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ANTIBIOGRAM ANALYSIS AND MOLECULAR CHARACTERIZATION OF MULTI-DRUG RESISTANT GENES (TETK AND GYRA) OF STAPHYLOCOCCUS AUREUS ISOLATED FROM DIABETIC FOOT ULCERS PATIENTS IN PESHAWAR

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Abstract

DFUs are one of the frequent complications of diabetic patients frequently resulting in the emergence of bacterial infections that make treatment difficult. The aim of this research was aimed at determining the antibiogram and molecular characterization of multidrug-resistant (MDR) genes, namely tetK and gyrA, in Staphylococcus aureus that was isolated on DFU patients in Peshawar, Pakistan. Sixty wound samples were taken out of DFU patients of Lady Reading Hospital, where more than 70% of respondents were males. Bacterial identification was done using biochemical tests (catalase and coagulase) and the samples were grown on the Mannitol Salt Agar (MSA). The Kirby-Bauer disc diffusion was utilized to identify the antibiotic susceptibility and PCR was done to identify the tetK and gyrA resistance genes. These findings indicated that rates of resistance to popular antibiotics were high with 100 percent resistance to ampicillin (100%), penicillin G (93%), and erythromycin (83%), with intermediate rates of resistance to tetracycline and ciprofloxacin. Further, the proportion of methicillin-resistant S. aureus (MRSA) and oxacillin-resistant of the isolates was 48 and 83 percent respectively. Molecular testing confirmed the existence of gyrA gene in 15 samples and tetK gene in 14 samples in which the two genes were important in antibiotic resistance. To sum up, the present paper highlights the increasing problem of antibiotic resistance to DFU infection. It highlights that persistent molecular monitoring and individualized treatment approaches should be used to provide effective treatment of infections among diabetic individuals.

Keywords: S. aureus, tetK, gyrAresistant genes, Resistant antibiotics.

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases that are characterized by chronically high blood glucose levels caused by the insulin synthesis, acting, or both deficiencies [1]. Depending on etiological factors and clinical manifestations, diabetes mellitus has four categories including type 1, type 2, gestational, and other special variants of the disease. Autoimmune destruction of 6310 percent of the pancreatic 5-cells is Type 1 diabetes which constitutes 5 10 percent of diabetes mellitus patients [2]. On the other hand, type 2 diabetes which is the most common form affecting approximately 90-95 percent of diabetics in the world is caused by insulin resistance and the progressive non-response of insulin production [3].

Diabetes has been on the increase in recent decades all over the world. The International Diabetes Federation (IDF) states that there are 463 million individuals with diabetes in the world in 2019, and that these numbers will grow to 578 million and 700 million in 2030 and 2045 respectively [4]. In Pakistan, DM was 19.9% in 2021, and was the third highest in the world. [5]. The migration to cities, inactivity, and poor diet are the primary causes of this alarming trend [6]. According to the World Health Organisation (WHO), DM has killed 1.6 million people across the globe in 2016 [7], and this imposes much pressure on the economy and health care systems. Diabetes care constituted 16 percent of the expenditure on

healthcare in 2019 in Pakistan. It will increase to $4.2 billion by 2030 [8].

Uncontrolled DM results in chronic hyperglycemia which damages several of the body organs including cardiovascular system, kidneys, eye, and the peripheral nerves [9] There are acute and chronic diabetes complications. An example of diabetic ketoacidosis and hypoglycemia are acute complications and diabetic retinopathy, nephropathy, neuropathy and peripheral artery disease are chronic complications. Also linked to DM are numerous emerging chronic complications including cancer, depression and dementia that contributes to further complications in health and economics [10].

One of the most common and harmful conditions of DM that may lead to devastating outcomes, including supplementation and death, is diabetic foot ulcers (DFUs). DFUs are estimated to lead to DFUs in 19-34 percent of patients with DM and 15 percent of those who suffer the condition amputated and 10 percent died in the first year of onset [11]. DFUs are increasing in Pakistan and this is aggravated by the inadequate healthcare facilities, lack of special care and patient education [12].

DFUs microbiological examination has revealed that it is polymicrobial and Staphylococcus aureus is one of the most common pathogens. These problems make the healing process harder as *S. aureus* in DFUs express various virulence factors that disrupt the wound healing process and enhance the intrusion of the tissues [13]. These virulence factors include cytotoxin including alpha toxin and beta toxin as well as biofilm formation, which protects the bacteria against host immune system, and antibiotic agents [14, 15]. Further, *S. aureus* has also acquired antimicrobial resistance (AMR) phenotypes, which include enzymatic inactivation, alteration of the target site, and resistance through efflux pumps [16, 17]. There is also a growing trend in the prevalence of methicillin-resistant S. aureus (MRSA) in DFUs, which complicates treatment because MRSA strains are very frequently co-carrying resistant to various antibiotics, which reduces the choice of treatment [18].

The study objectives will be confined to isolating and identifying S. aureus by pus sample of DFU patients, identify the antibiogram patterns of the isolates, identify the patterns of methicillin resistant S. aureus (MRSA) and methicillin susceptible S. aureus (MSSA), detect multi-drug resistance genes (tetK and gyrA) in S. aureus by multiplex PCR.

MATERIALS AND METHODS

The study is a cross-sectional study that was carried out between August and November, 2023, at Lady Reading Hospital, MTI Peshawar, Pakistan, and the study was accepted upon by the head of the Department of Diabetes and Endocrinology. Each patient was given informed consent in a written and oral format. A total of 60 diabetic foot ulcer (DFU) patients were sampled in the study. The eligibility criteria involved diabetic patients aged 20-70s and found in the outpatient department of the hospital with open foot ulcers. The other exclusion criteria included the presence of foot ulcers which were not associated with diabetes or patients who had used antibiotics in the last 48 hours.

DFU patients provided samples using the sterile swabs based on tight aseptic precautions. Before collecting, the ulcers were washed on a normal saline to remove any native flora and contaminants and any necrotic tissue excised. The swabs were stored in a gel-containing transportation medium and delivered to the research laboratory in the Abasyn University in Peshawar where they were stored at 4 o C until they could be reexamined. Identification of S. aureus was done using Mannitol Salt Agar (MSA). Such a selective media permits the growth of S. aureus whilst preventing the growth of other organisms. The media was prepared through the standard processes and autoclaved at 121 o C at 15 minutes to destroy germs. The isolated colonies were further confirmed by biochemical tests, such as the catalase and coagulase tests.

To determine the sensitivity of the S. aureus isolates to the antibiotics, the Kirby-Bauer disc diffusion method was performed on Mueller-Hinton agar (MHA). The drugs employed were in agreement with CLSI (2021). Some of the antibiotics studied included ampicillin, penicillin G, ceftriaxone, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, cefoxitin, and oxacillin.

In order to perform molecular analysis, the isolates of bacteria of interest were isolated using Qiagen DNA isolation kit. The presence of multiplex resistance genes *tetK* and *gyrA* was detected by means of multiplex PCR. Primer sequences of these genes were designed to amplify gyrA and tetK 280 and 360 bp respectively with the assistance of the commercially available PCR master mix. The amplification products were displayed at agarose gel electrophoresis with the help of appropriate DNA ladders to determine the size of products.

A Nano Drop spectrophotometer was used to quantify DNA to be able to analyze the PCR by getting the optimal DNA concentration. PCR cycle reagents entailed an initial denaturation phase of 94oC 5 minutes, and 35 cycles of denaturation, annealing and extension. The presence of the target genes was confirmed by the gel electrophoresis process and the gels were viewed with the help of a UV lamp. The findings of these studies helped in the genetic characterization of antibiotic resistance in isolates of *S. aureus* in DFU patients and helped in the appreciation of the relevance of resistance genes in the pathogenesis of diabetic feet infection.

RESULTS

A total of sixty wound specimens were collected from type 2 diabetic patients who had amputations due to bacterial infection, followed by complications of neuropathy and peripheral arterial disease. Seventy percent were males, and thirty percent were females

(Table 01). The wound of every patient was washed with normal saline to kill normal flora and other contaminants, and the dead tissue was removed.

Table 01. DFUs Samples distribution

S. No.	Gender	Frequency	Percentage
1.	Female	18	30%
2.	Male	42	70%
	Total	60	100%

The catalase test revealed that *S. aureus* was catalase-positive, as evidenced by the production of air bubbles upon adding 3% hydrogen peroxide to the colonies. In the coagulase test, the presence of coagulation in human plasma indicated that the isolates were coagulase-positive, confirming the identity of *S. aureus*

Antibiotic Susceptibility Test for *S. aureus* Isolates

The susceptibility of *S. aureus* strains to various antibiotics was assessed (Figure 2). The findings illustrate the varying degrees of susceptibility of *S. aureus* strains to the tested antibiotics. High levels of resistance were observed for several antibiotics, including ampicillin (100%), penicillin G (93%), and ciprofloxacin (48%). Intermediate susceptibility was noted for antibiotics such as penicillin G (7%), nilidixic acid (34%), ceftriaxone (28%), erythromycin (10%), tetracycline (17%), and trimethoprim-sulfamethoxazole (7%). While a minority of strains exhibited sensitivity to certain antibiotics, with ciprofloxacin (17%), trimethoprim-sulfamethoxazole (10%), and erythromycin (7%) showing some efficacy against *S. aureus* strains as summarized in table 02.

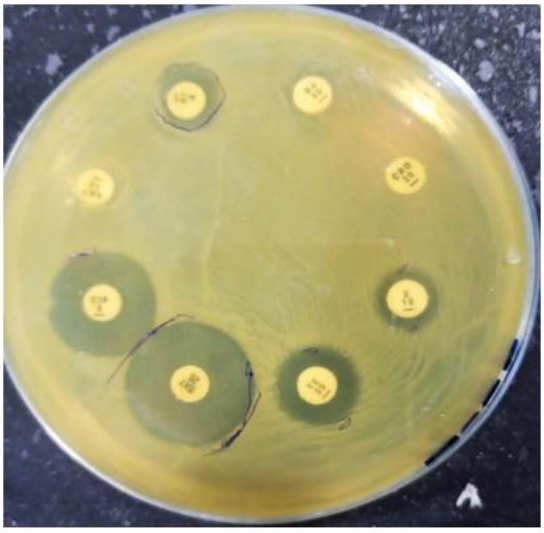

Figure 02: A representative picture of antibiotic sensitivity test S. aureus isolates

Table 02: Frequency of Antibiotic Resistance, Intermediate Susceptibility, and Sensitivity among *S. aureus* Strains

Antibiotic	Resistant	Intermediate	Sensitive
Ampicillin	100%	0%	0%
Penicillin G	93%	7%	0%
Nilidixic Acid	59%	34%	7%
Ceftriaxone	69%	28%	3%
Erythromycin	83%	10%	7%
Tetracycline	76%	17%	7%
Trimethoprim - Sulfamethoxazole	83%	7%	10%
Ciprofloxacin	48%	34%	17%

For the identification of MRSA and MSSA strains among the isolates, their susceptibility pattern against cefoxitin and oxacillin was determined. The results were interpreted as per CLSI guidelines 2021, which states that if zone of inhibition of any *S. aureus* strain is less than 21mm (showing resistance) then it will be MRSA and if it is more than 22mm then it will be MSSA (Figure 3). The results showed 48% of the strains were resistant to cefoxitin while 52% were sensitive similarly, 83% were resistant to oxacillin and only 17% were sensitive to it (Table 03).

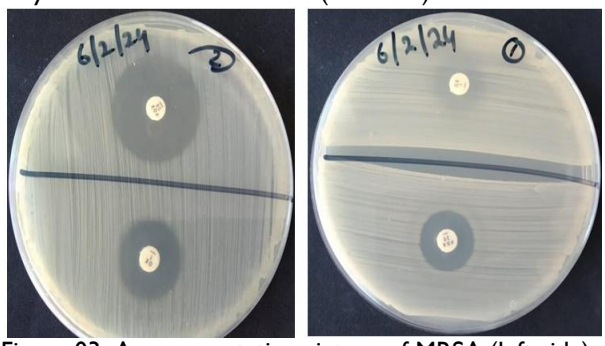

Figure 03: A representative picture of MRSA (left side), and MSSA (Right side)

Table 03: Identification of MRSA and MSSA Strains

Antibiotic	Resistant	Sensitive
Cefoxitin	48%	52%
Oxacillin	83%	17%

Polymarase Chain Reaction of the Target Genes

The PCR analysis targeting the resistance genes, tetK and gyrA, unveiled notable insights. Specifically, 15 samples confirmed the presence of the gyrA gene, while tetK was prevalent in 14 out of the 15 samples. However, one sample diverged from this trend, yielding a negative result for the tetK gene (Figure 3). The size of gyrA gene is 280bp while that of tetK gene is 360bp while 100bp ladder was used along with negative and positive controls. These combined results showed a comprehensive picture of S. aureus resistance dynamics and underscore the importance of both phenotypic and genotypic assessments in understanding bacterial resistance profiles. This suggests a notable prevalence of antibiotic resistance markers within the S. aureus strains from diabetic patients. Such molecular analyses are crucial for

monitoring antibiotic resistance patterns, especially in vulnerable populations, and guiding treatment strategies to ensure effective therapeutic outcomes.PCR tests were positive for S. aureus in a significant proportion of patients above the age of 50.

Figure 04: Gel images of clear bands of *gyr*A (280bp) and *tet*K (360bp) genes.

DISCUSSION

The threat posed by the development of antibiotic resistant strains of S. aureus poses an overwhelming challenge in clinical practice especially in diabetic patients who have an already existing predisposition of being predisposed to complications due to bacterial infections. The results of our study that showed a disturbing level of antibiotic resistance indicators in the S. aureus isolates of diabetic patients reflect the general tendency of the global increase in antibiotic resistance.

It is worth noting that PCR analysis revealed the presence of S. aureus in a considerable percentage of patients over the age of 50 and this highlights the predisposition nature of the elderly population to S. aureus infection. This is why specific interventions to help this high-risk population are urgently needed, which is supported by [19, 20] who also identified high rates of MRSA infection in older adults populations. The resistance of S. aureus to the effects of antibiotics has a long history of scientific research [21]. In our research, 15 of 29 samples were resistant to ciprofloxacin and tetracycline and indicate a tightening of the therapeutic indices in the treatment of S. aureus infections possibly because of mutations of antibiotic target genes and efflux pump pathways as observed [22].

Diabetic societies have their unique crisis in terms of the context of antibiotic resistance because the problem of the inability of metabolic regulation and weakened immunity is complicated [23]. All these data confirm this correlation, and suggest that the issue of antibiotic resistance of diabetic patients and S. aureus strains is a severe and pervasive problem that requires joint efforts, which also finds their reflection in [24, 25]. The synthesis of the molecular approach, which is mainly PCR has created a novel understanding of the dynamics of antibiotic resistance [26]. By identifying the genotype-phenotype gap revealed in our study, S. aureus antibiotic resistance genetic basis was investigated, in agreement with other investigations [27, 28] and others, and gyrA and tetK genes.

Overall, our findings put our study into the context of complicated interaction between antibiotic resistance and diabetic populations as a subset of general resistance study. That is why it is necessary to take multifaceted interventions and collaborate to address the growing public health threat of antibiotic resistance In addition, the socio-economic impact of the problem of antibiotic resistance could not be underestimated. Lob et al., 2016 exexplained the economic implications of antibiotic resistance, with interests in the increasing health care costs and challenges in devoting resources in the treatment of resistant diseases. Considering the present-day research results, the identified prevalence of the signs of antibiotic resistance in S. aureus strains among diabetes patients highlights the urgent need of resource-demanding interventions, including enhanced monitoring and stewardship programmes [29].

Ampicillin and Penicillin G did not succeed especially and the rates of resistance were 100 and 93 percent respectively. A more detailed analysis indicated that Nilidixic Acid was also not all right and the resistance rate has been at 59 percent and at 34 percent there are intermediate resistance samples. Similarly, Erythromycin, Tetracycline and Trimethoprim-Sulfamethoxazole were always found to have a range of resistance between 76 to 83. Interestingly, despite the enormous opposition, some of the sensitivities appeared. Erythromycin, Tetracycline and Trimethoprim-Sulfamethoxazole had 7, 7, and 10 percent sensitivities, respectively. The profile of Ciprofloxacin was slightly more balanced with 48 percent resistance rate and 17 percent of samples sensitive.

The global transmission of antibiotic resistance genes involves problems that are not restricted to the local level [30]. Such an international approach supports the results of our study that antibiotic resistance among vulnerable populations requires global collaboration to overcome its many issues. Placing these results in the context of the literature on antibiotic resistance, our research contributes to the need of multi-faceted

treatment and collaboration to overcome antibiotic resistance among populations at the risk.

CONCLUSION

The microbial composition and the antibiotic susceptibility profile of type 2 diabetes wound tissues are explored in this research. Microbiological culturing, biochemical, and antibiotics susceptibility and molecular analysis have proved the S. aureus infections and the alarming rate of antibiotic-resistance of this vulnerable group of patients. The results indicate that treating diabetic foot ulcer infections should be done through alternative treatment methods. Both MRSA strains and resistance genes suggest that the genetic basis of antibiotic resistance with a focus in phenotypic and genotypic evaluation of resistance is the problem. The research study can add to the body of knowledge of the microbial aetiology of diabetic patients and inform the practice and future research on the antibiotic resistance of diabetic foot ulcer management.

RECOMMENDATIONS

More research should be done to target large populations with the aim of capturing a very broad population, longitudinal investigation to capture the resistance dynamics and the intensive study of the resistance mechanisms in diabetic patients through the assistance of genomic analysis. Further collaboration with healthcare facilities to develop tailored antibiotic stewardship programs, alternative ways of treatment, and patient education are also significant steps in preventing the spread of antibiotic-resistant S. aureus infections and optimizing treatment outcomes of the susceptible group of patients.

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CONFLICTS OF INTEREST

Not Applicable.

AUTHOR CONTRIBUTION

All authors are contributed equally.

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None.

INFORM CONSENT

Informed consent was obtained from the patients.

ETHICAL CONSIDERATIONS

Ethical committee approval was obtained from Narayan Medical College & Hospital, a 1200-bedded hospital, and Sadar Hospital, a 250-bedded facility, both located in Sasaram, Bihar(IEC No: NNC/Dean-PO/25).

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