Molecular Identification of a Newly Isolated of *Niallia circulans* from Different Wounds in Erbil City/Iraq

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Abstract— **Molecular identification using 16S rRNA (PCR), sequencing, and the GenBank database, of newly isolated N. circulans, from gunshot wound among clinical wounds in several hospitals in Erbil, Iraq, and studying the antibiogram profile of isolates were the objectives of this study. A total of 157 clinical specimens from various wound infections were collected during (June 2022-September 2022). Molecular identification16S rRNA, morphological characterization and biochemical behavior identified a total of 60 (38.21%) Gram positive bacteria were detected, of which one (1.6%) N. circulans was newly reported in Iraq. The application of PCR in the field of genetic diagnostics has grown to the point where it is now acknowledged as the norm approach for identifying nucleic acids from variety number of sample and microbial types. The percentages of resistance among 20 different antimicrobials showed by N. circulans strain was 100% resist to 11 antibiotics Amoxicillin-Clavulanic acid, Ampicillin Chloramphenicol, Cefotaxime, Nalidixic acid, Nitrofurantoin, Norfloxacin, Rifampin, Streptomycin, Tetracycline and Trimethoprim. Antibiotic-resistant strains of the opportunistic human pathogens Niallia circulans (previously known as Bacillus circulans) have been isolated, raising concerns about the rising prevalence of antibiotic resistance in pathogenic bacteria globally. In conclusion, gunshot wound is a potential source of infection caused by newly isolated of N. circulans, treatment of nosocomial infections should be guided by antibiotic susceptibility testing.**

Index Terms— **Antimicrobials, Gunshot wound, *Niallia circulans,* PCR (16S rRNA), Susceptibility test.**

I. Introduction

Niallia circulans (formerly known as Bacillus circulans) bacteria are Gram-positive, motile, straight, round-ended, rods with a diameter of 0.6-0.8 μm. They may be slightly tapered or curved. They come singly, in pairs, and even in short chains. Endospores are ellipsoidal and found in enlarged sporangia either sub terminally or terminally. Bacillaceae spores measure 0.5-0.7 μm by 4-5 μm and belong to the order Bacillales. Niallia members were originally classified as Bacillus species. To shed light on the evolutionary relationships between Bacillus species, a number of studies employing comparative phylogenetic analyses have been published. As a result, many novel genera, including Brevibacillus, Alkalihalobacillus, Solibacillus, Virgibacillus, Alicyclobacillus, and Evansella, have been formed. The name Niallia is given in recognition of the many contributions made by the British microbiologist Professor Niall A. Logan of Glasgow Caledonian University to the systematics and applications of the Bacillus genus (Sarmiento-López et al., 2022, Saini et al., 2022).

Molecular Signature and Biochemical Properties This genus's members can be found in a variety of environments, including sewage, food, soil, and human waste. They are facultatively anaerobic. They are widely used in enzyme manufacturing, bioremediation, and wastewater treatment (Patel et al., 2022). It is also an opportunistic pathogen, capable of causing sepsis in immunocompromised persons. Analysis of the genomic sequences of Niallia species showed two conserved signature indels (CSIs), which offer a dependable method of molecularly distinguishing Niallia from additional Bacillaceae taxa and bacteria (Saini et al., 2022). In this study, a rod-shaped, gram-positive bacterium Niallia circulans was isolated from the gunshot wounds.

Wounds are disruptions in the cellular, functional and anatomical integrity of living tissue induced by chemical, physical, electrical, or microbiological assaults. Wound infection is a common type of nosocomial infection that affects many people throughout the world and can have a substantial influence on the treatment of many diseases because if the wound is not treated promptly, it can lead to more serious wound inflammation and ulceration. As a result, effective wound infection prevention is a critical component of clinical illness management. Drug-resistant bacteria have become a reasonably prevalent source of persistent infections, and wound infections claim the lives of many people each year. Wounds are generally can arise according to Some diseases like immune-compromised conditions, diabetes, ischemia and conditions like ageing malnourishment, local infection, local tissue damage due to gunshot, burn and bedsores, cuts or surgical incisions, often leads to delay in wound healing (Sharma et al., 2021, Alavi and Nokhodchi, 2022, Shariati et al., 2023, Gunasekaran et al.).

N. circulans isolated in this study in gunshot wound was resist to 11 antimicrobials among 20 different antimicrobials. The antibiotic sensitivity of microorganisms also changes with time, as multidrug resistant organisms evolve. Since the discovery of antimicrobial drugs, microbes have developed resistance to them through processes like as mutations and increased enzyme synthesis. Resistance to frequently used antibiotics is a major issue worldwide. A regular study of the causes of sepsis and associated antibiotic sensitivity patterns is

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critical in the establishment of efficient infection control programs and in directing empiric antibiotic treatment (Abdul-Rahman, 2019). The susceptibility tests done by disc diffusion technique, also known as the Kirby Bauer method was applied in this study carried out according to the Clinical and Laboratory Standard Institute guidelines (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS). Microorganisms significantly hinder wound healing. When infectious bacteria invade a host, the microbes create poisonous chemicals that destroy the host's tissues. These molecules, known as virulence factors, help germs to establish themselves in the host (Ali et al., 2017, Rabie et al., 2023).

The polymerase chain reaction (PCR) discovery in the 1980s is regarded as one of molecular biology's greatest overcomes. It was subsequently improved and now serves as the foundation for the many PCR-based approaches used in molecular diagnostics across diverse species and several types of samples (Moldovan and Moldovan, 2020). Molecular approaches, mostly based on PCR, have become a crucial tool in the diagnosis of infectious illnesses. Due to misidentifications, N. circulans in this article review is identified by molecular identification via conventional PCR amplification of the 16s rRNA gene and conventional biochemical assays. PCR permits the synthesis of DNA, allowing nucleic acid fragments to be selectively reproduced in a semiconservative method (Eghtedar Nejad et al., 2020). This technology can detect and identify tiny amounts of DNA. Several PCR-based approaches are currently available for determining the taxonomic status of bacterial isolates. Molecular approaches can also differentiate bacterial species, even when their shape is quite similar. For example, Bacillus spp. (Bacillus circulans and Niallia circulans) (Kuzdraliński et al., 2017, Eghtedar Nejad et al., 2020). Molecular techniques are known as suitable approachesfor identifying Niallia circulans. These techniques are pricey, but there is no denying their speed and precision.

II. MATERIALS AND METHODS

Isolation and Identification of Niallia circulans bacteria

From June to September 2022, 157 clinical samples were collected at several hospitals in Erbil, Iraq, including gunshot wounds, burns, surgeries, diabetic ulcers, and bedsores. All specimens were immediately inoculated onto blood agar and MacConkey agar and incubated at 37°C for 24 hours. N. circulans bacteria were isolated using standard microbiological procedures. The isolates were identified using conventional microbiological methods such as colony morphology, Gm stain, biochemical assays, enzymes, and hemolytic activity. Furthermore, to re-identify was utilized molecular identification utilizing the PCR (16S rRNA) gene, and the primers were 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT), then sequencing (Dos Santos et al., 2019, Ugbo et al., 2020). A web-based submission instrument (https://submit.ncbi.nlm.nih.gov) to assist with the submission procedure was utilized. Each sequence has been submitted to GenBank and accession number for all isolated bacteria were generated.

Test for antimicrobial sensitivity

Test for antimicrobial sensitivity was used to assess the sensitivity of isolated bacteria against 20 different antibacterials (Amikacin (AK), Amoxicillin-Clavulanic acid (AMC), Ampicillin (AMP), Ciprofloxacin (CIP), Chloramphenicol (C), Cefotaxime (CTX), Doxycycline (DOX), Gentamicin (CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MEM), Nalidixic acid (NA), Nitrofurantoin (F), Norfloxacin (NOR), Rifampin (R), Streptomycin (S), Tetracycline (TE), Tobramycin (TOB), Trimethoprim (TMP) and Vancomycin (VA)) by using disc diffusion method (Kirby Bauer) as explained by Clinical and Laboratory Standards Institute (CLSI) (Rabie et al., 2023).

Molecular study

A. Molecular identification of bacteria

Overnight-grown cultures of all identified bacteria were inoculated in BHI broth and cultivated in a shaker incubator at 37°C for 24 hours for sample preparation were utilized to isolate DNA using the Presto TM Mini gDNA bacterial kit (Gene aid-Taiwan). About 1 ml of BHI broth (containing up to 1×109 bacterial cells) was transferred to 1.5 ml microcentrifuge tubes.

Centrifuged for 1 minute at 14-16,000 rpm before discarding the supernatant. To re-suspend the cell pellet, add 180 µl of Guanidine Thiocyanate (GT) buffer and use a pipette or vortex. After that, add 20 µl of Proteinase K to the sample and incubate at 60°C for at least 10 minutes, inverting the tubes every 3 minutes. The GT buffer is used as a general protein denaturant, although it is most commonly used as a nucleic acid protector in DNA and RNA extraction from cells. Cells were lysed by adding 200 µl of GB buffer to samples, vortexing for 10 seconds, and incubating at 70°C for at least 10 minutes. The tubes were reversed every 3 minutes. At the same time, the elution buffer was preheated. Then, for DNA binders, 200 microliters of 100% ethanol were introduced to the samples and vigorously shaken. The mixture was moved to the GD column and centrifuged at 14–16,000 rpm for two minutes after The column was positioned in a 2 ml collecting tube. Following the removal of the 2 ml collected tube containing the flow-through, the GD column was positioned in a brand-new 2 ml collected tube. Furthermore, to wash the DNA The GD column was filled with 400 microliters of W1 buffer, and it was centrifuged for 30 seconds at 14–16,000 rpm. Place the GD column in a 2 ml collection tube and discard the flow-through from the collection tube after centrifugation. Before discarding the flow-through, add 600 µl of wash buffer (including ethanol) to the GD column and a centrifuge at 14–16,000 rpm for 30 seconds. The GD column was inserted back into the 2 mL collection tube. Centrifuge again for three minutes at a speed of 14,000–16,000 rpm to dry the column matrix. To elute, place the GD column that had dried in a sanitized 1.5 ml microcentrifuge tube and fill the column matrix's middle with 100 µl of hot elution buffer. To extract the pure DNA, it was centrifuged for 30 seconds at 14–16,000 rpm after being left for at least three minutes.

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B. Nanodrop spectrophotometer determination of DNA concentration and purity

Contains multiple stages. (1) The nanodrop spectrophotometer and associated software were turned on. (2) The Nanodrop program was opened, and the nucleic acid button was pressed. (3) The surface of the nanodrop spectrophotometer was cleaned with double-distilled water. (4) The blank was added with 1 µl of DNA hydration solution (elution buffer). (5) The blank was accessed by pushing the blank button. (6) The surface of the nanodrop spectrophotometer was cleaned with double-distilled water. (7) The first DNA sample was inserted as follows: 1 µl DNA template. (8) The concentration of DNA was obtained by pushing the measure button. (9) The surface of the nanodrop spectrophotometer was cleaned with double-distilled water. (10) Steps 7–9 were repeated for each sample. (11) DNA concentration measurements were done. (12) RNA and DNA have 260/280 ratios of around 1.8 and 2.0, respectively. The 260/230 purity ratio was a second measurement of DNA purity, with values for a "pure" nucleic acid typically ranging from 1.8 to 2.2 (Philippe and Deborah, 2010, Dhital and Mustapha, 2023).

C. Primers

The primers were provided by Macrogen (South Korea) in lyophilized form.

D. Determination of the melting temperature of primers

For primers with fewer than twenty-five nucleotides, the approximate melting point (Mohammed et al.) can be estimated by using the following equation:
Tm = 2(A+T) + 4(G+C), where G, C, A, and T represent the number of respective nucleotides in the primer (Parks and Torres, 2023)

E. Primer's preparation

The primers described in (Table 2.1) were processed to create a stock concentration of 100 µm by combining the concentrated lyophilized primer with certain amounts of nuclease-free water (PCR grade water), based on the primer production. To create a working primer concentration of 10 µM, 90 µl of nuclease-free water was mixed with 10 µl of stock primer. Each primer aliquot was kept at -20°C.

TABLE II.I
PRIMERS USED FOR AMPLIFICATION AND SEQUENCING OF 16S RRNA GENE

Target gene	Primer	Sequence (5'-3')	Amplicon size (bps)	References
16S rRNA	27F Forward	AGAGTTTGATCCTGGCTCAG	1400	(Dos Santos et al., 2019, Ugbo et al., 2020)
	1492R Reverse	TACGGYTACCTTGTTACGACTT		

F. Molecular identification using 16S rRNA gene sequencing

All isolated were identified by using 16S rRNA (PCR) (Table 3.2).

G. Amplification of DNA

An explanation of the PCR master kit

Master mix Taq Polymerase DNA 2x RED is a pre-made 2x reaction mix that includes magnesium chloride, dNTPs, NH4+ buffer system, and amplicon Taq DNA polymerase. For every reaction, fifteen microliters of the 2x master mix RED are needed. To effectively perform primer extensions and other molecular biology applications, a reaction volume of 30 µl was filled with primers, template, and nuclease-free water.

Amplification of 16S rRNA gene

PCR was used to amplify the 16S rRNA gene using universal primers listed in Table 2.1 (Ugbo et al., 2020). The 16S rRNA (PCR) reaction was run in a response volume of 30 µl. 1.5 µl of the forward and reverse primers, 2 µl of the DNA template, and 10 µl of nuclease-free water are included in the 15 µl master mix tube, as shown in Table 2.2. Before being used to identify bacteria, the 16S rRNA gene was amplified using DNA that had been fully denaturized for four minutes at 96°C in a thermal cycler. The PCR was then carried out using the following 35-cycle program: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds. With an end extension of 10 minutes at 72°C, and 35 cycles of these segments were repeated (Table 2.3). Eventually, PCR tubes were kept at -20°C till the additional analysis (Dos Santos et al., 2019, Ugbo et al., 2020).

TABLE II.II
PCR MIXTURE RESPONSE FOR THE 16S RRNA GENE (30 µL)

Components	Volume (µl)
PCR master mix	15
Primer F	1.5
Primer R	1.5
Genomic DNA template	2
Nuclease free water	10

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TABLE II.III
CONDITIONS FOR THERMOCYCLING AND THE PCR PROCEDURE

Name of gene	Initially denaturation	Denatured	Annealing	Extension	Cycles	Final Extension
16S rRNA	96°C /4 min.	94°C /30 sec.	57°C /30 sec.	72°C /60 sec.	35	72°C/10 min. Then 4 ˚C→∞

Protocol of PCR technique

A. DNA extract was utilized as a template in the PCR procedure. A reaction volume of 30 µl was used for the PCR.
B. Gently mixing the ingredients was done. Following a brief centrifugation, the tubes were sealed and put within the heat cycler.
C. Processed in the thermal cycler for 35 cycles.
D. The PCR result was found following the PCR procedure using gel electrophoresis.
E. The gel was photographed with the UV-illuminator.

H. Detection of DNA content

Agarose gel electrophoresis

Genomic DNA visualized by using gel electrophoresis with UV illuminator and safe stain (Parks and Torres, 2023).

Agarose preparation

To make agarose gel, combine 1 gm of agarose with 100 milliliters of 1× TAE buffer by swirling the mixture well. After allowing the mixture to cool to around 50–55°C and melting it for two to three minutes in the microwave, 5 µl of red safe dye was added for every 100 ml of agarose gel. The gel was allowed to solidify after the appropriate comb was placed in the tray and agarose was added progressively until it reached an inch of around 1 cm in the tank. After removing the comb, the gel is put into the electrophoresis chamber with the wells facing the cathode end. The gel had been covered with the running buffer until it was fully immersed. DNA was detected using a gel made of agarose at a concentration of 1%.

Sample preparation

Prior to electrophoresis, two µl of loading dye were combined with eight µl of DNA material. Subsequently, the liquid was extensively diluted by filling and pressing the pipette repeatedly.

Agarose electrophoresis

The electrophoresis tank was filled with buffer TAE 1X, the tray containing the 1% agarose was lowered into the container, and the buffer was a few milliliters above the agarose's surface. Ten microliters of sample were added to each well, and the first well was filled with five microliters of a standard molecular weight ladder DNA marker, which was used to determine the sample's size and molecular weight. The power supply was turned on, the gel reservoir was shut, and the voltage at which the gel worked was 5 Volts/cm. After that, the voltage was increased to between 75 and 100 volts, and the electrophoresis was allowed to run for the appropriate period of time. When the bromophenol blue reached three-quarters of the gel's length, the gel stopped flowing. After turning off the electricity, a UV transilluminator was used to collect DNA bands at 240–366 nm, and a Polaroid photo documentation camera was used to take pictures of the gel (Mishra et al., 2010, Bakr et al., 2021).

I. Sequencing, analysis, alignment and submission of 16S rRNA gene

16S rRNA gene sequencing and sequence analysis

After evaluating the purity of the PCR result using gel electrophoresis, it was submitted to Macrogen, a South Korean company, for DNA sequencing (http://www.macrogen.com/). Sequencing was done using primers 16S rRNA 27F (AGAGTTTGATCCTGGCTCAG) and 16S rRNA 1492R (TACGGYTACCTTGTTACGACTT) (Table 2.1) (Dos Santos et al., 2019, Ugbo et al., 2020). At the MACROGEN center in Seoul, Korea, sequencing was done using the Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystem's, USA). Using the Basic Local Alignment Search Tool (BLAST) program, which is available on the NCBI (National Centre for Biotechnology Information) website, one can compare and align laboratory or query sequences with other biological sequences to determine more similarity with isolates. BLAST applies the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of sequence editing were examined to determine how comparable the results were to those of neighboring species.

Sequence alignment and submission

The integrity of the sequenced data was assessed using Bio Edit v7.0.5. The online submission instrument, https://submit.ncbi.nlm.nih.gov, was used; it included wizards to help with the submission process. New sequence data that has been determined and reported by contributors is what the GenBank database was intended for. GenBank has received submissions for every sequence.

Nucleotide sequence accession numbers

The GenBank accession number for the 16S rRNA sequence generated in this study show in table (3.2)

III.RESULT

In present study N. circulans for first time recorded in Iraq depending on molecular study (PCR) using 16S rRNA, sequencing and submitted to NCBI, and the accession number

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was acquired (OQ380608).

A. Distribution and Identification of Niallia circulans bacteria

Of the 157 clinical sample processed from different wounds mentioned early during this study, depending on molecular study (PCR) using 16S rRNA gene sequence analysis, 38.21% (60/157) Gram positive and 1.66% (1/60) showed growth containing newly isolated N. circulans bacteria from gunshot wound. Because new bacteria described with detail. Was identified by inoculated directly on blood agar morphological characteristic was Creamy colored, slightly convex and gamma hemolytic. In addition, biochemical tests (Table 3.1) (Figure 3.1).

TABLE III.I

THE CULTURE CHARACTERISTICS, HEMOLYTIC ACTIVITY AND BIOCHEMICAL TESTS OF *Niallia circulans*

Isolated bacteria	Culture Media	Hemolytic activity	Biochemical Tests						
	Blood agar		**Catalase**	**Coagulase**	**Oxidase**	**Lipase**	**DNase**	**Protease**	**Urease**
Niallia circulans	**Creamy colored, slightly convex**	**Gamma**	+	**N/A**	+	-	+	+	-

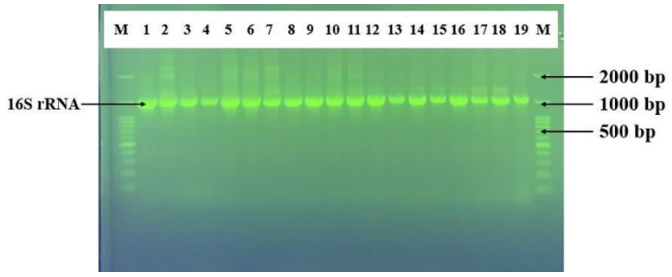

Fig. 3.1 Polymerase chain reaction products in agarose gel electrophoresis (1%) for the 16S rRNA gene. Lane M: Ladder (100 bps). Lane 1-19: Amplified PCR item of 16SrRNA gene (1400-1500bp) for all isolated bacteria.

B. Antimicrobial susceptibility

The present results showed that out of 20 tested antimicrobials, N. circulans was 100% resistant to 11 antimicrobials including Amoxicillin-Clavulanic acid, Ampicillin Chloramphenicol, Cefotaxime, Nalidixic acid, Nitrofurantoin, Norfloxacin, Rifampin, Streptomycin, Tetracycline and Trimethoprim, however, it was sensitive to other remaining antibiotics (Figure 3.2).

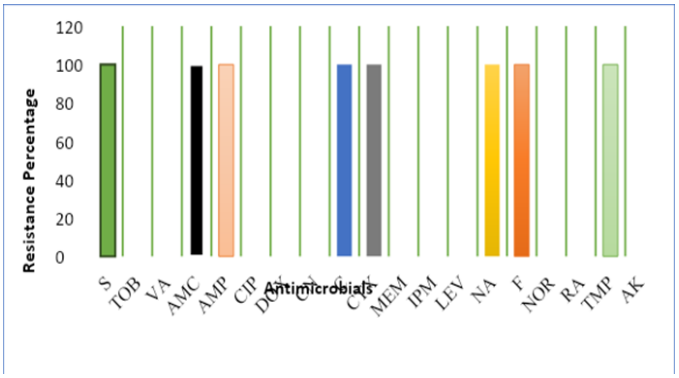

Fig. 3.2 Percentage of antimicrobial resistance of N. circulans..

C. *Molecular study*

To extract pure DNA from the N. circulans sample, genomic DNA isolation was done. Using PCR, the extracted DNA sample's 16sRNA was amplified. N. circulans' amplified 16s rRNA underwent purification and sequencing. The NCBI received the N. circulans sequences. According to Table 3.2, the sequence N. circulans' accession number was (OQ380608).

TABLE III.II
NEW RECORD OF ISOLATED *Niallia circulans* BACTERIA IN IRAQ

Isolated bacteria	No.	Accession Number
Niallia circulans	1	OQ380608

DISCUSSION

Compared to other habitats, wounds were discovered to be an abundant source for a variety of bacterial populations. Wounds present a challenge in medical care because of their increased susceptibility to bacterial infections. Furthermore, there is an urgent need for wound healing that is both quick and efficient while minimizing undesired scarring. The purpose of this study was to use molecular detection to look into novel bacteria found in wound isolates from patients who visited several hospitals in Erbil, Iraq. Using molecular studies (PCR) and 16S rRNA gene sequence analysis, 157 wound specimens were obtained for this study; of them, 38.21% (60/157) were Gm positive and 1.66% (1/60) shown growth comprising freshly recovered N. circulans

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bacteria from gunshot wounds. Former Bacillus species were members of the Niallia genus (strains of Niallia circulans, originally referred to as Bacillus circulans) Niall A. Logan, a professor of microbiology at Glasgow Caledonian University, is recognized for his various contributions to the systematics and uses of the Bacillus genus and is called Niallia (Sarmiento-López et al., 2022, Saini et al., 2022). Moreover, N. circulans is a Gram-positive, spore-forming, rod-shaped species (Hossain et al., 2023). (Khan et al., 2008, Kaftandzieva et al., 2012, Wan et al., 2021) Results matched ours in terms of the proportion of Gram-positive bacteria in wound infections: 36.25%, 42.11%, and 30%, respectively, of all isolates were Gram-positive bacteria. Iraq's recent finding of a novel strain of Gram-positive bacteria (N. circulans) in gunshot (Strains of N. circulans have been linked to a number of human illnesses, including wound and abscess infections and septicemia, particularly in immunocompromised patients) (Hossain et al., 2023). Similar to present study, N. circulans was firstly isolated by Logan in 1985 from the surgical wound infection (Russo et al., 2021). Although, unlike to this study, (Russo et al., 2021) obtained N. circulans from a variety of materials, such as food, sewage, dirt, and baby bile. Additionally, this bacteria was isolated from bee larvae's intestines. (Qin et al., 2021) showed a novel discovery of N. circulans was initially identified and detailed by Jordan in 1890, assigned as a novel and represents a notable development with implications for both microbiological research and clinical medicine. It is vital to comprehend the attributes of these bacteria in relation to gunshot wounds in order to develop efficacious therapeutic approaches and improve a more comprehensive understanding of wound infections. Significant contamination and widespread tissue loss are features of combat gunshot wounds. 80% of victims who passed away later from struggle injuries did so due to infectious complications. Consequently, one of the most important responsibilities in the treatment of combat injuries is the battle against wound infection (Krishtafor et al., 2023). The purpose of present study was to determine the newly isolated bacteria of combat gunshot wounds and its sensitivity to antibacterial drugs. In present study N. circulans was resist to 11 antibiotics among 20 antibiotics, Antibiotic-resistant strains of Niallia circulans, an opportunistic human infection, have been isolated; the rising prevalence of antibiotic resistance by pathogenic bacteria is a global problem. (Alebouyeh et al., 2011) showed that N. circulans was resist 8 antibiotics among 14 antibiotics. Identification of the bacteria for first time, raises questions about their pathogenicity, prevalence and potential impact on gunshot wound healing. For an explanation of the mechanism behind this effect, further experimental data will be needed.

CONCLUSION

This study report shows that there was presence of a novel N. circulans in gunshot wound and this isolate was multi-drug resistant. The most accurate approach for identifying N. circulans was the molecular technique. In order to use the bacteria from this study in a variety of sectors, more research on the acquired species is needed.

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