IDENTIFICATION OF TETA AND OPRL GENES OF PSUEDOMONAS AERUGINOSA ISOLATED FROM DIABETICFOOT ULCER'S PATIENTS FROM SELECTED AREAS OF PUNJAB

Zobia Khadam[*1], Hafiz Muhammad Aamir Farooq[2], Javeria Akhtar[3], Anmol Arif[4], Tabinda Akram[5], Maryam Afzal[6], Ayesha Raheem[7]

[*1]*Lecturer Department of MLT, Prime Care Institute of Health Sciences, Zafarwal*
[2]*Lecturer Department of Operation Theater Technology, Al-Shifa Institute of Health Sciences, Narowal*
[3]*Lecturer Department of Biochemistry, Al- Shifa Institute of Health Sciences, Narowal*
[4] Department of Allied Health Sciences Rashid Latif Khan University Lahore
[5,6]*Lecturer Department of Operation Theater Technology, Institute of Life Sciences Narowal*
[7]*Lecturer Department of Nursing, Prime Care Institute of Health Sciences, Zafarwal*

[*1]1663zobia.khadam@gmail.com, [2]aamirfarooq596@gmail.com, [3]javeria.akhtar1011@gmail.com, [4]anmol.arif@rlku.edu.pk, [5]tabindaakramakram@gmail.com, [6]maryambajwa724@gmail.com, [7]ayesharaheem082@gmail.com

DOI: https://doi.org/10.5281/zenodo.15023301

Keywords
Diabetic foot ulcers, P. aeruginosa, PCR, tetA and oprL genes

Article History
Received on 07 February 2025
Accepted on 07 March 2025
Published on 14 March 2025

Corresponding Author: *

Abstract
Diabetic Foot Ulcer poses a critical concern for healthcare organizations concerning diabetes. This issue presents a significant medical hurdle due to the widespread occurrence of bacterial infections that are resistant to multiple drugs. Among the various pathogens linked to Diabetic Foot Ulcers (DFUs), Pseudomonas aeruginosa stands out as a prevalent one, known for its resistance to multiple types of antibiotics. This research aims to analyze the prevalence of oprL and tetA genes in drug resistant strains of P. aeruginosa isolated from DFU's samples. These strains have been sourced from DFUs patients admitted in different hospitals of Punjab region. The study's dataset consists of 100 pus samples collected from individuals afflicted by diabetes and experiencing foot ulcers. All pus samples were cultured on cetrimide agar and further confirmed by different biochemical tests, then antibiotic susceptibility test was performed. Out of 100 samples, 25 were positive for P. aeruginosa. P. aeruginosa showed 100% resistance to ampicillin followed by tetracycline (74%), erythromycin (52%), gentamycin (69.7%), ceftazidime (24%), imipenem (44%), and ciprofloxacin (20%). Total 16 (69%) isolates of the P. aeruginosa showed multidrug resistance (MDR). The moleculer identification of genes in drug resistant isolates of P. aeruginosa was achieved using oprLand tetA primers. DNA Extraction and PCR analysis confirmed the presence of oprLand tetA genes in P. aeruginosa. Out of 25 (100%) isolated bacterial strains, oprL gene was identified in 23(92%) bacterial isolates followed by tetA gene that was detected in 21(84%) isolated strains of P. aeruginosa. From 21(84%) isolates in which tetA gene was detected, almost 15(68%) were tetracycline resistant. The findings from this study might contribute to a better understanding of the resistance mechanisms of P. aeruginosa in DFUs, facilitating the development of targeted therapeutic approaches and

effective antimicrobial stewardship programs.

INTRODUCTION

Diabetes Mellitus is a chronic metabolic disorder characterized by elevated blood sugar levels due to deficiencies in either the secretion of insulin, its action, or both. Insulin, a hormone produced by the pancreas, plays a crucial role in regulating blood glucose levels. According to a 2020 report from the World Health Organization (WHO), individuals with diabetes experience hyperglycemia, where their bodies either generate inadequate insulin or cannot effectively use it. This condition is categorized into three main types: type 1 diabetes, type 2 diabetes, and gestational diabetes.Type 1 diabetes is a prominent chronic ailment distinguished by malfunctioning beta cells that hinder the production of insulin. This condition significantly impacts the overall health of the population and contributes to increased morbidity (Wayan *et al*., 2021). Type 2 diabetes is regarded as one of the chronic metabolic disorders which has the reasonable effect on the quality of life and is a serious public health concern (Ozdemir *et al*., 2003). Almost 90% diabetic patients belong to the type 2 diabetes and it frequently affects old age people who have low insulin. If diet alone is unable to regulate blood sugar levels, an oral hypoglycemic medication is added to the programmed, followed by insulin, if needed. Another alternative is nutritional medical therapy. Patients of this type tend to be obese and have a strong family history of diabetes (Servan *et al.,* 2018). Due to neuropathy (nerve damage), vascular disease, or trauma, the diabetic patients are more chances to develop skin sores, especially severe ulcers. Diabetes patients frequently develop peripheral neuropathy, which causes nerve damage in the foot, as well as peripheral artery disease. Diabetes patients experience immune system dysfunction that has reduces its ability to fight against diseases. Foot ulcers are a common consequence among diabetics (Aynalem *et al.* 2018).

According to the International Diabetes Federation (IDF), the estimated number of adults with diabetes worldwide will increase from 463 million in 2019 to 700 million by 2045, the majority of whom will reside in low- and middle-income nations. Diabetes is one of the deadliest diseases, causing 1.6 million deaths annually, and by 2030, it's expected to overtake deadly disease as the seventh leading cause of death (Chen *et al.,* 2017). Diabetes mellitus has an impact on patients' health and quality of life, and there is a substantial danger of serious damage to numerous organs, including the blood vessels, nerves, and eyes. Diabetes is more common than ever, and its consequences are a significant public health issue (Srivastava *et al.,* 2019).

Diabetes patients are more prone to develop foot ulcers than those without the disease, making them a common consequence (Aynalem *et al*., 2018). Diabetes mellitus is commonly present in young and overweight people that are particularly susceptible to the disease (Lavery *et al*., 2003). WHO reported in 2019 that in the entire world, diabetes mellitus affects approximately 7% of the population and it is expected that in 2025, About 150 million people will experience diabetes mellitus worldwide Diabetes must be managed by maintaining appropriate blood glucose levels by doing regular walk, taking proper food and the use of glucose lowering medicine. In order to effectively manage diabetes, it's crucial to routinely check blood glucose levels as well as other health indicators (Asif *et al*., 2014).

Individuals diagnosed with type 2 diabetes often experience the development of foot ulcer wounds that manifest on the feet due to their diabetic condition. Left unattended, these ulcers can escalate, potentially necessitating the amputation of lower limbs. The initial stages involve elevated blood sugar levels, which impair the nerves and blood vessels in the feet. Consequently, patients may encounter challenges in sensing stimuli and undergoing proper healing processes. As a result, those with diabetes face an increased susceptibility to the emergence of foot ulcers, which, if infected, can lead to severe health complications. Diabetes increases 34% chances to develop diabetic foot ulcers foot. Around one in three diabetics may experience a diabetic foot ulcer over their lifetime. Amputation of a limb following infection of a DFU frequently results with substantial morbidity, emotional suffering, worse quality of life, and shorter life expectancy (Boulton *et al.*, 2017). In the United States, 38% of all the amputations were associated with diabetes (Syafril *et al.*, 2018). A diabetic neuropathy condition that damages motor, sensory, autonomic nerves and leave patients to develop diabetic foot ulcer. The primary consequence of diabetic foot ulcers can result in lower extremity amputation and even death, placing a significant cost on patients and healthcare systems (Zheng *et al.*, 2018). As comparison to patients without DFU, these risk factors can increase the death rate and shorten life expectancy in diabetic foot ulcer patients (Hamilton *et al.*, 2019).

Peripheral vascular disease, neuropathy, long-term diabetes, insulin use, age, cerebral vascular disease, retinopathy, nephropathy, inadequate glycemic management and other factors raise the chance of becoming an ulcer. These individuals having an increased cumulative glycemic burden, coronary artery disease, smoke, and have hypertension are most of the males (Tolossa *et al.*, 2020). Osteomyelitis (OM) stands out as the prevailing infection in cases of diabetic foot ulcers (DFUs), manifesting in over 20% of moderate infections and as high as 50% to 60% of severe infections. The presence of OM significantly increases the likelihood of amputation (Lazaro *et al.*, 2021).

Diabetic foot infections (DFIs) continue to be the most prevalent complication among individuals with diabetes, affecting approximately 60% of diabetic foot ulcers (DFUs). In some cases, hospitalization becomes necessary, and DFIs are frequently identified as the main trigger for amputations (Lavary *et al.,* 2018). As Diabetes Mellitus (DM) is anticipated to rise, DFUs will impose an even greater burden on healthcare systems worldwide and its cost is so high (*Sen et al.*, 2019). DFUs pose a major challenge for people with diabetes, as it is estimated that over 15% of diabetic patients will develop these ulcers during their lifetime. The impact of DFUs is profound, causing a considerable decline in quality of life, necessitating prolonged hospital exposure, in the United States that results in over seventy-thousand-foot amputations annually (Jeffcoate *et al.,* 2018). Patients with diabetes experience prolonged hospital stay primarily due to foot problems, surpassing other complications associated with the condition. Among diabetic patients, the primary cause of lower-extremity amputations is diabetic foot infections, particularly those that extend to the bone. These infections not only elevate the risk of mortality but also impose a substantial financial burden (Hussain *et al.*, 2018).

P. aeruginosa isone of the most important microorganisms that cause clinical problems resulting from high-resistance to antimicrobial agents. Though it is rarely found in the normal flora of humans, it is frequently isolated from patients with burns, cystic fibrosis, and neutropenia. According to research from Kenya, *Pseudomonas spp* were the most typical Gram-negative organisms recovered from DFU in some Middle Eastern nations. The *tet*A resistant gene has been described in some of these investigations, which explained antibiotic resistance patterns of *P. aeruginosa* isolates from DFUs in 2016. They discovered 129 *P. aeruginosa* isolates from DFUs, of which some possessed the *tet*A gene and 61% were tetracycline-resistant. Further they explained that 26.1% of *P. aeruginosa* isolates from DFUs had mutations in the *opr*L gene, which was associated with reduced susceptibility to antibiotics (Obeid *et al.*, 2018). *P. aeruginosa* should never be disregarded as minor in diabetic foot ulcers since it has the potential to cause serious tissue destruction in diabetics. Furthermore, as it can cause sepsis and loss of limbs it should never be regarded as pollutants or normal flora and always be considered a pathogen. Due to its recurring drug resistance, *P. aeruginosa* is a deadly and feared organism (Diggle *et al.,* 2020).

Alarmingly, more than 50% of the people who are facing amputation problem pass away within five years (Armstrong *et al.*, 2020). On a global scale, diabetic patients undergo lower limb amputations at an alarming rate of approximately one every 20 seconds. A diabetic foot ulcer is considered infected (with pus) when it exhibits at least two of the typical signs or symptoms of inflammation, such as pain or tenderness, warmth, redness, swelling, or the presence of purulent discharges

(Nugroho *et al.,* 2020). Effective management of infections necessitates meticulous focus on several aspects, including timely and accurate diagnosis of the condition, obtaining suitable specimens for culture, careful selection of initial and definitive antimicrobial therapy, prompt identification of the need for surgical interventions, and comprehensive provision of all required wound care (Lipsky *et al.*, 2021). It might result in an increase in morbidity and mortality. Despite being preventable, DFU costs the patient and the healthcare system a lot of money. A collaborative group of professionals focused on timely diagnosis, prevention, and successful treatment as a vital component of proper DFU management actions. Effective DFU management approaches include intensive prevention, early diagnosis, and powerful treatment by a multidisciplinary group of experts (Sneha *et al.*, 2022).The current study describes the prevalence of *Pseudomonas aeruginosa* associated diabetic foot ulcer in different regions of Punjab. Furthermore, we determined the prevalence of *opr*L and *tet*A genes in drug resistant strains of *Pseudomonas aeruginosa* isolated from diabetic foot ulcer's samples.

MATERIAL AND METHODS

Sample Collection:

Total 100 samples were collected from DFUs patients admitted in the Sir Ganga Ram and Mayo Hospital Lahore, Allama Iqbal Memorial Hospital Sialkot and private clinics of Multan. Samples were obtained from patients with diabetic foot ulcer and foot amputation with their consent. These samples were collected by swabculture technique; a sterile swab is gently inserted into the wound bed, rotated severaltimes, and then removed with caution to prevent contact with the surrounding skin.Samples wereshiftintoanicebox(Transportcontainer)andsend toMicrobiology laboratory for the isolation of selective bacteria i.e., *P. aeruginosa* by culturing method. Firstly, these samples were cultured on nutrient agar thenon selective media, Cetrimide agar.

Antibiotic Susceptibility Assay:

The disc diffusion experiment was carried out for assessing antibiotic sensitivity on agar. Ten antibiotics were used to test *Pseudomonas spp.*'s sensitivity. Into a sterile Eppendorf tube was added 300**µl** of PBS. This PBS solution was combined with a single bacterial colony that was isolated using a sterile loop. The bacterial suspension was vortexed until it reached a concentration of 0.5 McFarland. Using a sterile cotton swab, a bacterial lawn was created on the agar plate, which was then left to dry. Then, using sterile forceps, the antibiotic discs were picked up and put on the agar plate surface. Each disc was positioned with equal separation from the others in order to prevent contamination or reverse the effects of earlier antibiotics on the subsequent disc, the forceps were passed through the burner flame before taking up the second one. The plates were covered with antibiotics and incubated in an incubator for 24 hours at 37°C. We used a millimeter scale to assess the zone of inhibition. The classification of isolates as susceptible, resistant, or intermediate using CLSI (2022) break points.

DNA Extraction:

First of all, all samples were cultured on Cetrimide agar plate and kept them in incubator at specific and suitable conditions. After 24 hours, growth was appeared on the agar plate, then loop full colony of bacteria was picked and mixed with 300 micro liter PBS solution to in an Eppendorf tube. After mixing with PBS, suspension was placed in centrifuge tube for 5 minutes at 8500 rpm. After centrifugation colonies were settled down in the bottom. Then the supernatant was extract from the tube. Pellet was then re-suspended in 200µl of TE buffer. Then it was placed in water bath for 10 minutes at 99^{o}c. After heating the samples, samples were placed in refrigerator for 5 to 10 minutes. Then again samples were centrifuged for 5 minutes at 13000 rpm. At the end, supernatant was transferred into new Eppendorf tube and stored at -20^{o}c.

PCR Amplification of *Opr*L and *tet*A genes:

Primers used for PCR reaction were O*pr*L-F; 5'TTACTTCTTCAGCTCGACGCGAC5-3' and O*pr*L-R; 5'ATGGAAATGCTGAAATTCGGC-3',*tet*A-F; GTGAAACCCAACATACCCC and *tet*A-F; GAAGGCAAGCAGGATGTAG.Final solution reaction mix of 25µl was prepared for single PCR. Master Mix (2x) of Solis bio dyne was used. In PCR, 2µl of DNA along with the 5µl of ready to use master mix was added. 1.5µl of each forward and reverse primers were also added. In last 15µl of the autoclaved nuclease free water was added to prepare the final volume of 25µl.

Gel Electrophoresis of PCR Product:

The PCR products were analyzed by using gel electrophoresis During the gel electrophoresis process, TBE buffer (Tris-borate-EDTA) was utilized to facilitate the separation of nucleic acids. An agarose gel was employed to separate DNA fragments according to their respective sizes. To enable visualization, ethidium bromide was added, allowing the DNA to fluoresce when exposed to ultraviolet (UV) light.

To prepare the 3% agarose gel, 0.63g of agarose was added to 35ml of 1X TBE buffer, and the mixture was boiled in a microwave oven for 1 minute. Subsequently, it was allowed to cool down to 60°C. For visualization purposes, 2µl of ethidium bromide was added to the gel. The gel was then poured into a casting tray with pre-adjusted combs and left to solidify. After solidification, PCR products were loaded into the wells. Each sample consisted of 6µl of PCR product mixed with 3µl of loading dye. The gel was subjected to an electric current of 400mA and a voltage of 90 volts for a duration of 35 minutes. Following the gel electrophoresis run under these specific conditions, bands corresponding to the *opr*L gene were observed at 504 base pairs (bp), and the *tet*A gene bands appeared at 576 bp, using a 100bp ladder as a size reference. The bands of interest were visualized under a UV illuminator.

RESULTS AND DISCUSSION

Pseudomonas species are widely distributed in nature and can cause infections in animals. Among these species, *Pseudomonas aeruginosa* is the most well-known pathogen responsible for human infections. *P. aeruginosa* is a gram-negative bacillus and is considered an opportunistic pathogen. A severe consequence of diabetes mellitus that is spreading across the globe is called DFU (Diabetic Foot Ulcer). Swab samples were taken from 100 patients with DFUs for this particular investigation. According to the specimens' culture results, 25% of them had *Pseudomonas aeruginosa* growth shown in figure 1. Notably, Hitam *et al.* also showed in their study that individuals with diabetic foot infections (DFIs) had a similar percentage (28.8%) of polymicrobial infections (Hitam *et al.,* 2019).

Figure 1: Appearance of *Pseudomonas* on Cetrimide Agar. Green colored colonies produced on Cetrimide agar.

Numerous research has concentrated on figuring out the kinds of bacteria that have been isolated from diabetic foot ulcers, but they have not always linked these results to the prevalence of limb amputation or the survival rates of affected individuals. For instance, *Pseudomonas* was one of the most frequently isolated species from DFUs, according to a study (Kaimkhani *et al.*, 2018). The majority of the isolates (88%) were gram-negative bacteria, and *P. aeruginosa* was the most common bacterium (43%), according to culture data shiwn in Figure 2.

According to research from Kenya and Obeid *et al.* (2018), *Pseudomonas spp.* were the most typical Gram-negative organisms recovered from DFU in some Middle Eastern nations as shown in Figure 3& 4. The *tet*A resistant gene has been described in some of these investigations, which explained antibiotic resistance patterns of *P. aeruginosa* isolates from DFUs in the Journal of Medical Microbiology in 2016. They discovered 129 *P. aeruginosa* isolates from DFUs, of which some possessed the *tet*A gene and 61% were tetracycline-resistant. Further they explained that 26.1% of *P. aeruginosa* isolates from DFUs had mutations in the *opr*L gene, which was associated with reduced susceptibility to antibiotics (2015).

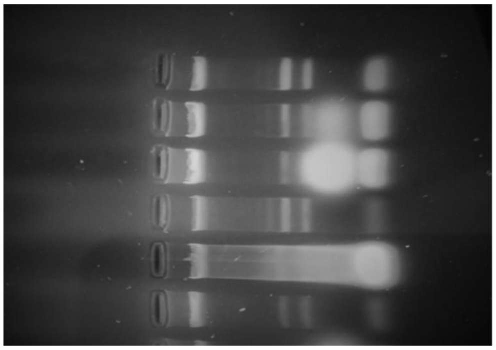

Figure 2: Gel electrophoresis of *opr*L and *tet*A gene (DNA extraction).

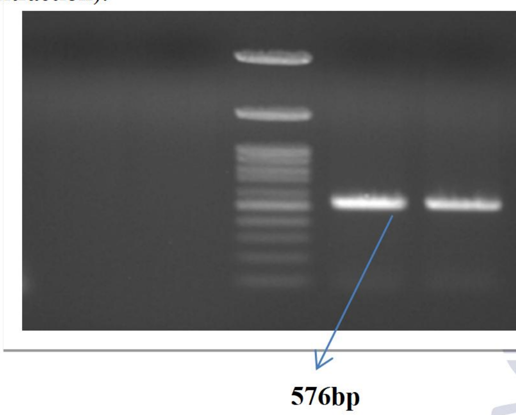

Figure 3: Amplification of *tet*A gene (576bp) through polymerase chain reaction (PCR).

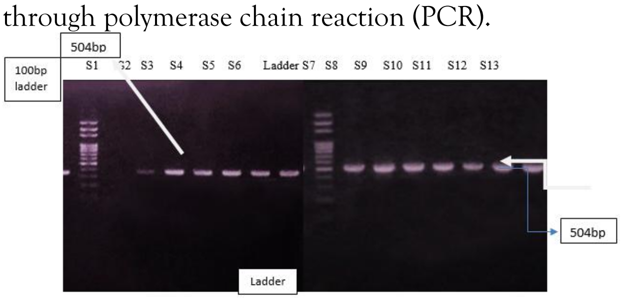

Figure 4: Amplification of O*pr*L gene (504bp) through polymerase chain reaction (PCR).

In order to ascertain the prevalence of *Pseudomonas* isolates, specifically *Pseudomonas aeruginosa*, in diabetic foot ulcer (DFU) patients, the current investigation was carried out. The study also sought to identify antibiotic resistance genes in the isolated bacteria and evaluate the profile of antibiotic resistance. Sialkot, Lahore, and Multan were the three locations in Punjab where a total of 100 samples were collected. Using sterile swabs, samples were taken from swabs and placed in phosphate buffer saline. These samples were then enriched overnight in buffered peptone water. After enrichment, the samples were streaked on selective medium (Cetrimide agar) and incubated overnight. The growth of green-colored colonies on the agar medium indicated the presence of *Pseudomonas aeruginosa*, which was further confirmed through gram staining and microscopy. The bacterial species appeared as short rods under the microscope. Various biochemical tests, including catalase, indole, triple sugar iron, and oxidase tests were performed. Out of the 100 DFU samples, 25 isolates tested positive for *Pseudomonas* based on different microbiological, cultural, and biochemical tests.

According to Sivanmaliappan *et al.*, a study on *Pseudomonas aeruginosa*, 55.5% of the strains tested were found to be multidrug resistant (MDR). They reported that all strains were resistant to Ampicillin, while 83.3% showed resistance to ciprofloxacin and 66.6% to Ceftazidime, gentamycin, and imipenem. Banar *et al.* also mentioned that among various drugs tested, Ceftazidime demonstrated higher activity against *P. aeruginosa* isolates. Kim *et al.*, 2020explored that *P. aeruginosa* was higher (23%) in DFI as compared to MRSA (6%) and MSSA (19%).

Twenty-five *P. aeruginosa* isolates were tested for antibiotic susceptibility, and Ampicillin was found to be 100% resistant, followed by Tetracycline (74%), Erythromycin (52%), Gentamycin (69.7%), Ceftazidime (24%), Imipenem (44%), and Ciprofloxacin (20%). Total 16 (69.7%) Of the *P. aeruginosa* isolates, showed multidrug resistance (MDR). Out of 25 (100%) isolates, *opr*L gene was identified in 23(92%) bacterial isolates followed by *tet*A gene that was detected in 21(84%) isolated strains of *P. aeruginosa.* From 21(84%) isolates in which *tet*A gene was detected, almost 15(68%) were tetracycline resistant shown in Figure 5.

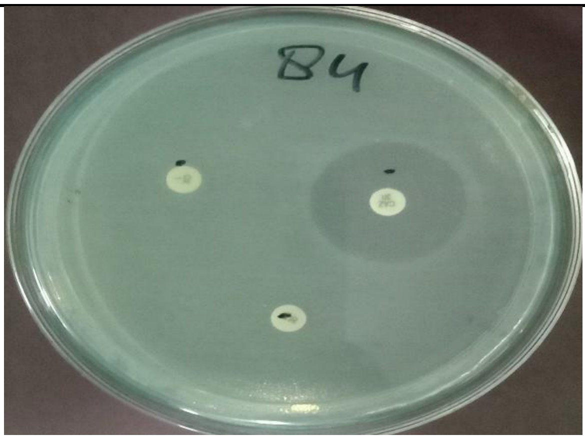

Figure 5: Antibiotic susceptibility testing by disc diffusion method.

Pseudomonas spp. were the most frequently isolated Gram-negative bacterium from DFU in some Middle Eastern nations, according to a study conducted in Lebanon (2020). The research also indicated that the best oral antibiotic regimen for outpatient treatment was the use of ciprofloxacin and amoxicillin/clavulanate. Another study carried out in Kenya in 2021 discovered that gram-negative bacteria, such as *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Klebsiella pneumonias,* and *Pseudomonas aeruginosa,* were the most common organisms isolated. These bacteria showed resistance to widely used antibiotics like tetracycline with tazobactam, ampicillin, ciprofloxacin, and ceftazidime.

Carro *et al.* (2022), conducted a study on diabetic foot ulcers (DFU) and found that gram-negative bacteria were the most commonly isolated microorganisms. Based on their findings, they recommended an empirical antibiotic therapy consisting of amoxicillin/clavulanate along with ciprofloxacin as the preferred treatment choice for managing DFU.

CONCLUSION

The presence of both *opr*L and *tet*A genes in *Pseudomonas aeruginosa* is a cause for concern due to the higher prevalence of multidrug-resistant strains found in clinical samples. To address this issue, it is crucial to regularly monitor and study the susceptibility patterns of antibiotics, while also exercising caution and using them judiciously. In this study, the successful detection of *P. aeruginosa* was achieved using *opr*L and *tet*A primers through PCR analysis, identifying 25 isolates. These isolates were further examined for resistance to different antibiotics: Ceftazidime (CAZ, 30 µg), Erythromycin (E, 15 µg), Gentamycin (CN, 10 µg), Tetracycline (TE, 30 µg), Ampicillin (AMP, 10 µg), Imipenem (IPM, 10 µg), and Ciprofloxacin. Study found that Ampicillin and Tetracycline's resistance has been increased and resistant genes are involved to maximum extent. Imipenem, ciprofloxacin, and Ceftazidime resistance is low as compared to other drugs used. Out of 25 (100%) isolates, *opr*L gene was identified in 23(92%) bacterial isolates followed by *tet*A gene that was detected in 21(84%) isolated strains of *P. aeruginosa.* From 21(84%) isolates in which tetA gene was detected, almost 15(68%) were tetracycline resistant. Further surveillance studies in Pakistan are prerequisite to document the rapid emergence and spread of multi-resistant *P. aeruginosa* in Diabetic Foot Ulcer. To get control on Foot amputations, we have to focus on more resistant genes as well as efflux pumps in *P. aeruginosa*.

REFERNCES

Addis, T., Araya, S., & Desta, K. (2021). Occurrence of multiple, extensive and pan drug-resistant Pseudomonas Aeruginosa and Carbapenems production from presumptive isolates stored in a biobank at Ethiopian public health institute. *Infection and Drug Resistance*, 3609-3618.

Adeyemo, A. T., Kolawole, B., Rotimi, V. O., & Aboderin, A. O. (2021). Multicentre study of the burden of multidrug-resistant bacteria in the aetiology of infected diabetic foot ulcers. African journal of laboratory medicine, 10(1), 1-10.

age MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *Syst Rev.* (2021) 10:1–1. doi: 10.1186/s13643-021-01626-4

Akhtar S, Nasir JA, Sarwar A, Nasr N, Javed A, Majeed R, et al. Prevalence of diabetes and pre-diabetes in Bangladesh: a systematic review and meta-analysis. *BMJ Open.* (2020)

10:e036086. doi: 10.1136/bmjopen-2019-03608

Aldana, P.C., Cartron, A.M. and Khachemoune, A., 2022. Reappraising diabetic foot ulcers: a focus on mechanisms of ulceration and clinical evaluation. *The International Journal of Lower Extremity Wounds*, *21*(3), pp.294-302.

American Diabetes Association. (2021). Diagnosis and classification of diabetes mellitus. Diabetes Care, 44(Supplement 1), S15-S33.

Armstrong, D. G. et al. Five-year mortality and direct costs of care for people with diabetic foot complications are comparable to cancer. *J. Foot Ankle Res.* **13**, 16 (2020).

Asif, M., (2014). The prevention and control the type-2 diabetes by changing lifestyle and dietary pattern. *Journal of education and health promotion*, *3*.

Atlas, D. (2015). International diabetes federation. *IDF Diabetes Atlas, 7th edn. Brussels, Belgium: International Diabetes Federation*, *33*(2).

Aynalem, S. B., & Zeleke, A. J. (2018). Prevalence of diabetes mellitus and its risk factors among individuals aged 15 years and above in Mizan-Aman town, Southwest Ethiopia, 2016: a cross sectional study. *International journal of endocrinology*, *2018*.

Baig, M. S., Banu, A., Zehravi, M., Rana, R., Burle, S. S., Khan, S. L., ... & Cavalu, S. (2022). An overview of diabetic foot ulcers and associated problems with special emphasis on treatments with antimicrobials. *Life*, *12*(7), 1054.

Bakker, K., Apelqvist, J., Lipsky, B. A., Van Netten, J. J., Schaper, N. C., & International Working Group on the Diabetic Foot (IWGDF). (2016). The 2015 IWGDF guidance documents on prevention and management of foot problems in diabetes: development of an evidence-based global consensus. Diabetes/metabolism research and reviews, 32, 2-6.

Balasubramanyam, A., Garza, G., Rodriguez, L., Hampe, C. S., Gaur, L., Lernmark, A., & Maldonado, M. R. (2006). Accuracy and predictive value of classification schemes for ketosis-prone diabetes. *Diabetes care*, *29*(12), 2575-2579.

Bialvaei, A. Z., & Samadi Kafil, H. (2015). Colistin, mechanisms and prevalence of resistance. *Current medical research and opinion*, *31*(4), 707-721.

Boulton, A., (2021). It's time to tackle the growing diabetes pandemic. *Diabetes research and clinical practice*, *182*

Breidenstein, E. B., de la Fuente-Nunez, C. & Hancock, R. E.(2011) *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol.* **19**, 419–426.

Carro, G. V., Carlucci, E., Priore, G., Gette, F., Llanos, M. D. L. Á., Dicatarina Losada, M. V., ... & Amato, P. S. (2019). Infecciones en pie diabético: Elección del tratamiento antibiótico empírico. *Medicina (Buenos Aires)*, *79*(3), 167-173.

Chang, M., & Nguyen, T. T. (2021). Strategy for treatment of infected diabetic foot ulcers. *Accounts of chemical research*, *54*(5), 1080-1093.

Diggle, S. P., & Whiteley, M. (2020). Microbe Profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat. *Microbiology*, *166*(1), 30.

Doll, M., Stevens, M., & Bearman, G. (2018). Environmental cleaning and disinfection of patient areas. International journal of infectious diseases, 67, 52-57.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. Emerging infectious diseases, 8(9), 881.

Ejaz, F., Ahmad, A., & Hanif, K. (2020). Prevalence of diabetic foot ulcer in lahore, Pakistan: a cross sectional study. Asian Journal of Allied Health Sciences (AJAHS), 34-38.

Gellatly, S. L., & Hancock, R. E. (2013). Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathogens and disease, 67(3), 159-173.

Glaudemans, A. W. J. M., Uçkay, I., & Lipsky, B. A. (2015). Challenges in diagnosing infection in the diabetic foot. *Diabetic Medicine*, *32*(6), 748-759.

Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., ... & Cheng, G. (2022). Bacterial multidrug efflux pumps at the frontline of antimicrobial resistance: An overview. Antibiotics, 11(4), 520.

Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., ... & Cheng, G. (2022). Bacterial multidrug efflux pumps at the frontline of antimicrobial resistance: An overview. *Antibiotics*, *11*(4), 520.

Hussain, Z., Thu, H. E., Shuid, A. N., Katas, H., & Hussain, F. (2018). Recent advances in polymer-based wound dressings for the treatment of diabetic foot ulcer: an overview of state-of-the-art. Current drug targets, 19(5), 527-550.

International Diabetes Federation. (2019). IDF Diabetes Atlas (9th ed.). Brussels, Belgium: International Diabetes Federation.

Jain, A., Gupta, Y., Agrawal, R., Jain, S. K., & Khare, P. (2007). Biofilms—a microbial life perspective: a critical review. Critical Reviews™ in Therapeutic Drug Carrier Systems, 24(5).

Jeffcoate, W. J., Vileikyte, L., Boyko, E. J., Armstrong, D. G., & Boulton, A. J. (2018). Current challenges and opportunities in the prevention and management of diabetic foot ulcers. Diabetes care, 41(4), 645-652.

Ji, X., Jin, P., Chu, Y., Feng, S., & Wang, P. (2014). Clinical characteristics and risk factors of diabetic foot ulcer with multidrug-resistant organism infection. The international journal of lower extremity wounds, 13(1), 64-71.

Karthik, L., Kumar, G., Keswani, T., Bhattacharyya, A., Chandar, S. S., & Bhaskara Rao, K. V. (2014). Protease inhibitors from marine actinobacteria as a potential source for ant malarial compound. *PloS one*, *9*(3), e90972.

Khademi, F., Maarofi, K., Arzanlou, M., Peeri-Dogaheh, H., & Sahebkar, A. (2021). Which missense mutations associated with DNA gyrase and topoisomerase IV are involved in Pseudomonas aeruginosa clinical isolates resistance to ciprofloxacin in Ardabil? *Gene Reports*, *24*, 101211.

Lavery, L. A., Armstrong, D. G., Murdoch, D. P., Peters, E. J., & Lipsky, B. A. (2007). Validation of the Infectious Diseases Society of America's diabetic foot infection classification system. Clinical infectious diseases, 44(4), 562-565.

Lavery, L. A., Armstrong, D. G., Wunderlich, R. P., Tredwell, J., & Boulton, A. J. (2003). Diabetic foot syndrome: evaluating the prevalence and incidence of foot pathology in Mexican Americans and non-Hispanic whites from a diabetes disease management cohort. Diabetes care, 26(5), 1435-1438.

Lavery, L. A., Armstrong, D. G., Wunderlich, R. P., Tredwell, J., & Boulton, A. J. (2003). Diabetic foot syndrome: evaluating the prevalence and incidence of foot pathology in Mexican Americans and non-Hispanic whites from a diabetes disease management cohort. *Diabetes care*, *26*(5), 1435-1438.

Lázaro-Martínez, J. L., García-Madrid, M., García-Álvarez, Y., Álvaro-Afonso, F. J., Sanz-Corbalán, I., & García-Morales, E. (2021). Conservative surgery for chronic diabetic foot Osteomyelitis: Procedures and recommendations. Journal of Clinical Orthopedics and Trauma, 16, 86-98.

Lee, V. T., & Pukatzki, S. (2018). Symbiotic Role of Pseudomonas aeruginosa in Disease and Maintenance of Immunity. Microbiology and molecular biology 82(2).

Lipsky, B. A., Berendt, A. R., Cornia, P. B., Pile, J. C., Peters, E. J., Armstrong, D. G., ... & Senneville, E. (2012). Infectious Diseases Society of America 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. Clin Infect Dis, 54(12), e132-e173.

Malekian, A., Djavid, G. E., Akbarzadeh, K., Soltandallal, M., Rassi, Y., Rafinejad, J., ... & Totonchi, M. (2019). Efficacy of maggot therapy on Staphylococcus aureus and Pseudomonas aeruginosa in diabetic foot ulcers: a randomized controlled trial. *Journal of Wound Ostomy & Continence Nursing*, *46*(1), 25-29.

Matta-Gutierrez, G., Garcia-Morales, E., Garcia-Alvarez, Y., Álvaro-Afonso, F. J., Molines-Barroso, R. J., & Lazaro-Martinez, J. L. (2021). The influence of multidrug-resistant bacteria on clinical outcomes of diabetic foot ulcers: a systematic review. Journal of Clinical Medicine, 10(9), 1948.

Miere, F., Teuşdea, A. C., Laslo, V., Cavalu, S., Fritea, L., Dobjanschi, L., ... & Vicas, S. I. (2021). Evaluation of in vitro wound-healing potential, antioxidant capacity, and antimicrobial activity of Stellaria media (L.) Vill. Applied Sciences, 11(23), 11526.]

Moradali, M. F., Ghods, S., & Rehm, B. H. (2017). Pseudomonas aeruginosa lifestyle: a paradigm for adaptation, survival, and persistence. *Frontiers in cellular and infection microbiology*, 7, 39.

Morin, C. D., Déziel, E., Gauthier, J., Levesque, R. C., & Lau, G. W. (2021). An organ system-based synopsis of Pseudomonas aeruginosa virulence. *Virulence*, *12*(1), 1469-1507.

Moura Neto, A., Zantut-Wittmann, D. E., Fernandes, T. D., Nery, M., & Parisi, M. C. R. (2013). Risk factors for ulceration and amputation in diabetic foot: study in a cohort of 496 patients. Endocrine, 44, 119-124.

Mutonga, D. M., Mureithi, M. W., Ngugi, N. N., & Otieno, F. C. (2019). Bacterial isolation and antibiotic susceptibility from diabetic foot ulcers in Kenya using microbiological tests and comparison with RT-PCR in detection of S. aureus and MRSA. *BMC Research Notes*, *12*(1), 1-6.

Nugroho, P. S., Tianingrum, N. A., Sunarti, S., Rachman, A., Fahrurodzi, D. S., & Amiruddin, R. (2020). Predictor Risk of Diabetes Mellitus in Indonesia, based on National Health Survey. Malaysian Journal of Medicine & Health Sciences, 16(1).

Obeid, M., Moughames, E., Aboulhosn, P., Madi, R., Farah, M., Feghali, J., ... & Husni-Samaha, R. (2018). Epidemiology and susceptibility profiles of diabetic foot infections in five hospitals in Lebanon. *The Journal of Infection in Developing Countries*, *12*(05), 347-351.

Ozdemir, M., Buyukbese, M. A., Cetinkaya, A., & Ozdemir, G. (2003). Risk factors for ocular surface disorders in patients with diabetes mellitus. *Diabetes research and clinical practice*, *59*(3), 195-199.

Pouget, C., Dunyach-Remy, C., Pantel, A., Schuldiner, S., Sotto, A., & Lavigne, J. P. (2020). Biofilms in diabetic foot ulcers: Significance and clinical relevance. Microorganisms, 8(10), 1580.

Römling, U., & Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *Journal of internal medicine*, *272*(6), 541-561.

Seder, N., Rayyan, W. A., O'la Al-Fawares, M. H., & Bakar, A. (2023). Pseudomonas aeruginosa Virulence Factors and Ant virulence mechanisms to Combat Drug Resistance; A Systematic Review. *mortality*, *10*, 11.

Sen, C. K. (2019). Human wounds and its burden: an updated compendium of estimates. Advances in wound care, 8(2), 39-48.

Serván, P. R. (2018). Diet recommendations in diabetes and obesity. *Nutrition hospitalaria*, *35*(Spec No4), 109-115.

Silva, A., Silva, V., Igrejas, G., & Poeta, P. (2020). Carbapenems and Pseudomonas aeruginosa: Mechanisms and epidemiology. In Antibiotics and Antimicrobial Resistance Genes in the Environment (pp. 253-268). Elsevier.

Strateva, T., & Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. *Annals of microbiology*, *61*(4), 717-732.

Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. Diabetes atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* (2022) 183:109119. doi: 10.1016/j.diabres.2021.109119

Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. Diabetes atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* (2022) 183:109119. doi: 10.1016/j.diabres.2021.109119

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B. B., ... & 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.

Thacharodi, A., & Lamont, I. L. (2022). Aminoglycoside-modifying enzymes are sufficient to make Pseudomonas aeruginosa clinically resistant to key antibiotics. *Antibiotics*, *11*(7), 884.

Thacharodi, A., & Lamont, I. L. (2022). Aminoglycoside-modifying enzymes are sufficient to make Pseudomonas aeruginosa clinically resistant to key antibiotics. *Antibiotics*, *11*(7), 884.

Tolossa, T., Mengist, B., Mulisa, D., Fetensa, G., Turi, E., & Abajobir, A. (2020). Prevalence and associated factors of foot ulcer among diabetic patients in Ethiopia: a systematic review and meta-analysis. *BMC public health*, *20*, 1-14.

Wahyuni, S.N., Sudarvatno, B. and Pramudyantoro, A., (2022), December. Prediction Analysis of Diabetes Mellitus Based on Machine Learning Algorithm. In *2022 5th International Seminar on Research of Information Technology and Intelligent Systems (ISRITI)*. 209

World Health Organization. (2020). Diabetes. Retrieved from https://www.who.int/health-topics/diabetes#tab=tab_1

Xie, X., Bao, Y., Ni, L., Liu, D., Niu, S., Lin, H., & Luo, Z. (2017). Bacterial profile and antibiotic resistance in patients with diabetic foot ulcer in Guangzhou, Southern China: focus on the differences among different Wagner's grades, IDSA/IWGDF grades, and ulcer types. International journal of endocrinology, 2017.

Xie, X., Lu, C., Wu, M., Liang, J., Ying, Y., Liu, K., ... & Jing, C. (2020). Association between triclocarban and triclosan exposures and the risks of type 2 diabetes mellitus and impaired glucose tolerance in the National Health and Nutrition Examination Survey (NHANES 2013–2014). Environment international, 136, 105445.

Zhang P, Lu J, Jing Y, Tang S, Zhu D, Bi Y. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis. *Ann Med.* (2017) 49:106–16. doi: 10.1080/07853890.2016.1231932

Zubair, M., Malik, A., & Ahmad, J. (2010). Clinico-bacteriology and risk factors for the diabetic foot infection with multidrug resistant microorganisms in north India. Biol Med, 2(4), 22-34.].