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Molecular detection of *oprL* gene in *Pseudomonas aeruginosa* associated with surgical site infections in Bauchi, Nigeria

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ABSTRACT

Background: *Pseudomonas aeruginosa* (*P. aeruginosa)* associated with surgical site infections (SSIs) is often multidrug-resistant and associated with delayed wound healing, morbidity, and deaths. Molecular detection of *P. aeruginosa* is often required for specific diagnosis for optimal care. Although SSIs are prevalent in North-East Nigeria, there is a lack of studies on molecular confirmation targeting virulence genes. This study aimed to isolate and characterize *P. aeruginosa* isolates from surgical wound swabs and then confirm them with a PCR assay targeting the *oprL* gene. **Materials and methods:** The study was a cross-sectional study that analyzed 250 post-surgical wound swabs (n = 250) from two hospitals in Bauchi State, Nigeria. The inclusion criteria include fever and purulent pus from the incision site within 30 days of surgery. The conventional analyses were bacterial colonial morphology on Cetrimide agar, Gram staining, and biochemical characterization. The PCR assay was conducted with *oprL* as the target gene. **Results:** *P. aeruginosa* was serially identified in 5 out of 250 swabs, with a prevalence of 2.0%. All the isolates had the *oprL* gene with an amplicon size of 504bp. **Conclusion:** The prevalence of *Pseudomonas aeruginosa* isolates associated with SSIs was 2.0% from the traditional assays. The PCR assay of the isolates detected the *oprL* gene in all the isolates. The study confirms the increasing evidence of the specificity of the *oprL* virulence factor for detecting *Pseudomonas aeruginosa* associated with SSIs. It also recommends further studies on the antibiogram assays associated with *P.aeruginosa* with the *oprL* gene to optimize the treatment of SSIs.

Introduction

Pseudomonas aeruginosa (*Ps. aeruginosa*) is a ubiquitous Gram-negative opportunistic bacillus that is commonly associated with nosocomial infections [1-3]. The bacterium is particularly pathogenic and equipped with various virulence factors that confer on it a wide range of antibiotic resistance [4]. It is adapted to survive in diverse environments like minimal nutritional requirements, and elevated temperatures of up to 42^0C. Its water-soluble pigments include pyocyanin (from "pyocyaneus" meaning "blue pus") which is characteristic of suppurative infections caused by *Ps. aeruginosa*. Pyocyanin is a redox-active (oxidant) phenazine that increases the bioavailability of iron by scavenging for it and enhances virulence through oxidative stress [5,6].

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Ps. aeruginosa infections are favored by immune-compromised conditions, including Acquired Immune Deficiency Syndrome, AIDS, Diabetes Mellitus, etc. An alteration in the integrity of epithelial tissues, including surgical site infections, also favors the pathogenesis [7]. Surgical site infections (SSIs) occur near or at the incision site and/or deeper underlying tissue spaces and organs within 30 days of a surgical procedure (or up to 90 days for implanted prosthetics). It may be superficial incisional when it involves the skin and subcutaneous tissue of the incision; deep incisional when it involves the deep soft tissue for example, fascia, muscle; and organ/space when it involves any part of the anatomy other than the incision that was opened or manipulated during an operation [8].

Pseudomonas aeruginosa associated with SSIs usually occurs after some alteration of the normal skin defense or architecture by the surgical incision. The pathogenesis of *Ps. aeruginosa* associated with SSIs initiates with the introduction of the bacilli from their reservoirs to the surgical site. The reservoirs include water pools, fruits, flowers, and unsterilized medical and hospital instruments and devices [9]. Described three distinct stages of the pathogenesis of SSIs: (1) bacterial attachment on compromised epithelial tissues. This is aided by pili and flagella and subsequent colonization through extracellular polysaccharide slime called biofilm [10-12], (2) local invasion of the whole epidermis tissue, and possibly the subcutaneous tissue. This is aided by flagella attachment and biofilms and extracellular enzymes [13]. It impairs skin epithelial repair mechanisms and resists phagocytosis by the host immune system and (3) disseminated systemic disease, by blood-borne transfer of the infection, to other distant organs. The secondary infections may be urinary tract infections (UTIs), otitis externa, meningitis, brain abscess, osteomyelitis, endocarditis, and Ecthyma gangrenosum [14-16]. The pathogenesis of *Ps. aeruginosa* is linked to extracellular and cell-mediated virulence factors such as *toxA, exoU,exoS, exoY,lasA,lasB ,oprD, oprL* and *oprI.* These virulence genes may be intrinsic genes or encoded in plasmids. They play important functions in tissue invasion, destruction and spread in the host body. [17-20]. The virulence factors are coordinated by bacterial cell-to-cell communication systems, called quorum sensing [21].

The drug resistance of *Ps. aeruginosa* is associated with the *oprl* and *oprL* genes. The outer membrane proteins of *Ps. aeruginosa* play important roles in the interaction of the bacterium with the environment [22]. The L-peptidoglycan-associated lipoprotein and I-lipoproteins are two outer membrane proteins of *P*s. *aeruginosa* that alter membrane permeability and utilize efflux mechanisms to confer antibiotic resistance for *Pseudomonas* species and *Pseudomonas aeruginosa,* respectively. These L and I outer membrane proteins are coded by the *oprL* and *oprI* genes, respectively. While the *oprI* is present in all *Pseudomonas* species, the *oprL* is specifically present in *Ps. aeruginosa.* Therefore, the *oprL* gene is a fairly specific [23,24] and accurate [25,26] gene for molecular identification of *Ps. aeruginosa* in clinical samples, including surgical wound swabs. Other factors that contribute to antibiotic resistance of the bacteria are the biofilm matrix and acquired antimicrobial resistance genes.

The detection of *Ps. aeruginosa* associated with SSIs is conventionally conducted through phenotypical identification of the colonial morphology, Gram staining, and biochemical characterization tests of *Ps.aeruginosa* [27,28]. However, these traditional methods are often lengthy and unreliable and may not always specifically identify *Ps. aeruginosa* from other closely related microbes [22,29]. Therefore, Molecular techniques, such as polymerase chain reaction (PCR), which are rapid and reliable have been developed for the identification of *Ps. aeruginosa* [22,30] and other microbial pathogens, Various PCR-based diagnostic methods targeting specific genes been developed to enhance the accuracy of *Ps. aeruginosa* diagnosis, especially in wound infections where early and precise identification is critical to effective treatment. The virulence gene *oprL* is a major constituent of outer membrane lipoproteins of the bacteria and is utilized as a marker for identifying *Ps. aeruginosa*-associated infections.

The investigation of human infections like *Ps. aeruginosa* and its prevalence within a hospital is essential for the articulation of effective preventive measures [31]. The World Health Organization, WHO, recommends extensive research on hospital-specific SSIs for infection control and to build national data on Hospital Acquired Infections (HAIs) [32-34]. Globally, the rates of SSI infections associated with *Ps. aeruginosa* vary among patients, health facilities, and geographical regions. In low- and middle-

income countries, 11% of patients who undergo surgery develop SSIs [35]. This heavy burden of the bacteria underscores the need for more studies on *Ps. aeruginosa* associated with SSIs and the drug susceptibility pattern that is peculiar to the environment [36,37]. In a systematic review and meta-analysis, [38] found that the pooled cumulative incidence of SSIs in the 6 geopolitical regions of Nigeria was 14.5% (95% confidence interval [CI]: 0.113-0.184). These SSIs included those caused by *Ps. aeruginosa*. The highest prevalence was reported from the northeastern geopolitical zone of Nigeria (27.3%**,** 95% CI: 0.132-0.481). This heavy disease burden of SSIs underscores the need for knowledge of the infecting bacterial pathogens and the drug susceptibility pattern that is peculiar to any setting [36,37]. It underscores the need to conduct this study in a hospital setting in northeastern Nigeria. The study was conducted in two selected hospitals in Bauchi State, north-eastern Nigeria. These hospitals - Specialist Hospital Bauchi and New General Hospital Bayara- render enormous services for out and in-patients, including those managed for SSIs. However, such molecular confirmation after the traditional methods of detecting *Ps. aeruginosa* infection associated with SSI had not been conducted at these facilities in the past. The study was therefore conducted to have empirical evidence of this molecular method and optimize the management of *Ps. aeruginosa* associated with SSIs. Its findings might also be a reference for other primary or secondary studies on infection control.

This paper reports the prevalence of *Ps. aeruginosa* strains from surgical site wounds that were identified through the conventional identification of *Ps. aeruginosa.* It highlights the molecular confirmation (using RCR) of the positive strains using *oprL* as the target gene. The report on the prevalence, distribution, and associated factors of SSI linked to *Ps. aeruginosa* strains and on the drug resistance pattern of the *Ps. aeruginosa* strains are presented in other study reports.

Methods

The study design

The study was a cross-sectional, hospital-based study

Study area and sample size determination

The study was conducted in two selected hospitals in the Bauchi local government of Bauchi State, Nigeria. These are the New General Hospital, Bayara and the Specialist Hospital, Bauchi, Bauchi State, Nigeria. The sample size for the study was determined using the formula by [39]. A prevalence of 19% reported for Ps. *aeruginosa* in surgical site infection by [37], was utilized for estimating the minimum sample size.

$n=Z^2Pq/L^2$

Where n = number of samples

Z = standard normal deviate at 95% CI = 1.96;

P = 19% [37] = 0.19 q = 1 – 0.19 = 0.81;

L = allowable error of 5% (0.05).

$n = 1.96^2 * 0.19*0.81/0.05^2 = 0.59122224/0.0025 = 236.48 \sim 237$

A larger sample size of 250 surgical swabs was utilized for the study. The inclusion criteria for the participants were as recommended by the Centers for Disease Control, CDC. The study included consented patients who had surgeries and were on admission within 30 days of the surgeries and who had purulent discharge from the surgical wound sites or/and at least had one of the following: pain, tenderness, or high temperature (greater than 40^0C) [40]. Patients who had received antibiotics two weeks before the surgery or did not consent to the study were excluded.

Sample collection and processing

Two hundred and fifty surgical site wound swabs were collected from Bauchi Specialist Hospital (205 samples) and New General Hospital, Bayara patients (45 samples) within the five months, January 2019 and May 2019. The demographic data of participants included health facility, ward, age, sex, surgery type, duration in hospital, and occupation.

Bacterial isolation and identification

A total of two hundred and fifty (250) surgical wound swab samples were collected during wound review or wound dressings at the wards. The swabs were labeled and transported in sterile ice packs to the Abubakar Tafawa Balewa University Teaching Hospital's (ATBUTH's) Microbiology Unit for sample analysis. The sample analysis was three steps: identification of culture on Cetrimide agar, Gram staining, and biochemical analysis of isolates [27,28].

For the phenotypic identification of culture on Cetrimide agar, the swabs were inoculated into Cetrimide Agar and incubated at 37°C for 48 hours. After 48 hours of incubation, the culture plates were examined for colonial morphology, shape, color, and odor characteristics of *Ps. aeruginosa.*

The next step was the Gram staining of the pure isolates of *Ps. aeruginosa*. The Gram staining procedure was conducted.

The final step in the sample analysis was the biochemical characterization of the isolates using Oxidase and Catalase tests. In the Oxidase test, a colony of the test organism was picked with a wire loop and smeared on the oxidase strip paper. The strip was impregnated with the substrate tetramethyl-p-phenylenediamine dihydrochloride. The smeared area of the oxidase strip paper was observed for the color change to deep blue or purple (indophenols) within 10 seconds, for a positive reaction. In the Catalase test**,** there was an observation for bubbles of gas (positive reaction) when a colony of the *Ps. aeruginosa* isolates was added to two drops of 3% hydrogen peroxide (H_2O_2) solution placed on the ends of a clean glass slide. Positive samples from these three steps were further analyzed with Polymerase Chain Reaction (PCR) [9,27].

Confirmation of isolates using Polymerase Chain Reaction (PCR)

The PCR analysis was conducted at the Central Research Laboratories of Uthman Dan Fodio University, Sokoto, Nigeria. The PCR analysis was conducted with *oprL*, with an amplicon size of 504, as the target gene. The primer sequence for the PCR is shown in **Table 1**.

The primers were obtained from Inqaba Biotec West Africa Ltd. (Co. Reg. No: RC1232028). The Catalogue number of the Primers is: 17949735-0001.

The three steps in this molecular confirmation using PCR were: DNA extraction, amplification, and Gel electrophoresis

DNA extraction

To minimize contamination, the procedure was carried out on separate benches. DNA extraction was conducted using a QIAGEN DNA extraction kit. The kit's details are QIAcube® (110 V), Catalogue no:90006292.

The DNA extraction involved three processes: collection of cells, lysing bacteria, and DNA purification.

I. Collection of cells: One milliliter of incubated bacterial culture was pipetted into a 2.0 ml sterile Eppendorf tube. This was centrifuged for 5 minutes at 7500 rpm. The supernatant was discarded into a 50 ml conical tube with 10% commercial bleach marked "biohazard waste" [42-44].

ii. Lysing bacteria: 180 µl of Buffer ATL (alkyltransferase-like protein, ATL) was added to the mixture, followed by the addition of 20 µl of Proteinase K. The bacterial cell pellet was resuspended by 'vortexing' and incubated for 30 minutes. This was followed by the addition of 200 µl of Buffer AL and then mixed by vortexing [42-44].

iii. DNA purification: The mechanism of DNA purification is the selective absorption of DNA to the silica of the spin column. The debris and proteins were the discarded materials.

The lid of a 'DNeasy' spin column was uniquely labeled (recorded identifier) for each of the 5 samples. Using a P1000 pipette, the full volume of pre-treated bacterial cells was transferred to the corresponding spin column. This was centrifuged at 8000 rpm for 1 minute, and then the collection tube and its contents were discarded. The DNeasy spin column was placed into a new 2.0ml collection tube. Next, there was the addition of 500 µl of Buffer AW, and then the mixture was centrifuged at 8000 rpm for 1 minute. Next, the collection tube and its contents were discarded. The DNeasy spin column was again placed into a new 2.0 ml collection tube. There was an addition of 500 µl of Buffer AW2(DNA Washing solution), and then the mixture was centrifuged at 13000 rpm for 3 minutes. The collection tube and its contents were discarded and the spin column was examined to ensure that no liquid remained on the spin column. The spin column was placed in a new, sterile 1.5ml elution tube labeled with a unique, recorded identifier. There was an addition of 100 µl of Buffer EB (Elution Buffer) to the spin column. The Buffer EB was pre-incubated at 37°C to improve elution. The new mixture was incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for 1 minute. The eluate was the purified DNA [42-44].

PCR amplification

Eight (8) microliters of extracted DNA were dispensed in an aliquot of 0.2 mL of nuclease-free microtubes.

Preparation of Reaction mix: The cocktail was prepared 1.5ml nuclease-free microfuge tube by these steps. First, 5 x 12.5µl of Qiagen Toptaq PCR master mix was added. Then, there was the addition of 5 x 0.5 µl of 20mM of Forward Primer of *oprL* Gene to all the tubes. Next is the addition of 5 x 0.5 µl of 20mM of Reverse Primer of *oprL* Gene. The

next step was the addition of 2.5 µl of coral load and then the addition of 1 µL of Nuclease-free water. The whole cocktail was carefully mixed. Then, 14.2µl (71 µl /5) of the cocktail was dispensed into each of the 5 PCR tubes (containing the template) and carefully mixed.

The PCR tubes were capped and transferred to the Applied Biosystem 9700 Thermocycler. The initial denaturation was at 95ºC for 5 minutes. This was followed by 30 cycles of denaturation at 95 °C. The annealing temperature was at 57ºC for 30 seconds and the extension at 72ºC for 1 minute. The final extension was at 72ºC for 10 minutes. Cooling was at 4ºC for four minutes [42-44].

Gel electrophoresis

The amplified products were resolved with 1% Agarose gel electrophoresis stained with ethidium bromide and photographed using an Ultraviolet transilluminator.

Preparation of 1% Agarose gel. One gram of Agarose powder was measured out and placed in a clean beaker and then 100ml of water was added and then mixed. Next, 2ml of 1X Tris Acetate Ethylene Diamine Tetra Acetate (TAE buffer) was added, and then the mixture was heated for 3 minutes in a microwave. Next, 5µl of ethidium bromide was added. Next, the gel is cast and cooled in the electrophoresis chamber. Two hundred and fifty milliliters of 1X TAE were added to the chamber. Next, the samples were loaded into the microwells using the micro-pipette. The electrophoresis was conducted at 80 volts for 45 minutes visualized under the ultraviolet illuminator [42-44].

Results

Socio-demographic data

From **Table 2**, the majority of the patients were males (57.2%). Most (35.6%) of the patients were in the age bracket 21-30 years of age, compared to the age bracket of 51 to 60, with the least figure (3.6%). The majority (47.6%) of the patients had no formal education compared to about 5%, who had tertiary education. Most (34%) of the patients were traders, while the least occupations were football players and hotel managers.

Phenotypic identification

Culture morphology, Gram-staining, and biochemical tests. The isolates that were identified as *Ps. aeruginosa* had a colony morphology characterized by a blue-green smooth colony with smooth edges and an elevated appearance. The colonies had the smell of 'grapefruit'. On Gram staining, they were Gram-negative rods **(Figure 1)**. They were all Oxidase and Catalase positive.

Figure 1 shows the microscopic view of Gram-stained *Ps. aeruginosa* with numerous pink rods or curved structures on a pink background. This is characteristic of a Gram-negative bacterium.

The Prevalence of *Pseudomonas aeruginosa* isolates

From **Table 3, 5** isolates out of 250 (2.0%) were identified as *Ps. aeruginosa*. All five samples were obtained from Specialist Hospital Bauchi while no positive sample was obtained from New General Hospital Bayara. These isolates were further analyzed with PCR assay.

Gel Electrophoresis Graph of *oprL* gene with an amplicon size of 504 bp

From **Figure 2**, all the five (5) isolates (A-E) showed the amplicon size of 504bp. Therefore, the PCR confirmed that all five isolates, that were morphologically and biochemically identified, had the *oprL* virulence gene.

Table 1. Primer sequence

Target gene	5'-sequence-3'	Size of Target gene [41]
oprL-F	ATGGAAATCTGAAATTCGGC	504 bp
oprL-R	CTTCTTCAGCTCGACGCGACG	

Table 2. Socio-demographic data

Variables	**Frequency (%)**
Sex	
Male	143(57.2)
Female	107(42.8)
Total	250(100)
Age	
1-10	10(4.0)
11-20	62(24.8)
21-30	89(35.6)
31-40	61(24.4)
41-50	19(7.6)
51-60	9(3.6)
Total	250(100)
Education Status	
No formal education	119(47.6)
Primary	88(35.2)
Secondary	31(12.4)
Tertiary	12(4.8)
Total	250(100)
Occupation	
Unemployed	9(3.6)
Domestic work	43(17.2)
Student	46(18.4)
Manual labour	9(3.6)
Trading	85(34.0)
Hotel management	1(0.4)
Professional Soccer	1(0.4)
Public relations	3(1.2)
Civil service	14(5.6)
Driving	11(4.4)
Farming	28(11.2)
Total	250(100)

Table 3. Prevalence of *Ps. aeruginosa* associated with SSIs in selected hospitals in Bauchi, Nigeria.

Variables	**No. Tested n=250**	**No. Positive (%)**	**X^2**	**df**	**P-value**
Specialist Hospital					
Bauchi	**205**	**5(2.4%)**	**1.12**	**1**	**0.029**
New General Hospital					
Bayara	**45**	**0(0.0%)**			

Key: X^2=Chi Square; P-value < 0.05 is Statistically significant; p-value= and > 0.05

Figure 1. Microscopic view of Gram-negative rods of *Ps.aeruginosa*

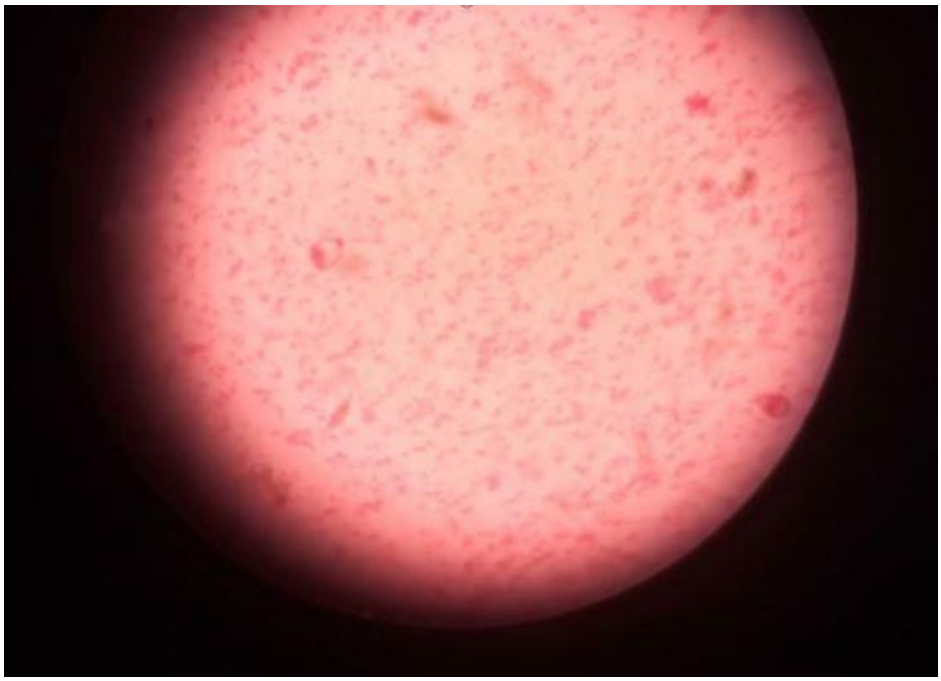
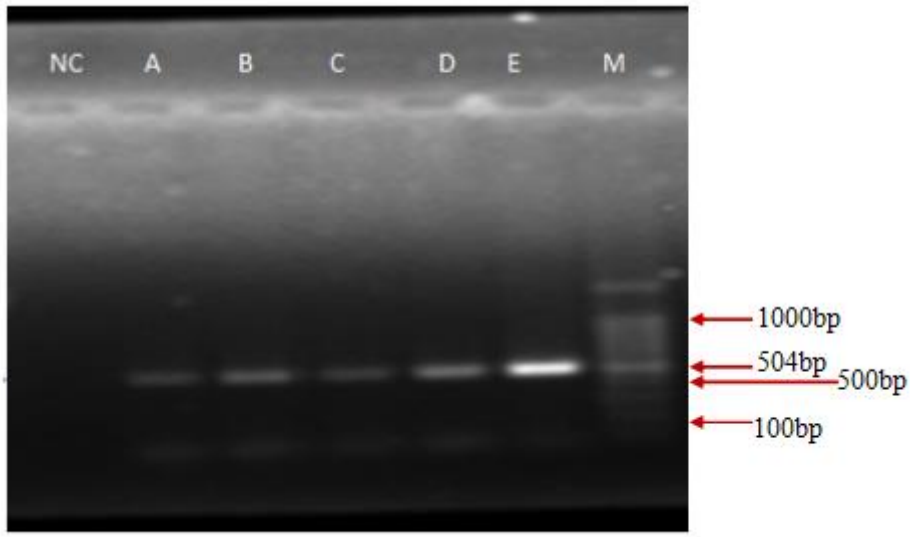


Figure 2. Gel electrophoresis of *oprL* gene with amplicon size of 504 bp



Key:
Lane NC = Negative control
Lanes A-E = Samples
Lane M = 100bp molecular marker

Discussion

The use of molecular techniques in confirming *Ps. aeruginosa* isolates associated with surgical site infection, offers several advantages over the conventional methods. An intrinsic virulence gene, the *oprL* gene, which is often present in the outer membrane of *Ps.aeruginosa*, was utilized for accurate confirmation of *Ps.aeruginosa* strains identified by the traditional method [45-48].

The study analyzed 250 surgical site samples from two hospitals in Bauchi, Nigeria. The study found that 5(2.0%) of the wound swabs were infected with *Ps.aeruginosa*.. In this study, the

prevalence of *Ps. aeruginosa* isolates was 2.0%. This is comparable to the 2.75% reported by [49]. It is however much lower than the 19.0% reported in the study by [37] in Nguru, Yobe State,in North-Eastern Nigeria. Similarly, the overall prevalence is lower than the 19.4% reported by [50] in three selected hospitals in Sokoto, North-Western Nigeria, and 29.6% reported by [51] in India. The variations in the prevalences may be due to the *Ps.aeruginosa* gene diversity which varies among populations studied, geographic regions, and immune statuses of individuals [35]. Most of the patients were males, in the active population, 21 to 30 years, mostly trade and possibly exposed to the environment.

The reported prevalences at Specialist Hospital Bauchi and New General Hospital Bayara were 2.4% and 0.0% respectively. New General Hospital Bayara had a lower prevalence than Specialist Hospital Bauchi probably because of lower patient density compared to the latter which is located in Bauchi capital metropolis and attends to a larger number of patients.

The positive isolates were all confirmed through PCR, with the *oprL* gene, with an amplicon size of 504, as the target gene. The PCR results showed that 5 (100%) of 5 *Ps. aeruginosa* isolates were positive for *oprL* genes. Previous studies have reported the effectiveness of targeting the *oprL* gene for molecular confirmation of conventional biochemical tests for *Ps. aeruginosa* identification [45-48], all reported a 100% confirmation of *Ps. aeruginosa* culture-positive isolates with PCR using *oprL* target gene. The finding from this study reinforces the notion that molecular techniques can be a valuable adjunct or alternative to conventional methods, particularly in cases where accuracy is paramount. It also reaffirms the reliability of *oprL* gene on the account of the high degree of specificity of the target gene in the molecular detection of *Ps.aeruginosa* in the wound and other clinical samples [48,52]. The 100% molecular confirmation in this study contrasts that of [50] who reported discrepancies between culture-positive isolates and the PCR analysis using the *oprL* gene as the target gene. This difference between this study and the study by [43] might be due to the gene mutation, degree of contamination, and immune status of individual patients [53].

OprL gene is one of the virulence factors wielded by multidrug-resistant *P.aeruginosa* associated with SSIs. These virulence factors co-exist and affect pathogenicity, multidrug-resistance and extreme drug resistance (XDR)[54]. The bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes of *P.aeruginosa* isolates were among the extended-spectrum β-lactamase-producing *P.aeruginosa* associated with multidrug-resistance [55]. Abdulhaq et al. reported that the psl_A gene is associated with biofilm formation and multidrug-resistance in clinical isolates of *P.aeruginosa* [56]. Biofilm formation and multidrug -resistance of *P.aeruginosa* were similarly reported by Olaniran et al. [57]. Therefore multiplex PCR assays of *P.aeruginosa oprL* gene and other virulence factors is recommended for the surveillance of these virulence factors and the drug susceptibility pattern of the *P.aeruginosa* isolates associated with SSIs. Molecular assays of MDR *P.aeruginosa* associated with SSIs are involved in aimed at providing novel therapies in the management of *P.aeruginosa* associated with SSIs. Vaccines, nanoparticles, bacteriophages that target and infect *P.aeruginosa*, and their combination therapies are promising therapy for combatting MDR *P.aeruginosa* infections [58]. Studies have reported successfully use of Phage phPS127[59] and podovirus vB_PaeP_PS28 [60] to lyse *P.aeruginosa*. In a related study, *P.aeruginosa* outer membrane vesicles (PA-OMVs) conjugated with the diphtheria toxoid (DT) formulated with alum adjuvant (PA-OMVs-DT + adj) was associated with an increase in antibodies against *P.aeruginosa* of wound infection in a mice model [61].

Although the present study is limited to molecular confirmation of *Ps. aeruginosa* isolates using the *oprL* gene, it provides supporting evidence for the broader body of literature. By utilizing the PCR technique, the accuracy of species identification is significantly improved. This is especially crucial in settings where accurate identification is essential for appropriate patient management, such as hospitals and clinical laboratories.

Conclusion

The study found a prevalence of 2.0% (2/250) of *Ps. aeruginosa* associated with SSIs in the selected hospitals in Bauchi, Nigeria. It also found a 100% molecular confirmation of all the *Ps. aeruginosa* isolates using the *oprL* target gene for all the five isolates that were identified through phenotypic and biochemical analysis of surgical wound swabs. This study adds to the growing body

of evidence supporting the efficacy of molecular methods for accurate species identification. It also demonstrates the importance of integrating molecular techniques into routine identification protocols. Future studies could explore the feasibility of incorporating these techniques into clinical practice, considering cost-effectiveness and potential impacts on patient outcomes.

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Conflicts of interest

The authors declare that they do not have any conflict of interest.

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