program. Antimicrob Agents Chemother. 2008 Feb 1 ;52(2):570–3.
28. Bogdanovich T, Adams-Haduch JM, Tian G-B, Nguyen MH, Kwak EJ, Muto CA, et al. Colistin-resistant, Klebsiella pneumoniae carbapenemase (KPC)-producing Klebsiella pneumoniae belonging to the international epidemic clone ST258. Clin Infect Dis. 2011 Aug;53(4):373–6.
29. Roy S, Datta S, Viswanathan R, Singh AK, Basu S. Tigecycline susceptibility in Klebsiella pneumoniae and Escherichia coli causing neonatal septicaemia (2007-10) and role of an efflux pump in tigecycline non-susceptibility. J Antimicrob Chemother. 2013 May ;68(5):1036–42.
30. Roy S, Viswanathan R, Singh AK, Das P, Basu S. Sepsis in neonates due to imipenem-resistant Klebsiella pneumoniae producing NDM-1 in India. J Antimicrob Chemother. 2011 Jun;66(6):1411–3.