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Incidence of Multi-Drug-Resistant Bacterial Pathogens from Pus Samples of Diabetic Foot Ulcer Patients Collected at a Tertiary Care Hospital in North Kerala, India

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Abstract

Background: Diabetes mellitus is a prevalent metabolic disorder, particularly affecting individuals over 45, with 529 million people worldwide impacted. Uncontrolled diabetes can lead to complications, including diabetic foot ulcers (DFU), which are responsible for 20% of morbidity and 25% of mortality in diabetic patients, especially in developing countries. Pathogenic bacteria cause infections in DFUs, and recovery is influenced by bacterial resistance to antibiotics. This study aimed to determine drug resistance rates of pathogens in DFUs and their impact on morbidity in a tertiary care hospital in North Kerala, India.
Materials and Methods: Pus samples from DFU patients were collected between 2018 and 2022, and pathogens were identified through biochemical tests. Antibiotic resistance was assessed using the Kirby-Bauer disc diffusion method.
Results: Of 3008 samples, 2920 showed significant bacterial growth. The majority of samples were from patients aged 51–60 years. Among the positive samples, 85.9% were males, and 14.1% were females. The bacterial distribution was 66.8% Gram-negative and 33.2% Gram-positive. Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumoniae were the most common pathogens. Of 621 Staphylococcus aureus isolates, 48.9% were methicillin-resistant. Among the 1949 Gram-negative isolates, 8.3% were extended-spectrum beta-lactamase (ESBL) resistant, and 5.6% were carbapenem-resistant.
Conclusion: The study concludes that the increasing resistance of DFU pathogens complicates treatment. Culturing and identifying causative organisms and understanding their susceptibility are crucial for effective management of infections in diabetic patients.

Keywords: Diabetes Mellitus, Drug Resistance, DFU, Staphylococcus Aureus.

Introduction

Diabetes mellitus is the most common metabolic disorder resulting from defects in insulin secretion, insulin action, or both [1]. Insulin, produced by the beta cells of the pancreas, helps utilize glucose from digested food as a source of energy [2]. Diabetes is classified into four types: Type I, Type II, Type III, and Type IV [1,2]. Type I diabetes accounts for about 5-10% of diagnoses and typically manifests in childhood [2]. Type II diabetes, which usually develops after the age of 40, constitutes around 90-94% of cases [3]. Worldwide, approximately 529 million people are affected by diabetes mellitus, and this number is projected to double by 2050 [4]. Uncontrolled diabetes can lead to several complications, including diabetic foot ulcers (DFU), diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy [5,6]. Among these, DFU is the most significant complication, contributing to the highest morbidity and mortality rates, especially in developing countries [7].
Diabetic foot ulcers (DFU) are associated with several pathological complications, including neuropathy, peripheral vascular diseases, foot ulceration, and

infections. These infections can lead to gangrene and may ultimately necessitate limb amputation [8]. Infections can be caused by pathogenic bacteria from the external environment or bacteria from the skin's microflora [9]. The extent of the infection depends on the number of microorganisms present in the wound, while the healing process is influenced by the type of bacterial strain and its pathogenicity [10]. Mortality following amputation is now higher than that associated with many malignancies [11,12]. Therefore, preventing amputation is crucial in managing DFU [11]. Timely diagnosis and prompt treatment are essential to avoid amputation and improve patient outcomes [13].

Proper management of infections in diabetic foot ulcers (DFU) requires the appropriate selection of antibiotics based on culture and sensitivity reports [14]. Effective, definitive therapy should include antimicrobial treatment, proper anti-diabetic care, and patient education on properly managing their lesions to improve overall outcomes [15]. Several studies have highlighted that infections with multidrug-resistant organisms have become a significant problem in DFU cases [16]. These infections increase hospital stay durations and treatment costs and can ultimately lead to amputation [17]. Given the growing prevalence of multidrug-resistant organisms, ensuring the accurate use of antimicrobial agents has become a national priority [18].

The rise in antimicrobial resistance is critical in developing countries like India. There is insufficient data on the prevalence of multidrug-resistant infections and their outcomes in patients with diabetic foot ulcers (DFU) in this region [19]. This study aims to identify the pathogens causing DFU and determine their antimicrobial susceptibility patterns in patients at a tertiary care hospital in North Kerala.

Materials and Methods

This is a retrospective study conducted at MES Medical College and Hospital, a 600-bed tertiary care hospital in the Malappuram district of North Kerala, India. A total of 3,008 sample data points were systematically collected from the microbiology clinical laboratory records from January 2018 to December 2022. The wound surface was thoroughly cleaned with saline to minimize the risk of isolating normal skin commensals instead of pathogens. Specimens were then collected by scraping the ulcer base, performing wound curettage, aspirating pus, and obtaining necrotic tissue and bony fragments (Fig. 1).

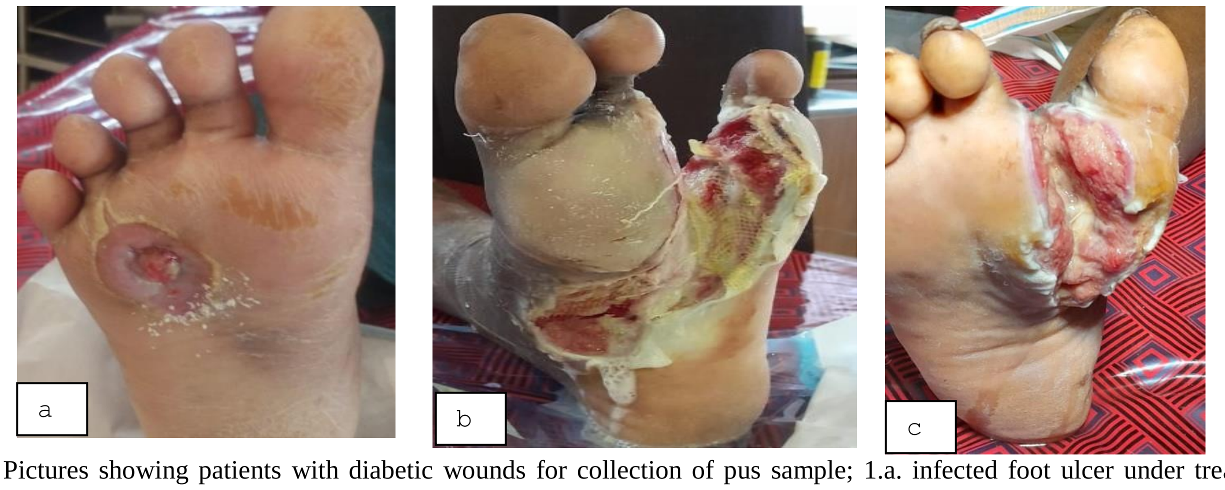

Fig.1. Pictures showing patients with diabetic wounds for collection of pus sample; 1.a. infected foot ulcer under treatment; 1.b. infected foot ulcer with amputation of two fingers; 1.c. infected foot ulcer with amputation of a finger by MRSA infection

Bacterial identification method: Pus samples were initially screened using Gram staining. The samples were then sub-cultured onto MacConkey and blood agar and incubated at 37°C. The isolates were identified at the species level using standard microbiological methods, as outlined by the Clinical Laboratory Standards Institute (CLSI) [19,20].

Antimicrobial susceptibility testing (AST): Antibiotic susceptibility testing (AST) was performed using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar plates with Oxoid antibiotic discs. The antibiotic panel included gentamicin (10 µg), clindamycin (2 µg), linezolid (30 µg), erythromycin (15 µg), ampicillin (10 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), amoxicillin-clavulanic acid (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), amikacin (10 µg), doxycycline (30 µg), vancomycin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), and colistin (10 µg), as recommended by the Clinical and Laboratory Standards Institute (CLSI) [19,20]. Standard reference strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus* aureus ATCC 29213 were also included in the assay for quality control. The antibiotic susceptibility of each isolate was interpreted as sensitive, intermediate, or resistant, according to CLSI guidelines [17].

Identification of multidrug-resistant organisms: Organisms that exhibited non-susceptibility to at least one antimicrobial agent in three or more classes of antimicrobial agents were classified as multidrug-resistant (MDR) pathogens. These MDR organisms were further categorized based on their specific resistance profiles into methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase (ESBL) producers, and carbapenem-resistant *Enterobacteriaceae*.

Staphylococcus aureus was considered methicillin-resistant if the zone of inhibition using a cefoxitin disc (30 µg) was ≤21 mm, while a zone diameter of ≥22 mm was considered sensitive, by CLSI guidelines [21]. Gram-negative bacilli were further tested for extended-spectrum beta-lactamase (ESBL) production using the double-disc diffusion method with ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10 µg). An increase in the zone of inhibition of ≥5 mm with ceftazidime/clavulanic acid compared to ceftazidime alone was considered a positive result for ESBL production [22]. Resistance to carbapenems and colistin in Gram-negative *Enterobacteriaceae* was classified as carbapenem-resistant *Enterobacteriaceae* and colistin-resistant organisms, respectively.

Multiple Antibiotic Resistance (MAR) Index Detection: Commonly used antibiotics in human therapy were selected, as these are typically administered either orally or by injection to treat various diseases [23]. The Multiple Antibiotic Resistance (MAR) index for each isolate was determined using the formula: MAR = a/b, where 'a' represents the number of antibiotics to which the test isolate showed resistance, and 'b' represents the total number of antibiotics for which the isolate's susceptibility was evaluated [24].

Results

A total of 3008 pus samples from diabetic foot ulcers were analyzed over a five-year period (January 2018 – December 2022), of which 2920 were culture-positive. The inclusion criteria were patients with DFU who visited or were referred to our tertiary care hospital, while the exclusion criteria were patients who did not meet the definition of DFU. The percentage of positive cases ranged between 96.4% and 97.3% each year, indicating that approximately 97% of DFU cases were bacterial culture-positive with significant growth.

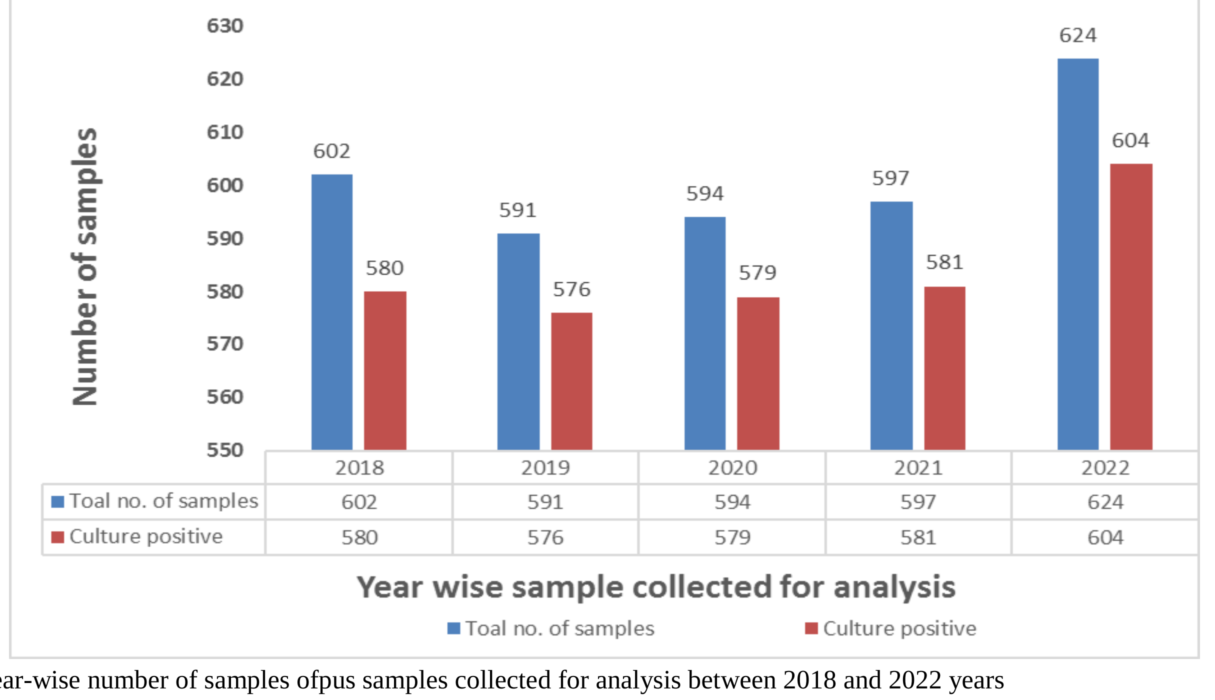

Fig. 2. Year-wise number of samples ofpus samples collected for analysis between 2018 and 2022 years

Isolation of bacteria from diabetic foot ulcer pus samples: The samples were streaked onto selective agar plates and observed for cultural characteristics such as growth, color, and colony morphology. After 24 hours of aerobic incubation, 2920 samples showed significant bacterial growth out of 3008 diabetic foot ulcer samples, as illustrated in Graph 1. The number of hospital patients remained relatively stable each year, with no notable decrease in cases. However, a slight increase in affected individuals was recorded over the study period.

Age-wise distribution: Samples from diabetic foot ulcers were collected, and growth on culture media, along with cultural characteristics and colony color, were recorded. The most samples were collected from patients aged between 51 and 60 years [25]. The age-wise distribution of samples is presented in Table 1.

Table 1. The samples were analyzed gender-wise and age-wise

Year	2018		2019		2020		2021		2022	
Age group	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
<1	0	0	0	0	0	0	0	0	0	0
1 to 10	0	0	0	0	0	0	0	0	0	0
11 to 20	0	0	0	0	0	0	0	0	0	0
21 to 30	0	0	0	0	0	0	0	0	0	0
31 to 40	17	0	18	0	15	0	21	0	18	0
41 to 50	102	16	117	17	107	14	112	13	114	19
51 to 60	168	43	169	34	166	39	170	39	172	46
61 to 70	143	23	147	21	146	21	145	19	149	23
71 to 80	48	7	39	0	53	9	48	4	47	5
81 to 90	13	0	11	0	9	0	13	0	11	0
Total no. of cases	**491**	**89**	**501**	**72**	**496**	**83**	**509**	**75**	**511**	**93**
					Total					**2920**

Gender-wise analysis of samples: Out of the 3008 samples received and analyzed in the Microbiology Clinical Laboratory, 2920 showed culture-positive results. Among these culture-positive samples, males accounted for 85.9% (2508 samples), while females accounted for 14.1% (412 samples), as shown in Table 2 [18].

Gram staining and cultural characteristics revealed that 33.2% (971) of the isolates were Gram-positive, while 66.8% (1949) were Gram-negative organisms [22,26]. Thus, Gram-negative bacteria were the predominant isolates in diabetic foot ulcers (Fig. 3). Despite the higher number of Gram-negative pathogens, the most frequently encountered pathogen was Methicillin-resistant *Staphylococcus aureus* (MRSA), followed by *Pseudomonas aeruginosa* (P. aeruginosa).

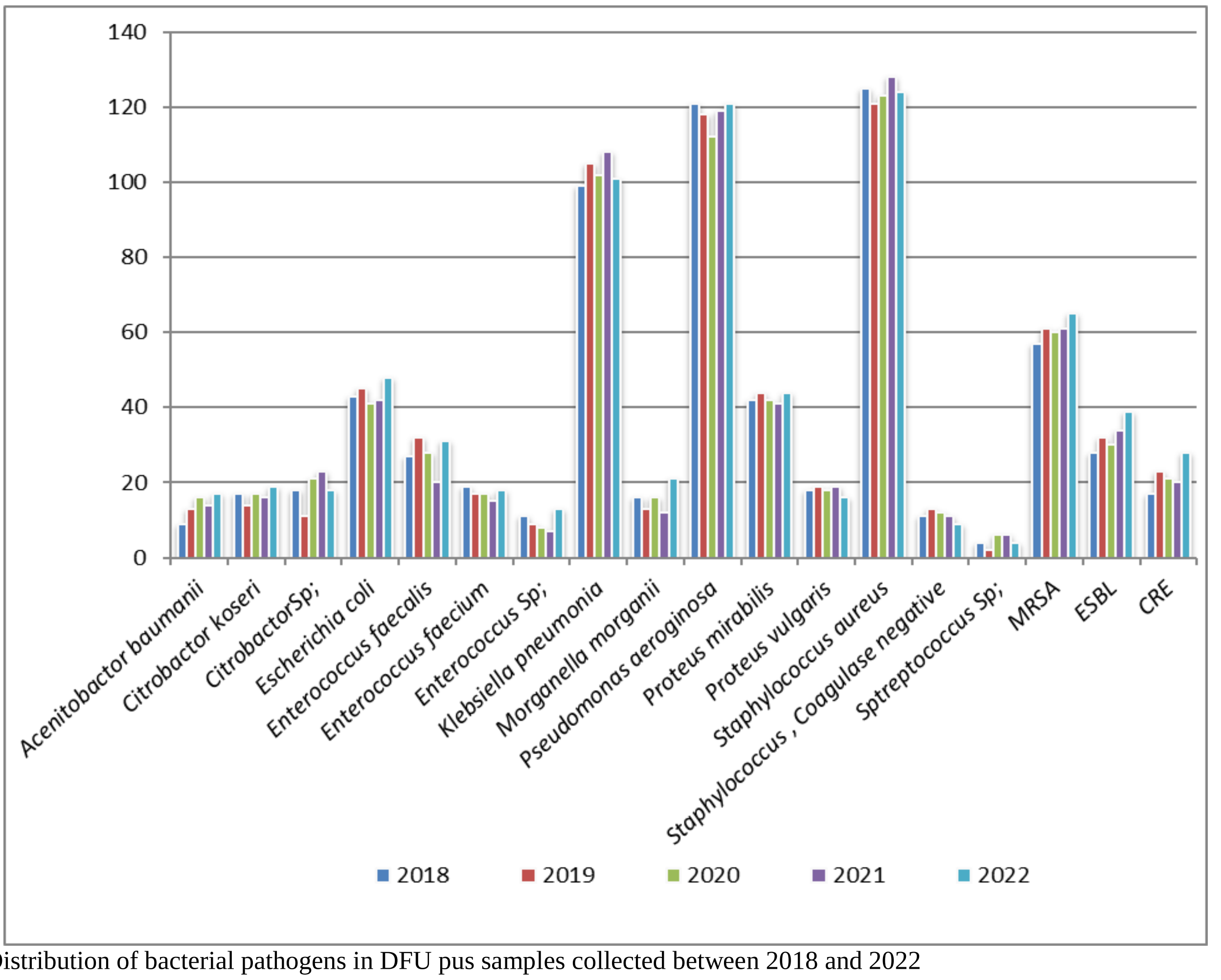

Fig. 3. Distribution of bacterial pathogens in DFU pus samples collected between 2018 and 2022

Organisms isolated from diabetic foot ulcers: The most predominant organisms identified each year in diabetic foot ulcers were *Staphylococcus aureus* (~120 to 130 cases per year), followed by *Pseudomonas aeruginosa* (~110 to 120 cases per year), *Klebsiella pneumoniae* (~100 to 110 cases per year), and others, as shown in Fig. 3 [27].

Emergence of multi-drug-resistant organisms: The isolates were tested for antibiotic sensitivity against common antibiotics, and most organisms showed susceptibility to these drugs. Among the 621 *Staphylococcus aureus* isolates, 304 (48.9%) were methicillin-resistant [18]. Of the 1949 Gram-negative pathogens, 163 (8.3%) were identified as extended-spectrum beta-lactamase (ESBL) producers, and 109 (5.6%) were carbapenem-resistant *Enterobacteriaceae*, as shown in Table 3. These results are concerning.

Table 2. Distribution of multi-drug-resistant organisms

Organism	2018	2019	2020	2021	2022	Total
Mrsa	57	61	60	61	65	**304**
Esbl	28	32	30	34	39	**163**
Cre	17	23	21	20	28	**109**

Multiple Antibiotic Resistance (MAR) Index calculation: The isolates' Multiple Antibiotic Resistance (MAR) index was determined. Bacteria with a MAR index ≥ 0.2 are considered to originate from high-risk sources of contamination where multiple antibiotics are commonly used [28, 29]. In our study, *Acinetobacter baumannii* showed the highest MAR index of 0.8, indicating a strong resistance pattern to commonly used antibiotics, followed by *Klebsiella pneumoniae* with a MAR index of 0.5. Other isolates had MAR index scores ranging from 0.1 to 0.4, as shown in Table 3.

Table 3. MAR Index score of isolates

Organism	Total number antibiotics used	Number antibiotic resistance	MAR Index
Acenitobactorbaumanii	17	19	0.8
Citrobactorsp.	8	17	0.5
Klebsiella pneumonia	8	17	0.5
Citrobactorkoseri	7	17	0.4
Proteus vulgaris	5	17	0.3
Escherichia coli	4	17	0.2
Enterococcus faecalis	1	6	0.2
Enterococcus faecium	1	6	0.2
Staphylococcus aureus	2	12	0.2
Morganellamorganii	4	17	0.2
Proteus mirabilis	3	17	0.2
Streptococcus sp.	1	12	0.1
Pseudomonas aeroginosa	1	9	0.1
Enterococcus sp.	1	7	0.1

Discussion

The prevalence of organisms in diabetic foot ulcers from 2018 to 2022 was analyzed. Gram-negative organisms were the most frequently isolated, with Staphylococcus aureus being the predominant Gram-positive organism. The distribution of Gram-negative and Gram-positive organisms was 66.8% and 33.2%, respectively. Gram-negative (GNB) and Gram-positive (GPB) contribute to infections in diabetic foot ulcers. The incidence of bacterial infections depends on factors such as the nature of the wound, its location, cleanliness, and other co-morbidities associated with diabetes mellitus, including diabetic neuropathy and uncontrolled diabetes. In this study, Gram-negative organisms were more prevalent than Gram-positive organisms, consistent with findings from a study conducted in the endocrinology ward at the All-India Institute of Medical Sciences [19].

In the current study, the majority (64.5%) of diabetic foot ulcers (DFU) were observed in elderly individuals aged between 51 and 70 years. The increased prevalence among the elderly can be attributed to several factors, including a longer duration of diabetes mellitus, the presence of multiple co-morbidities, and a reduced immune response with age [21, 30]. In the modern world, diabetes mellitus is increasingly recognized as a condition with metabolic, vascular, and neuropathic components that are interrelated [31].

The study was conducted among patients with diabetic foot infections over a five-year period. Each year, the study found that more than 80% of diabetic foot ulcers were infected with microorganisms [9, 21]. This highlights the severity of diabetic foot infections, as the percentage of culture positivity has not decreased over time. One possible reason for this persistent high rate of infection could be a lack of awareness about the potential consequences of untreated infections, which may ultimately lead to amputation. This issue is especially prevalent in developing countries like India, where public awareness and education about diabetes complications are limited.

The samples collected from patients with diabetic foot infections were analyzed to determine the occurrence among males and females. In this study, the male-to-female ratio was 6.1:1, with 2508 male cases compared to 409 female cases [32]. The higher incidence in males may be attributed to greater exposure to trauma, particularly from heavy manual labor or physical activities [33].

The collected samples were analyzed for isolates and the predominance of organisms. This study identified Staphylococcus aureus as the most predominant organism, followed by Gram-negative isolates such as Pseudomonas aeruginosa and Klebsiella pneumoniae. These findings are consistent with the study conducted by Dr. Asima Banu et al. on the spectrum of bacteria associated with diabetic foot ulcers and biofilm formation [34].

Diabetic foot infections pose one of the greatest challenges in many developing countries. Treating these infections is increasingly difficult due to the emergence of multidrug-resistant strains [35]. The study highlights the high prevalence of multidrug-resistant strains, such as MRSA and ESBL producers, among diabetic foot ulcers, significantly complicating treatment and management [6].

Among the 621 Staphylococcus aureus isolates, 304 were identified as MRSA (Methicillin-resistant Staphylococcus aureus). The prevalence of MRSA in this study was similar to that reported by Dr. Asima Banu et al. on the spectrum of bacteria associated with diabetic foot ulcers and biofilm formation: A prospective study [34].

Over the study's five years, the infection rate with MRSA has been increasing (Table 3). This trend indicates a lack of an optimal antimicrobial regimen in the region and insufficient awareness about multidrug-resistant organisms [36]. If not addressed as a serious issue, this could significantly contribute to the mortality rate in the country.

In this study, Gram-negative organisms were the most frequently isolated group, with approximately 40-50% being ESBL-producing bacteria. This finding is consistent with many studies' reports [19, 37]. An increasing rate of carbapenem-resistant Enterobacteriaceae (CRE) was also observed [1]. The rise in CRE is particularly concerning, as carbapenems are considered one of the most effective antimicrobial agents [1]. The growing prevalence of drug-resistant strains such as ESBL and CRE highlights a serious challenge in the treatment of diabetic foot ulcers, especially in developing countries like India, Jordan, and others [21].

The Multiple Antibiotic Resistance Index (MARI) was analysed to assess antibiotic resistance and associated health risk factors. Bacteria with a MAR index ≥ 0.2 are considered to originate from high-risk sources of contamination where multiple antibiotics have been used [28]. The MAR index of the isolated organisms in this study is presented in Table 3. The MARI analysis revealed that 11 isolates (78.57%) had a MAR index greater than 0.2, while 3 isolates (21.43%) had a MAR index less than 0.2. Notably, three isolates showed a MAR index of 1, indicating resistance to all the antimicrobials tested. This finding is consistent with a study conducted by Raminder Sandhu et al. [24]. Organisms with MAR indices ≥ 0.2 suggest the presence of multidrug-resistant genes, which likely originate from environments where the misuse or overuse of antibiotics is prevalent [28].

Conclusion

The study reveals that diabetic foot infections have become a significant social and economic burden. These infections can be caused by a wide range of organisms, with Gram-negative bacteria being the most frequently isolated, followed by Gram-positive bacteria. The predominant organism identified was *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and others. The resistance patterns observed in these pathogens pose a serious threat in clinical settings, potentially leading to complications such as osteomyelitis, gangrene, limb amputation, and even mortality in diabetic patients. These outcomes highlight the importance of identifying risk factors in diabetic foot ulcers (DFU). Although the pathogens causing diabetic foot infections are generally sensitive to most routinely used antibiotics, resistance to certain drugs is increasingly observed throughout the study period. This growing resistance underscores the need for careful culture, identification of the causative agent, and a thorough understanding of its susceptibility pattern to ensure appropriate management of diabetic foot ulcers.

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Conflict of interest

None declared.

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Ethical Considerations

Ethical approval for the present study was granted by the standard protocols of the Committee for Control and Supervision of Experiments on Animals and Humans (CPCSEA).

Code of Ethics

The Institutional Ethics Committee of MES Medical College Hospital, Kerala, adhered to these protocols and approved (MESMCH/MES-IRB/Aug-2023, dated 14th August 2023).

Authors' Contributions

K Rajalakshmy: Collection of sample and clinical data, processing, recording of results and manuscript writing; P Saravana Kumari: Work plan, curation of the data, validation, editing of manuscript, and communications; Syed Mustaq Ahmed: Data validation, ethical clearance, and quality assurance.

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