VOLUME 10 ISSUE 2 2024

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 10, No. 2, 1171-1176 (2024)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Detection of Biofilm Forming Methicillin-Resistant *Staphylococcus aureus* from Diabetic Foot Ulcer Patient Pus Sample and its Antibiotic Susceptibility Test

Jamith Basha Abdul Wahid[1]*, Muhannad Alruwaili[1] and Balasubramanian A.[2]

[1]Faculty of Applied Medical Sciences, Northern Border University, Saudi Arabia
[2]PG Research Department of Microbiology, V.H.N.S.N. College, Virudhunagar, Tamil Nadu, India

**Corresponding Author*

Received: 2nd November, 2024; **Accepted:** 22nd November, 2024; **Published online:** 25th November, 2024

https://doi.org/10.33745/ijzi.2024.v10i02.114

Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a great risk to patients with wounds. Significant increase in both mortality and morbidity in humans has been reported in patients infected with MRSA due to the development of biofilm. MRSA are frequently resistant to a wide variety of antibiotics, and this is more pronounced in those having ability to form a biofilm. The pus samples were obtained from the wounds of diabetic foot ulcer patients in Government Hospital, Virudhunagar, India. 40 pus samples were inoculated onto nutrient agar, blood agar and mannitol salt agar. The aim of the study was to evaluate the detection of biofilm forming MRSA from collected pus sample and their antibiotic susceptibility tests.

Keywords: Methicillin-resistant *Staphylococcus aureus*, Pus, Foot Ulcer, Biofilm, Antibiotic susceptibility tests

Citation: Jamith Basha Abdul Wahid, Muhannad Alruwaili and Balasubramanian A.: Detection of biofilm forming Methicillin-Resistant *Staphylococcus aureus* from diabetic foot ulcer patient pus sample and its antibiotic susceptibility test. Intern. J. Zool. Invest. 10(2): 1171-1176, 2024.

https://doi.org/10.33745/ijzi.2024.v10i02.114

Introduction

Diabetes mellitus (DM) is a long-lasting and progressive disorder of the endocrine system, characterized by persistent high blood sugar levels (Fekadu *et al.*, 2019). One of the most serious complications associated with this condition is diabetic foot infection (DFI) (Inzucchi *et al.*, 2015; Anwar *et al.*, 2020), which is caused by various bacterial species (Hinojosa *et al.,* 2016). Among diabetic complications, foot-related issues have the most significant clinical impact, with 85% of limb amputations preceded by non-healing ulcers (Moore *et al.,* 2021). Research indicates that roughly 25% of diabetic patients will experience one or more diabetic foot ulcers (DFUs) in their lifetime (Ena *et al.*, 2021). Chronic, non-healing wounds such as DFUs, pressure ulcers, and venous

leg ulcers pose major global healthcare challenges (Bowers *et al.*, 2020). When the protective skin barrier is compromised, underlying soft tissues become vulnerable to bacterial infections, potentially leading to osteomyelitis if joints or bones are involved (Maheswary *et al.*, 2021). DFUs significantly affect mortality and morbidity rates (Rubio *et al.*, 2020) and negatively impact patients' quality of life by reducing physical, physiological, and social functioning (Polikandrioti *et al.*, 2020). The most commonly isolated microorganisms in DFUs include species of *Staphylococcus*, *Streptococcus*, *Enterococci*, Enterobacteriaceae and *Pseudomonas aeruginosa* (Alfonsi *et al.*, 2022).

Recent studies have shown that one of the most prevalent colonizers among DFUs is *S. aureus* (Elbehiry *et al.*, 2021). *Staphylococcus aureus* belongs to a gram-positive group with a cocci shape. It was found in grape-like clusters. Frequently, *S. aureus* is mostly found in humans who are the favorable for maintenance and infection. Especially, *S. aureus* has characteristic of biofilm formation. When S*. aureus* enters into the circulatory system, it avoids the detection by immune system, binds to a specific surface including infection area and forms biofilm to survive in host. Moreover, its biofilm can be created on both biotic and abiotic surface, *S. aureus* shows resistance to antibiotics that becomes a problem in treatment. Some infectious diseases related to the *S. aureus* biofilm formation were arthritis, endocarditis, and cystic fibrosis (Costa *et al*., 1999).

Bacteria could have characteristics to form biofilm. The extracellular polymeric matrix is made from the combination of exopoly-saccharides, proteins, teichoic acids, enzymes, and extracellular DNA (Melchior *et al*., 2006). According to previous studies, the matrix's structure is changed from strains to strains due to the environment and the conditions. Biofilm may have a 3-dimension structure, but sometimes a single layer only. By living in community, biofilms have various benefits and advantages from its parts and one of them is resistant to the immune system and antibiotics. The aim of the present study was to evaluate the detection of biofilm forming MRSA from collected pus sample and their antibiotic susceptibility tests.

Materials and Methods

Sample collection

The pus samples were obtained from the wounds of diabetic foot ulcer patients in Government Hospital, Virudhunagar, India. Totally 40 pus samples were inoculated onto nutrient agar, blood agar and mannitol salt agar. *S.aureus*, was identified by standard microbiological techniques including Gram stain, Catalase, Coagulase test and DNase test. Biofilm formation by these isolates was detected by two *in vitro* methods: tissue culture plate (TCP) method and tube adherence method (TM).

Antimicrobial susceptibility test

Antimicrobial susceptibility tests of the clinical isolates against different antimicrobials were performed in Muller Hinton agar (MHA) using the standard disk diffusion technique (modified Kirby-Bauer method) and interpreted as per clinical and laboratory standard Institute guidelines. The following antimicrobial agents were tested: ampicillim (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (15 µg), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg).

Isolates were considered multidrug resistance (MDR) based on the guidelines recommended by the joint initiative of the European Center for Disease Control and prevention. According to those guidelines, the isolates showing non-susceptible to at least one agent in one or more antimicrobial categories were identified as MDR.

Detection of methicillin resistant Staphylococcus aureus (*MRSA*)

All the isolates identified as *S.aureus* were further screened for methicillin resistant using the cefoxitin and methicillin antibiotic disk. The *S. aureus* culture was inoculated on to MHA agar

Table 1: Cultural characterization of *Staphylococcus aureus*

S. No.	Medium	Colony morphology
1.	Mannitol salt agar	Yellow color colonies
2.	Blood agar	Golden yellow color colonies with clear hemolysis (β-hemolysis)
3.	Nutrient agar	Yellow color colonies

Table 2: Biochemical characterizations of *Staphylococcus aureus*

S. No.	Tests	Results
1	Oxidase	Negative
2	Catalase	Positive
3	Methyl red	Positive
4	Vogas-proskauer	Positive
5	Indole	Negative
6	Citrate utilization test	Positive
7	Coagulase	Positive
8	DNase	Positive

plates by lawn culture. Cefoxitin (30 µl) was placed on the agar plate and incubated overnight at 37°C. On the following day, the zone of inhibition was measured, and ≤ 21 mm in diameter was considered as MRSA (Doebbeling, 1995).

Detection of Biofilm formation of Staphylococcus aureus

Biofilm was formed in sterile flat-bottomed 96 well polystyrene microlitter plates based on the previous studies. One colony was inoculated in 5 ml of LB broth and cultured at 37°C for 18 to 20 h. The bacteria were diluted at 1: 100 with appropriate medium: (1) LB, (2) LB with 1% of glucose, (3) LB with 1% glucose and 0.1% K_2HPO_4, (4) LB with 0.1% K_2HPO_4 (5) LB with 0.1 5% Na_2HPO_4 and added 200 µl of diluted bacteria into the wells of 96 well plate. 200 µl of bacteria in 0.9% NaCl was put into one well to be used as a control. The plates were incubated at 37°C for 24, 48, 72 h (Singh *et al*., 2011). After incubation the broth in each well of the plates was discarded, then the wells were washed three times with sterile phosphate buffer (PBS). The wells were heated at 60°C for 60 min. The wells were stained with 50 µl of crystal violet for 10 to 15 min at room temperature. The crystal violet was removed by micropipette, and then the wells were washed with 200 µl of sterile distilled water for three times. The plates were dried at room temperature before adding 200 µl of 95% ethanol. The plates were incubated for 15 to 20 min at room temperature and measured the OD value at 630 nm wavelength. The assay was performed three times.

Results

Isolation and characterization of Staphylococcus aureus

Staphylococcus aureus strains were isolated from pus samples. Mannitol salt agar and blood agar was used as a differential and selective media for recovering strains from the sample. *Staphylococcus aureus* produce golden yellow color colonies in mannitol salt agar and β-hemolytic, golden yellow color colonies in blood agar (Table 1).

Table 3: Antibiotic sensitivity patterns of isolated *Staphylococcus aureus*

S. No.	Antibiotics	Concentration (µg)	Diameter of zone of Zone of inhibition (mm)
1	Ampicillin	10	Nil (R)
2	Chloramphenicol	30	23 (S)
3	Tetracycline	30	14 (R)
4	Rifampicin	5	26 (S)
5	Vancomycin	30	16 (R)
6	Gentamicin	10	10 (R)
7	Ciprofloxacin	5	28 (S)
8	Streptomycin	25	15 (R)
9	Cefoxitin	30	17 (R)
10	Ofloxacin	2	28 (S)
11	Amaxacillin	25	Nil (R)
12	Teicoplanin	30	31 (S)
13	Methicillin	30	10 (R)

S: Sensitive; R: Resistance

Table 4: Absorbance of *Staphylococcus aureus* at wavelength 630 nm after incubation for 24, 48 and 72 h in difference medium

Time (h)	LB	LB + glucose	LB+glucose+ K_2HPO_4	LB+K_2HPO_4	LB + Na_2HPO_4	Control
24	1.85	2.17	3.1	1.4	1.5	0.1
48	1.87	3.7	4.5	1.5	2.0	0.2
72	2.1	4.5	6.8	1.7	2.1	0.2

The gram nature of *Staphylococcus aureus* was observed under microscope. *Staphylococcus aureus* appears Gram positive and cocci in shape.

Biochemical characterization of Staphylococcus aureus

Staphylococcus aureus were subjected to various biochemical tests. The results are expressed in Table 2.

Antibacterial susceptibility test

The isolated bacterial strain was further subjected to antibacterial susceptibility test with different antibacterial agent. The results are illustrated in Table 3.

Biofilm formation assay

The Biofilm formation of *Staphylococcus aureus* strain was investigated at 24, 48 and 72 h in different medium. The bacterium produces the highest amount of Biofilm at 24 h in medium containing glucose and K_2HPO_4. The supplemented medium stimulates the Biofilm formation.

Discussion

Nowadays many microorganisms have developed resistance against antibiotics. *Staphylococcus aureus* has developed the resistance against methicillin. Methicillin resistance *Staphylococcus aureus* (MRSA) developed resistance not only to methicillin and but also to other β-lactam antibiotics. *Staphylococcus aureus* is the major cause of nosocomial infections. *Staphylococcus* infections are very difficult to cure because *S. aureus* produces Biofilm and resistance against almost all clinical available antibiotics (Jency *et al*., 2014).

In the present investigation, the Biofilm forming *Staphylococcus aureus* isolated from pus sample of diabetic foot ulcer patients admitted in Government Hospital, Virudhunagar, India. The

isolated *Staphylococcus aureus* was identified later and characterized biochemically.

Dima Owais *et al.* (2024) screened the antimicrobial susceptibility testing for 32 different *S. aureus* isolates to determine their antimicrobial profile against 15 antimicrobials. *S. aureus* isolates showed high rates of resistance to oxacillin (95.2%), clindamycin (68.7%), erythromycin (65.6%), cefazolin (60.2%), levofloxacin (50.2%), ciprofloxacin (48.1%), gentamycin (46.9%), and ampicillin (45.3%). Whereas antibiotics that are effective and sensitive were linezolid (3.7%), rifampin (5.1%), chloramphenicol (6.7%), norfloxacin (14.1%), teicoplanin (15.6%), amikacin (18.8%), tigecycline (31.2%).

From the ulcer wounds of 213 diabetic foot patients, researchers isolated 354 micro-organisms, with an average of 1.7 organisms per wound. Polymicrobial infections were more prevalent (n = 132; 50.7%) than monomicrobial infections (n = 81; 31.2%). The majority of isolates were Gram-negative bacteria (n = 192; 54.2%), while aerobic Gram-positive Cocci made up 162 (45.8%) of the total. *Staphylococcus aureus* emerged as the most commonly isolated aerobe (n = 106; 29.9%), followed by *Pseudomonas aeruginosa* (n = 91; 25.7%) (Sekhar *et al*., 2018).

The isolated *Staphylococcus aureus* was further analyzed for Biofilm formation. The Biofilm formation detected by tissue culture plate method, tube adhesion method similar observation was made by Christensen *et al*. (1982). In Congo red agar medium Biofilm forming *Staphylococcus aureus* grow in block color colony. It is also reported by Freeman *et al*. (1989). Dima Owais *et al.* (2024) reported that the TCP method revealed 10 (31.25%) isolates as strong biofilm producers, 14 (43.75%) as moderate biofilm producers, and 8 (25%) as non-biofilm producers. Among the 32 *S. aureus* isolates, 24 (75%) were capable of biofilm formation, while 23 (71.9%) exhibited multi-drug resistance. Statistical analysis demonstrated a significant correlation between an isolate's biofilm-producing ability and its multi-drug resistance (p= 0.015).

Neopane *et al.* (2018) reported that among 43 clinical S. aureus isolates from wounds, biofilm formation was detected in 30 (69.8%) isolates using the TCP method and in 28 (65.1%) isolates using TM. The TCP method identified 3 (6.97%) isolates as strong biofilm producers, 12 (27.90%) as moderate producers, and 15 (34.88%) as weak producers. In comparison, TM identified 2 (4.65%) isolates as strong producers, 10 (23.25%) as moderate producers, and 16 (37.20%) as weak producers. When both methods were employed, 2 (4.65%) isolates were classified as strong biofilm producers, 10 (23.25%) as moderate producers, and 13 (30.23%) as weak producers.

The *Staphylococcus aureus* biofilm formation assay was performed with different culture media. The high amount of Biofilm formation was observed in LB medium containing glucose and K_2HPO_4 after 24 h incubation. The similar trends were reported by Periasamy *et al*. (2012).

Conclusion

Methicillin-resistant *S. aureus* (MRSA) poses a great risk to patients with wounds. Significant increase in both mortality and morbidity in humans has been reported in patients infected with MRSA due to the development of biofilm. This study concluded the biofilm forming MRSA found in the pus sample of foot ulcer Patients. Further studies for treating biofilm forming MRSA by valuable medicinal plants and their bioactive molecules were designed for drug synthesis.

Acknowledgements

The authors extend their appreciation to the Dean, Scientific Research at Northern Border University, Arar, KSA for funding this research work through the project number "NBU-FFR-2024-1329-08".

References

Alfonsi V, Gorgoni M, Scarpelli S, Zivi P, Sdoia S, Mari E and De Gennaro L. (2022) Changes in sleep pattern and dream activity across and after the COVID-19

lockdown in Italy: A longitudinal observational study. J Sleep Res. 31(2): e13500.

Anwar K, Hussein D and Salih J. (2020) Antimicrobial susceptibility testing and phenotypic detection of MRSA isolated from diabetic foot infection. Int J Gen Med. 13: 1349-1357.

Bowers S and Franco E. (2020) Chronic wounds: evaluation and management. American Family Physician 101(3): 159-166.

Christensen GD, Simpson WA, Bison AL and Beachey EH. (1982) Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun. 37(1): 318-326.

Costa GM, Pizzi C, Leone C,. Borghi A, Cordioli E and Bugiardini R. (1999) Thrombosis of a mitral valve prosthesis resulting from *Staphylococcus epidermidis* endocarditis. Cardiologia 44: 675-678.

Doebbeling B. (1995) The epidemiology of methicillin-resistant *Staphylococcus aureus* colonization and infection. J Chemother. 7 (3): 99-103.

Elbehiry A, Marzouk E, Moussa IM, Alenzi A, Al-Maary KS, Mubarak AS, Alshammari HD, Al-Sarar D, Alsubki RA, Hemeg HA, Kabli SA and Attala OA. (2021) Multidrug-resistant *Escherichia coli* in raw milk: Molecular characterization and the potential impact of camel's urine as an antibacterial agent. Saudi J Biol Sci. 28(4): 2091-2097.

Ena J, Carretero-Gomez J, Arevalo-Lorido JC, Sanchez-Ardila C, Zapatero-Gaviria A and Gomez-Huelgas R. (2021) The association between elevated foot skin temperature and the incidence of diabetic foot ulcers: a meta-analysis. Int J Lower Extremity Wounds 20(2): 111-118.

Fekadu G, Bula K, Bayisa G, Turi E, Tolossa T and Kasaye HK. (2019) Challenges and factors associated with poor glycemic control among type 2 diabetes mellitus patients at Nekemte Referral Hospital, Western Ethiopia. J Multidiscipl Healthcare 12: 963-974.

Hinojosa CA, Boyer-Duck E, Anaya-Ayala JE, Nunez-Salgado A, Laparra-Escareno H, Torres-Machorro A and Lizola R. (2016) Impact of the bacteriology of diabetic foot ulcers in limb loss. Wound Repair Regeneration 24(5): 923-927.

Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M and Matthews DR. (2015) Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 38(1): 140-149.

Maheswary T, Nurul AA and Fauzi MB. (2021) The insights of microbes' roles in wound healing: A comprehensive review. Pharmaceutics 13(7): 981.

Melchior MB, Vaarkamp H and Fink-Gremmels J. (2006) Biofilms: a role in recurrent mastitis infections? Vet J. 171(3): 398-407.

Moore Z, Avsar P, Wilson P, Mairghani M, O'Connor T, Nugent L and Patton D. (2021) Diabetic foot ulcers: treatment overview and cost considerations. J Wound Care 30(10): 786-791.

Neopane P, Nepal HP, Shrestha R, Uehara O andAbiko Y. (2018) *In vitro* biofilm formation by *Staphylococcus aureus* isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. Int J Gen Med. 11: 25-32.

Owais D, Al-Groom RM, AlRamadneh TN, Alsawalha L, Khan MSA, Yousef OH and Burjaq SZ. (2024) Antibiotic susceptibility and biofilm forming ability of *Staphylococcus aureus* isolated from Jordanian patients with diabetic foot ulcer. Iranian J Microbiol. 16(4): 450.

Periasamy S, Joo HS, Duong AC, Bach TH, Tan VY, Chatterjee SS, Cheung GY and Otto M. (2012) How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc Natl Acad Sci USA. 109(4): 1281-1286.

Polikandrioti M, Vasilopoulos G, Koutelekos I, Panoutsopoulos G, Gerogianni G, Babatsikou F and Toulia G. (2020) Quality of life in diabetic foot ulcer: associated factors and the impact of anxiety/depression and adherence to self-care. Int J Lower Extremity Wounds 19(2): 165-179.

Rubio JA, Jiménez S and Lázaro-Martínez JL. (2020) Mortality in patients with diabetic foot ulcers: causes, risk factors, and their association with evolution and severity of ulcer. J Clin Med. 9(9): 3009.

Sekhar S, Unnikrishnan MK, Rodrigues GS, Vyas N and Mukhopadhyay C. (2018) Antimicrobial susceptibility pattern of aerobes in diabetic foot ulcers in a South-Indian tertiary care hospital. The Foot 37: 95-100.