Comparative analysis of Phenotypic and Genotypic Characterization of *E. coli*and *Klebsiella pneumoniae* in Diabetic Foot Ulcer

Dr.Dipak S. Kale[1], Dr.Kailas Datkhile[2] ,Dr .Geeta S. Karande[3] ,Manasi V. Yadav[4], Snehal Deshmukhe[5]

Krishna institute of medical sciences, Krishna Vishwa Vidya peeth, karad 415339.

KEYWORDS

DFU, AST,ATCC, CLSI, ESBL, MBL.

ABSTRACT

Background:

Diabetic foot ulcers (DFUs) are a common complication in people with diabetes, often leading to severe infections caused by microorganisms, including *Escherichia coli* and *Klebsiella pneumoniae*.

Objectives:

This study is to conduct a comparative examination of the phenotypic and genotypic characteristics of these two bacterial pathogens isolated from DFUs. The collected samples were from diabetic patients with foot ulcers for analysisand identified the bacterial isolates through standard microbiological techniques, including culture, Gram staining, and biochemical tests. Subsequently, molecular methods, such as polymerase chain reaction (PCR) and sequencing, were employed to analyze specific genetic markers connected to virulence and antibiotic resistance.

Results:

Our results demonstrated distinct phenotypic traits between *E. coli* and *K. pneumoniae*, with varying biochemical profiles and antibiotic sensitivity patterns. Genotypically, we identified key virulence factors in both species, including extended-spectrum beta-lactamase (ESBL) genes and other resistance determinants. The comparative analysis demonstrated significant variations in the prevalence of these genetic traits, which correlate with the clinical outcomes observed in patients with DFUs.

Conclusion:

Understanding the phenotypic and genotypic distinctions between *E. coli* and *K. pneumoniae* in the context of DFUs supplies critical insights into their pathogenicity and resistance mechanisms. This understanding can influence the design of targeted therapeutic strategies and improve management protocols for diabetic patients, ultimately enhancing patient outcomes and reducing the impact of diabetic foot infections.

INTRODUCTION:

Diabetes has the most of pressing global health challenges of the 21st century, ranking as the ninth leading cause of death worldwide, with 1.6 million lives lost in 2019 alone. (Pradeepa and Mohan, 2021; Reed et al., 2021; Sun et al., 2022; Tinajero and Malik, 2021). Diabetes syndrome leads to several harmful metabolic effects, which can trigger pathophysiological complications such as foot ulcers, neuropathy, atherosclerosis, etc. (Grunfeld, 1992). Approximately 12-25% of diabetic patients are at a higher risk of developing foot ulcers due to poor circulation, nerve damage, and delayed healing processes. (Boyko et al., 1996; Singh, 2005). Diabetic foot ulcers (DFUs) are a complex condition, often accompanied by cellulitis or osteomyelitis, driven by the immune system and infecting bacteria (Williams et al., 2004), and can lead to severe consequences for on health, economy, and psychology. When DFUs become infected with bacteria, the condition worsens, and patients are typically advised to seek hospitalization. It is estimated that between 44-68% of patients who develop osteomyelitis while hospitalized may eventually require amputation of the affected area due to the intensity of the infection (Pecoraro et al., 1990; Van Asten et al., 2016). To prevent additional complications, treatments such as antibiotics, neuropathic medications, growth factors, and inflammatory modulators have been proposed (Boulton et al., 2005; Karri et al., 2016). One of the key challenges in treating DFUs is the bacterial colonization and the progress of antibiotic resistance.

MATERIAL AND METHODS:

This study wasobservational-analytical studyconducted in theMicrobiology Department, Molecular Biology and Genetics, Krishna Hospital & Medical Research Center, Krishna Vishwa Vidyapeeth (Deemed to be Univesrsity) over a 3 years period from May 2021- June 2024.

Inclusion criteria:

The age of patients between 40-82 years diagnosed with diabetes and DFU.

The patients includedin this study who had been suffering from DFUs for at least six months.

Patients with DFUs of grade XXX were involved in the study.

Exclusion criteria:

*E. coli and K. pneumoniae*isolates from other clinical samples were excluded.
Participants who chose not to consent to the study were excluded.

Sample collection:

In total, 252 diabetic patients were detected for bacterial infections in DFUs. The data on age, sex, socio-economic status of the patients was recorded. Diabetic history and physiological conditions of each patient were recorded. Blood tests including sugar (glucose) level and HbA1C were performed before bacterial sampling. In addition, urea, creatinine, potassium, and sodium levels were measured in compliance with established standard.

Microbiological procedure:

Bacterial infections were detected in the pus samples collected from the ulcers. The pus samples were taken from the ulcer with two sterile cotton swabs.One swab was applied for

Gram staining, while the second was cultured on both Blood agar and MacConkey agar.The inoculated plates were kept at 37°C for incubation. The following day, the plates were checked for colony morphology, and Gram staining along with biochemical characterization was performed.Total of 91 *E. coli* isolates and 161 *K. pneumoniae*isolates were identified.

Data presentation analysis:

The data from the present study were analyzed and presented in graphical and tabular formats using Microsoft Excel. Heat maps were created using the SR plot online tool.Principal Component Analysis (PCA) was applied to evaluate the differences in antibiotic resistance and the prevalence of genes linked to ESBL and MBL production between E. coli and K. pneumoniae, utilizing the PAST version 4.10 software.(Hammer and Harper, 2001).

Antibiotic susceptibility testing:

Antibiotic sensitivity of all*Escherichia coli* and *Klebsiella pneumoniae*isolates to Ampicillin, Amoxicillin, Piperacillin, Cefuroxime, Ceftriaxone, Cefoperazone, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, and Ciprofloxacin was studied by Kirby-Bauer disc diffusion following guidelines established by CLSI 2021 M45-A.

Genetic characterization of beta lactamase production:

The genomic and plasmid DNA from 252 *Escherichia coli* and*Klebsiella pneumoniae*isolates were extractedby using the HipurA bacterial genomic DNA purification Kit and HipurA Plasmid DNA miniprep purification kit (HiMedia).Beta-lactamase producing genes (TEM_{ESBL}, SHV_{ESBL}, $CTX\text{-}M_{ESBL}$,$NDM\text{-}1_{bla}$, KPC_{bla}, $OXA\text{-}48_{bla}$, and VIM_{bla}) were amplified through Polymerase Chain Reaction (PCR) using designated primers.PCR amplification was conducted using in 20µL reaction mixture containing 1X PCR assay buffer (10 mM Tris HCl (pH 8.3), 1.5 mM MgCl2, 50 mM of KCl, 200 µM each dNTP, and 1U of Taq DNA polymerase; Merk Millipore), 0.2 nmole of each primer, and 200 ng of purified DNA template of each sample.The PCR amplification was conducted using a Master Cycler gradient PCR machine(Eppendorf). The amplified products were subjected to 2.0% agarose gel electrophoresis in 1X TAE buffer, stained with ethidium bromide (10 mg/ml), and visualized under a UV transilluminator before being photographed using a gel documentation system. (Bio- Rad Laboratories).

Klebsiella pneumoniae ATCC 700603 was used as positive control strain for TEM_{ESBL}, SHV_{ESBL}, and $CTX\text{-}M_{ESBL}$. *Klebsiella pneumoniae* ATCC BAA-2146 and *K.pneumoniae* ATCC BAA-1705 strains werepositive controls for $NDM\text{-}1_{bla}$ and KPC_{bla}, respectively. The PCR product of $OXA\text{-}48_{bla}$ positive isolate (307 bp size) was confirmed by DNA sequencing and further used as positive control. Control for $VIM_{bla}$to be added.

OBSERVATION AND RESULTS:

COMBINATION OF ESBL AND MBL GENES IN *Escherichia Coli*AND *Klebsiella Pneumoniae*ISOLATES

Combination of ESBL genes in*Escherichia coli (91)* and *Klebsiella pneumoniae(161)* isolates

The ESBL gene combinations in *E. coli* and *K. pneumoniae* isolates indicated that all three genes (TEM-ESBL, SHV-ESBL, and CTX-M-ESBL) were present in 20.49% of K. pneumoniae isolates and 9.89% of E. coli isolates (Table 1). The combinations of any two ESBL genes studied in the present research were more prevalent in *Klebsiella pneumoniae* isolates compared to *E. coli* isolates.

Table 1 Combination of ESBL genes in *Escherichia coli*and in*Klebsiella pneumoniae* isolates.

ESBL genes	*E. coli*	*K. pneumoniae*
TEM+ SHV+CTXM	9 (9.89)	33 (20.49)
SHV+CTXM	14 (15.38)	41 (25.46)
TEM+ CTXM	20 (21.97)	62 (38.50)
TEM+ SHV	15 (16.48)	54 (33.54)

The analysis of MBL genes revealed no significant difference in their prevalence between the isolates (Table 2). The combination of all four genes included in the present study were present in single isolate of *E. coli* and*K. pneumoniae*. The combination of NDM-1_{bla} and KPC_{bla} was most prevalent in both *E. coli* (35.16 %) and *K. pneumoniae* (18.63 %) followed by NDM-1_{bla} +OXA-48_{bla} and KPC_{bla} +OXA-48_{bla}.

The analysis of the combination of ESBL and MBL genes in the study revealed that all the genes were present in a single *E. coli* isolate (Table 3). The combination of TEM_{ESBL}, $CTXM_{ESBL}$, NDM-1_{bla}, and KPC_{bla} was prevalent in 5.49 % *E. coli* isolates and 8.69 % *K. pneumoniae* isolates. Thecombination of various ESBL and MBL genes in *E. coli* and *K. pneumoniae* isolates is summarized in Table 3.

A comparison of antibiotic resistance and the prevalence of β-lactam genes was conducted between *E. coli* and *K. pneumoniae* isolates.

The comparison of the antibiotic resistance between *E. coli* and*K. pneumoniae*revealed that *K. pneumoniae*isolates were more resistant to Amoxicillin, Ceferoxime, Ceftriaxone, Cefoperazone, Meropenem, Amikacin, and Gentamicin than *E. coli* isolates. Principal component analysis showed no significant difference in antibiotic resistance between *Escherichia coli* and *Klebsiella pneumoniae*(Table 4).

The comparison of the proportion of the β-lactam producing genes between *E. coli* and *K. pneumoniae* revealed that TEM and SHV were more prevalent in *E. coli* isolates while NDM-1 and KPC were more prevalent in*Klebsiella pneumoniae*isolates.

Principal component analysis showed no difference in the prevalence of ESBL and MBL genes between *E. coli* and *Klebsiella pneumoniae* isolates (Table 5).

Table 2. Combination of MBL genes in *E. coli* and *K. pneumoniae*isolates.

MBL genes	*E. coli*	*K. pneumoniae*
NDM-1+ KPC+OXA-48+VIM	1 (1.09)	1 (0.62)
NDM-1+ KPC+OXA-48	7 (7.69)	7 (4.34)
NDM-1+ KPC	32 (35.16)	30 (18.63)

NDM-1+ OXA-48	8 (8.79)	13 (8.07)
KPC+OXA-48+VIM	1 (1.09)	3 (1.86)
KPC+OXA-48	8 (8.79)	13 (8.07)
OXA-48+VIM	1 (1.09)	5 (3.10)
NDM-1+ KPC+VIM	7 (7.69)	3 (1.86)
KPC+VIM	10 (10.98)	8 (4.96)
NDM-1+ OXA-48+VIM	1 (1.09)	2 (1.24)
NDM-1+VIM	7 (7.69)	11 (6.83)

Table 3. Combination of the ESBL and MBL genes in *E. coli* and *K. pneumoniae* isolates.

ESBL genes	MBL genes	*E. coli*	*K. pneumoniae*
TEM+ SHV+CTXM	**NDM1+ KPC+OXA48+VIM**	1 (1.09)	0
SHV+CTXM	**NDM1+ KPC+OXA48+VIM**	0	0
TEM+ CTXM	**NDM1+ KPC+OXA48+VIM**	0	0
TEM+ SHV	**NDM1+ KPC+OXA48+VIM**	1 (1.09)	1 (0.62)
TEM+ SHV+CTXM	**NDM1+ KPC+OXA48**	1 (1.09)	1 (0.62)
SHV+CTXM	**NDM1+ KPC+OXA48**	2 (2.19)	1 (0.62)
TEM+ CTXM	**NDM1+ KPC+OXA48**	2 (2.19)	4 (2.48)
TEM+ SHV	**NDM1+ KPC+OXA48**	1 (1.09)	4 (2.48)
TEM+ SHV+CTXM	**NDM1+ KPC+VIM**	1 (1.09)	3 (1.86)
SHV+CTXM	**NDM1+ KPC+VIM**	1 (1.09)	2 (1.24)
TEM+ CTXM	**NDM1+ KPC+VIM**	2 (2.19)	2 (1.24)
TEM+ SHV	**NDM1+ KPC+VIM**	1 (1.09)	2 (1.24)
TEM+ SHV+CTXM	**NDM1+ OXA48+VIM**	1 (1.09)	0
SHV+CTXM	**NDM1+ OXA48+VIM**	1 (1.09)	0
TEM+ CTXM	**NDM1+ OXA48+VIM**	1 (1.09)	0
TEM+ SHV	**NDM1+ OXA48+VIM**	1 (1.09)	1 (0.62)
TEM+ SHV+CTXM	**KPC+OXA48+VIM**	1 (1.09)	2 (1.24)
SHV+CTXM	**KPC+OXA48+VIM**	1 (1.09)	2 (1.24)
TEM+ CTXM	**KPC+OXA48+VIM**	1 (1.09)	2 (1.24)
TEM+ SHV	**KPC+OXA48+VIM**	1 (1.09)	3 (1.86)
TEM+ SHV+CTXM	**NDM1+ KPC**	1 (1.09)	6 (3.72)
SHV+CTXM	**NDM1+ KPC**	3 (3.29)	6 (3.72)
TEM+ CTXM	**NDM1+ KPC**	5 (5.49)	14 (8.69)
TEM+ SHV	**NDM1+ KPC**	2 (2.19)	9 (5.59)
TEM+ SHV+CTXM	**KPC+VIM**	2 (2.19)	3 (1.86)
SHV+CTXM	**KPC+VIM**	2 (2.19)	3 (1.86)
TEM+ CTXM	**KPC+VIM**	4 (4.39)	3 (1.86)
TEM+ SHV	**KPC+VIM**	3 (3.29)	7 (4.34)
TEM+ SHV+CTXM	**OXA48+VIM**	2 (2.19)	4 (2.48)

SHV+CTXM	**OXA48+VIM**	1 (1.09)	2 (1.24)
TEM+ CTXM	**OXA48+VIM**	1 (1.09)	2 (1.24)
TEM+ SHV	**OXA48+VIM**	1 (1.09)	2 (1.24)

Table 4. The principal component analysis of antibiotic resistance in *E. coli* and *K. pneumoniae* isolates.

	Eigenvalue	**%variance**
PC1	1.06	45.13
PC2	0.28	11.84
PC3	0.21	9.12
PC4	0.16	6.85
PC5	0.14	5.76
PC6	0.11	4.65
PC7	0.09	4.01
PC8	0.09	3.70
PC9	0.07	3.09
PC10	0.06	2.47
PC11	0.04	1.77
PC12	0.04	1.62

Table 5. The principal component analysis of the prevalence of genes in *E. coli* and *K. pneumoniae* isolates.

	Eigenvalue	% variance
PC 1	0.39	27.23
PC 2	0.30	21.04
PC 3	0.20	14.15
PC 4	0.18	12.50
PC 5	0.15	10.72
PC 6	0.12	8.04
PC 7	0.09	6.32

Fig 1. The *E. coli* and *K. pneumoniae* isolates focused on antibiotic resistance and the prevalence of β-lactam genes.

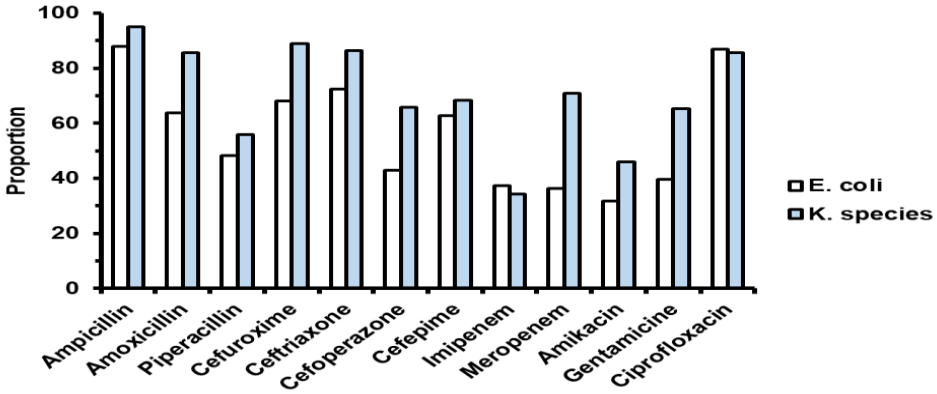


Fig 2.Principal component of antibiotic resistance in *E. coli* and *K. pneumoniae* isolates

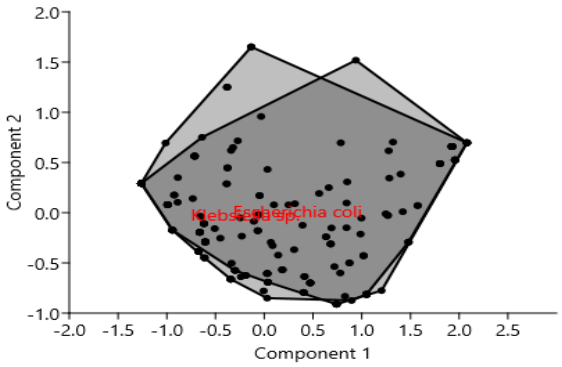


Fig 3.Comparison of the prevalence β-lactam producing genes in *E. coli* and *K. pneumoniae*

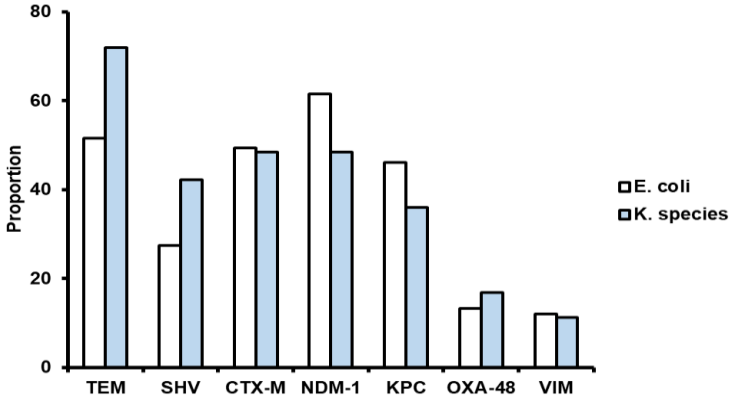
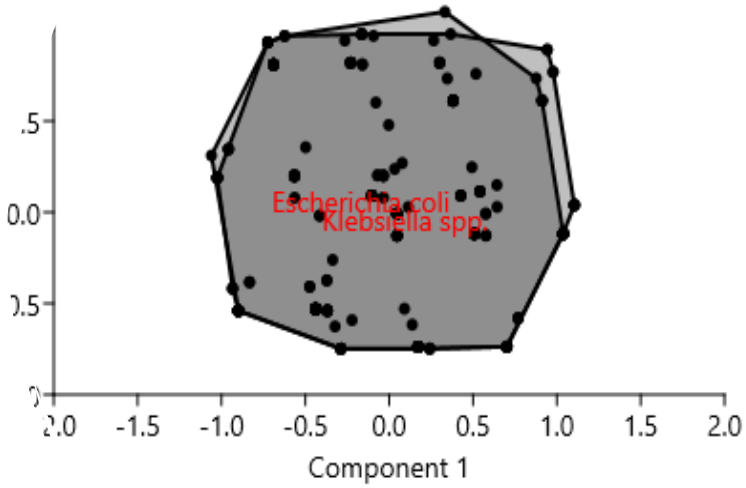


Fig 4.Principal component analysis of the prevalence of ESBL and MBL genes in *E. coli* and *K. pneumoniae* isolates



DISCUSSION:

To develop effective strategies to control the infections in DFUs, the antibiotic resistance in inhabitant bacteria and their genetic basis need to be evaluated (Blair et al., 2015; Martinez, 2014). The evaluation of the antibiotic resistance mechanism in bacteria is the preliminary step toward understanding the genetic basis of antibiotic resistance. In this thesis, I first reviewed India studies related to the etiology and microbiology of DFUs and provide comprehensive results on the ESBL and MBL production in two common pathogens, *E. coli* and *Klebsiella pneumoniae*,in relation to their genetic composition

Comparison of *E. coli* and *Klebsiella pneumoniae* isolates regarding antibiotic resistance and the prevalence of genes.

The study found no significant difference in the proportion of ESBL and MBL-producing *E. coli* and *Klebsiella pneumoniae* isolates from DFUs. The proportion of ESBL and MBL positive *E. coli* and *K. species* isolates was comparable. No marked difference in the antibiotic resistance of *E. coli* and *Klebsiella* species isolates to the antibiotics tested in the present study. Moreover, there was no variation in the prevalence of genes associated with ESBL and MBL production between *E. coli* and *Klebsiella* species isolates. The comparison between *E. coli* and *Klebsiella* species isolates suggests that antibiotic resistance, along with the genes responsible for ESBL and MBL production, is widespread in these isolates from Maharashtra, regardless of the species.

Additionally, a significant proportion of Indian population is diabetic and composed of diverse ethnic groups. Proportion of diabetic patients and associated complications also vary in different geographic zones (Kale et al., 2023; Pradeepa and Mohan, 2021). Recently, Shukla et al. (2023) studied the state-wise distribution of the genes involved in ESBL and Carbapenem resistance in the genomes of the *K. pneumoniae* isolates from India and revealed that CTX-M$_{ESBL}$ is the most prevalent gene followed by TEM$_{ESBL}$, OXA$_{bla}$, and NDM$_{bla}$. The present study demonstrated that the TEM$_{ESBL}$are most of prevalent gene in *K. pneumoniae* isolates followed by CTX-M$_{ESBL}$ and NDM in the western Indian state Maharashtra. These results suggest that there are significant variations in the geographic distribution of the genes involved in antibiotic resistance.

DFUs may have mono-microbial or poly-microbial infections (Kale et al., 2023). In the poly-microbial infections, bacteria cooperate and establish symbiotic relations which confer the maintenance of the infection and sometimes the production of toxins (Noor et al., 2015a). In poly-microbial infection, each species of bacteria may have different gene pool and able to secrete variety of enzymes which could contribute to the antibiotic resistance and delayed wound healing (Noor et al., 2015a; Wright, 2007). Beta-lactamases can be found in the outer membrane vesicles or extracellular vesicles of the bacterial cell wall and extra cellular spaces around producers. These beta-lactamases can protect producers and entire poly-microbial colony as well from antibiotics (Orazi and O'Toole, 2019). Moreover, the biofilm formation by pathogenic bacteria in diabetic foot infections is also an emerging factor responsible for the antibiotic resistance (James et al., 2008; Mah and O'Toole, 2001). The bacteria inhabiting poly-microbial colony are also able to exchange genes through horizontal transfer and produce chemicals that alter antibiotic tolerance, drug sensitivity, and cell wall (Orazi and O'Toole, 2019; Reygaert, 2018). Therefore, to understand the complete scenario, antibiotic resistance in different bacterial isolates from DFUs and their combined gene pool need to be evaluated.

The relation between the prevalence of the genes involved in beta-lactam antibiotic resistance and their geographic distributions need to be further investigated considering the grade of urbanization, and ethnicity of the population. Further, the possibility of the

involvement of other genes in antibiotic resistance cannot be denied as the prevalence of the genes (presently investigated) involved in ESBL and MBL antibiotic resistance was similar in all isolates irrespective of the presence of these antibiotic resistance mechanism. Contrastingly, Chaudhry et al. (2016) reported that 100% ESBL positive *K. pneumoniae* isolates were carrying associated genes (TEM_{ESBL}, SHV_{ESBL}, and $CTX\text{-}M_{ESBL}$).

SUMMARY:

The study shows that the majority of the *E. coli* and *K. pneumoniae* isolates.

E. coli and *K. species* isolates with respect to the presence of ESBL and MBL production in the isolates and the patient sex from which the isolates were extracted. Overall, $TEM_{ESBL}$are the prevalent gene in all isolates followed by $NDM\text{-}1_{bla}$, $CTX\text{-}M_{ESBL}$, KPC_{bla} and SHV_{ESBL} while $OXA\text{-}48_{bla}$ and VIM_{bla} were the least prevent gene in all isolates. TEM_{ESBL} and $CTX\text{-}M_{ESBL}$ genes were more prevalent in ESBL –ve isolates than ESBL +ve isolates. The prevalence of TEM_{ESBL} gene was high in MBL +ve isolates than MBL –ve isolates.

CONCLUSION:

The combination of any two ESBL genes was more prevalent in *Klebsiella pneumoniae*, isolates than *E. coli* isolates. There was no considerable difference in the prevalence of MBL genes in both *K. species* than *E. coli* isolates. Also, there was no difference in the antibiotic resistance and the prevalence of the genes involved in ESBL and MBL production between *E. coli* and *K. species* isolates, suggesting that the involvement of other factors in the antibiotic resistance in these isolates. These results highlight the necessity of the monitoring of antibiotic resistance in common pathogens over the time and evaluation of their genetic basis of antibiotic resistance.

CONSENT:

As per international standard or university standard, patient's informed consent and ascent was taken.

DATA AVAILABILITY:

The article contains the appropriate and proper data obtained during the experiment which supports the research article's result, discussion and conclusion.

HUMAN AND ANIMAL RIGHTS:

This research is derived from studies that excluded the use of animals or human.

FUNDING:

None.

CONFLICT OF INTEREST:

This manuscriptis free of any conflicts of interest.

REFERENCES:

1. Pradeepa, R., Mohan, V., 2021. Epidemiology of type 2 diabetes in India. Indian J Ophthalmol 69, 2932. https://doi.org/10.4103/ijo.IJO_1627_21
2. Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B.B., Stein, C., Basit, A., Chan, J.C.N., Mbanya, J.C., Pavkov, M.E., Ramachandaran, A., Wild, S.H., James, S., Herman, W.H., Zhang, P., Bommer, C., Kuo, S., Boyko, E.J., Magliano, D.J., 2022. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Research and Clinical Practice 183, 109119. https://doi.org/10.1016/j.diabres.2021.109119
3. Tinajero, M.G., Malik, V.S., 2021. An Update on the Epidemiology of Type 2 Diabetes. Endocrinology and Metabolism Clinics of North America 50, 337–355. https://doi.org/10.1016/j.ecl.2021.05.013
4. Grunfeld, C., 1992. Diabetic foot ulcers: etiology, treatment, and prevention. Adv Intern Med 37, 103–132.Gupta, S., Mujawdiya, P., Maheshwari, G., Sagar, S., 2022. Dynamic Role of Oxygen in Wound Healing: A Microbial, Immunological, and Biochemical Perspective. Archives of Razi Institute 77. https://doi.org/10.22092/ari.2022.357230.2003
5. Boyko, E.J., Ahroni, J.H., Smith, D.G., Davignon, D., 1996. Increased Mortality Associated with Diabetic Foot Ulcer. Diabet. Med. 13, 967–972. https://doi.org/10.1002/(SICI)1096-9136(199611)13:11<967::AID-DIA266>3.0.CO;2-K\
6. Singh, N., 2005. Preventing Foot Ulcers in Patients With Diabetes. JAMA 293, 217. https://doi.org/10.1001/jama.293.2.217
7. Williams, D.T., Hilton, J.R., Harding, K.G., 2004. Diagnosing Foot Infection in Diabetes. Clinical Infectious Diseases 39, S83–S86. https://doi.org/10.1086/383267
8. Peterson, E., Kaur, P., 2018. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Front. Microbiol. 9, 2928. https://doi.org/10.3389/fmicb.2018.02928
9. Van Asten, S.A.V., La Fontaine, J., Peters, E.J.G., Bhavan, K., Kim, P.J., Lavery, L.A., 2016. The microbiome of diabetic foot osteomyelitis. Eur J ClinMicrobiol Infect Dis 35, 293–298. https://doi.org/10.1007/s10096-015-2544-1
10. Boulton, A.J., Vileikyte, L., Ragnarson-Tennvall, G., Apelqvist, J., 2005. The global burden of diabetic foot disease. The Lancet 366, 1719–1724. https://doi.org/10.1016/S0140-6736(05)67698-2
11. Karri, V.V.S.R., Kuppusamy, G., Talluri, S.V., Yamjala, K., Mannemala, S.S., Malayandi, R., 2016. Current and emerging therapies in the management of diabetic foot ulcers. Current Medical Research and Opinion 32, 519–542. https://doi.org/10.1185/03007995.2015.1128888
12. Hammer, O., Harper, D., 2001. PAST: Palentological statistics software package for education and data analysis. Palaeontologia Electronica 4, 1–9.Hills, A.P., Arena, R., Khunti, K., Yajnik, C.S., Jayawardena, R., Henry, C.J., Street, S.J., Soares, M.J., Misra, A., 2018. Epidemiology and determinants of type 2 diabetes in south Asia. The Lancet Diabetes & Endocrinology 6, 966–978. https://doi.org/10.1016/S2213-8587(18)30204-3
13. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; CLSI document M100-S31.20.

14. Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V., 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13, 42–51. https://doi.org/10.1038/nrmicro3380
15. Martinez, J.L., 2014. General principles of antibiotic resistance in bacteria. Drug Discovery Today: Technologies 11, 33–39. https://doi.org/10.1016/j.ddtec.2014.02.001
16. Kale, D., Karande, G., Datkhile, K., 2023. Diabetic foot ulcer in India: Aetiological trends and bacterial diversity. Indian J EndocrMetab 27, 107. https://doi.org/10.4103/ijem.ijem_458_22
17. Shukla, S., Desai, S., Bagchi, A., Singh, P., Joshi, M., Joshi, C., Patankar, J., Maheshwari, G., Rajni, E., Shah, M., Gajjar, D., 2023. Diversity and Distribution of β-Lactamase Genes Circulating in Indian Isolates of Multidrug-Resistant Klebsiella pneumoniae. Antibiotics 12, 449. https://doi.org/10.3390/antibiotics12030449
18. Noor, S., Zubair, M., Ahmad, J., 2015a. Diabetic foot ulcer—A review on pathophysiology, classification and microbial etiology. Diabetes & Metabolic Syndrome: Clinical Research & Reviews 9, 192–199. https://doi.org/10.1016/j.dsx.2015.04.007
19. Wright, G.D., 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol 5, 175–186. https://doi.org/10.1038/nrmicro1614
20. Orazi, G., O'Toole, G.A., 2019. "It Takes a Village": Mechanisms Underlying Antimicrobial Recalcitrance of Polymicrobial Biofilms. J Bacteriol 202. https://doi.org/10.1128/JB.00530-19
21. James, G.A., Swogger, E., Wolcott, R., Pulcini, E. deLancey, Secor, P., Sestrich, J., Costerton, J.W., Stewart, P.S., 2008. Biofilms in chronic wounds. Wound Repair and Regeneration 16, 37–44. https://doi.org/10.1111/j.1524-475X.2007.00321.x
22. Mah, T.-F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9, 34–39. https://doi.org/10.1016/S0966-842X(00)01913-2
23. Reygaert, W., 2018. An overview of the antimicrobial resistance mechanisms of bacteria.AIMS Microbiology 4, 482–501. https://doi.org/10.3934/microbiol.2018.3.482