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Isolation, Characterization and Antimicrobial Susceptibility Pattern of *Pseudomonas aeruginosa* from Tsetse Flies Captured in Yankari Game Reserve, Nigeria

Youssouf M. Mfopit, Daniel M. Achukwi, Mohammed Mamman, Soerge Kelm, Emmanuel O. Balogun, Mohammed N. Shuaibu, and Junaidu Kabir

ABSTRACT

***Background*: Tsetse flies are a cyclical vector of trypanosomiasis. Their microbiota is involved in their vector competence and may help in developing novel disease control tools. Pseudomonas aeruginosa is reported to be ubiquitous in the natural environment, humans, and animals. It has been used for biocontrol in plants.**

***Methods*: Twenty-five live tsetse flies, collected from Yankari Game Reserve, Nigeria, were dissected under sterile conditions. The midgut was incubated successively in standard culture media. Suspected isolates were then subjected to biochemical tests. The 16S rRNA gene sequence was used to confirm the genotype. The positive isolate was also tested for susceptibility to 17 antimicrobials.**

***Results*: Eight (32%) of the 25 flies tested were positive for *P. aeruginosa*. They were positive for oxidase, catalase, citrate, and motility tests and negative for urease, indole, Methyl Red tests. Analysis of 16S rRNA gene confirmed the identity of the isolate, and the phylogenetic relationship with other strains was established. The isolate was sensitive to fluoroquinolones and intermediate to chloramphenicol. Drug resistance was observed against aminoglycosides, penicillin, erythromycin, clindamycin and imipenem**

***Conclusion*: The presence of *P. aeruginosa* in tsetse gut contributes to the repertoire of cultivable tsetse gut bacteria. It is crucial to investigate whether it could play a role in modulating the fly vector's competence.**

Keywords: Antibiotic Susceptibility, Characterisation, *Glossina*, Nigeria, *Pseudomonas aeruginosa*.

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Y. M. Mfopit*
Institute of Agricultural Research for Development, Cameroon
ACENTDFB, Ahmadu Bello University, Nigeria
(email: youssoufmfopit@yahoo.fr)
D. M. Achukwi
TOZARD Research laboratory, Cameroon
M. Mamman
Department of Veterinary Pharmacology and Toxicology. ACENTDFB, Ahmadu Bello University, Nigeria
S. Kelm
Centre for Biomolecular Interactions Bremen, University of Bremen, Germany
E. O. Balogun
Department of Biochemistry, ACENTDFB, Ahmadu Bello University, Nigeria
M. N. Shuaibu
Department of Biochemistry, ACENTDFB, Ahmadu Bello University, Nigeria
J. Kabir
Department of Veterinary Public Health and Preventive Medicine, ACENTDFB, Ahmadu Bello University, Nigeria

Corresponding Author

I. Introduction

Tsetse flies are cyclical vectors of African trypanosomes, responsible for Human African Trypanosomiasis (HAT) and Animal African Trypanosomiasis (AAT). More than a surface of 1.18 million km^2 are still at risk of *Trypanosoma brucei gambiense* infection and 56 million people are estimated to be at different levels of risk of contracting HAT while the animal form is one of the biggest constraints to livestock production [1].

Control measures are based on vector control and drug administration which are associated with numerous challenges such as cost, toxicity, and resistance [2]. For vector control, the implementation and field sustainability of developed tools remains difficult [3]. Thus, alternative therapeutic and preventive strategies must be pursued.

The microbiota of tsetse flies is of interest because of their unique lifestyle and its potential for disease control. Microbiota can influence the vector competence of their hosts by means of direct interaction with the parasite. This can occur through inhibitory bioactivity of secreted enzymes or toxins. So far, tsetse flies are known to harbour four symbiotic bacteria: *Wigglesworthia glossinidia*, *Sodalis glossinidius*, *Wolbachia*, and *Spiroplasma* [4]. In addition to the endosymbionts, the tsetse gut also harbours a diversity of bacteria acquired from the environment. Previous studies have shown that these bacterial populations vary considerably depending both on the tsetse species or subspecies and the geographic origin of the flies [5].

Pseudomonas sp has been found in the gut of tsetse flies captured in different localities [6]–[9]. They are ubiquitous in the natural environment, humans, and animals and are known to possess complex genetic systems including super integrons and diverse metabolic and virulence gene cassettes. Infections caused by *Pseudomonas aeruginosa* are increasing both – in hospitals and the general community, and it has been reported as one of the main causes of nosocomial infections and an important opportunistic human pathogen that causes severe infections, particularly among immune-compromised patients [10], [11]. These infections are difficult to cure and often require combination therapy due to antibiotic resistance which has become an increasing problem. A varying degree of resistance to known antipseudomonal antibiotics has been reported in different areas of the world [10]–[12].

Pseudomonas sp has been used for biocontrol in plants [13], [14]. They possess a large number of cell-associated and extracellular virulence factors including *alg*D genes, *exo*S, *tox*A, *las*B, *plc*H, *plc*N, and the extracellular neuraminidase genes [15]. *Pseudomonas aeruginosa* produces a wide range of compounds with antimicrobial activity. These compounds result from secondary metabolism from various pathways including the polyketide and shikimic-chorismic acid pathways [16].

These factors and other enzymes and toxins produced by *Pseudomonas* sp could interact with the trypanosome in the tsetse gut, thus affecting the survival of the parasite and, therefore, influencing the vector competence of the fly. Studies have shown substances with antimicrobial activity secreted in the secondary metabolism of microorganisms that could be applied in the management of human, animal, and plant diseases [17].

In order to improve our understanding of the colonization of the tsetse gut by environmental microbes, we attempted to isolate and characterize *Pseudomonas aeruginosa* from tsetse flies in Nigeria.

II. Materials and Methods

A. Study Area

Tsetse flies were captured in Yankari Game Reserve situated in Bauchi State, Nigeria, within the Sudan savannah vegetation zone. It covers an area of 2244 km^2 and is located between latitude 9° 45.240′ N and longitude 10° 30.448′ E. Flies were transported to the bacteriology laboratory of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

B. Sample Collection

Biconical traps were used to catch tsetse flies [18], then transported to the laboratory where they were identified using entomological identification keys based on morphological characters [19]. The collected flies were dissected using a light stereomicroscope under sterile conditions. The individual insect was surface sterilized by submersion in 5% bleach for 1 min, then in 70% ethanol for 5 min, and rinsed in sterile phosphate-buffered saline (PBS) for 5 min. The gut of each insect was then carefully removed using clean forceps and homogenised in 200 µl of sterile PBS. Care was taken to disinfect dissection tools after each use during dissection to prevent contamination successively using Sodium hypochlorite (5%), ethanol (70%), and sterile water. A part of the homogenised gut sample will be preserved in a microtube containing RNA later for molecular investigations while another part will be used for bacteria isolation.

A. Inoculation and Culture

One hundred microliters of homogenised gut were mixed with 3 mL of an enrichment medium: nutrient broth and incubated overnight at 37 °C in aerobic condition. The overnight culture was first streaked on a nutrient agar plate and incubated overnight at 37 °C. The suspected colonies from each sample characterised by irregular shape, large and low elevation, greenish-blue pigment production, were subcultured on selective medium cetrimide agar. The positive colonies (growth and greenish pigment production) were subcultured on MacConkey agar. The suspected non-lactose fermenting and colourless colonies were then subcultured on blood agar and on Mueller Hinton agar to observe for haemolysis (beta haemolysis) and pigmentation (greenish) respectively. All the inoculated plates were incubated at 37 °C for 18-24 hours and growth was evaluated.

Suspected isolates were identified based on colony morphology, haemolysis, as well as pigment production on these media, were further identified by their Gram stain reaction and conventional biochemical tests: oxidase test, catalase, Urease, citrate utilization, indole production, urea hydrolysis, gas formation, MR-VP test, Triple Sugar Ion test (TSI), Sulphur Indole Motility test (SIM).

B. Antibiotic Susceptibility

Antibiotic susceptibility tests were performed by Kirby-Bauer disc diffusion technique [20] on Mueller-Hinton Agar. Briefly, the inocula was prepared by re-suspending about 3 colonies in 3 mL sterile normal saline and adjusting the turbidity of bacterial suspension to 0.5 McFarland's turbidity standard. A sterile swab was dipped into the inoculum, after the excess inoculum was removed, the swab was streaked all over the surface of the medium three times. Finally, the swab was passed around the edge of the agar surface. The inoculum was allowed to dry for 3 minutes to 5 minutes at room temperature with the lid closed. Then the antimicrobial-impregnated disks were placed on the surface of the agar using an antibiotic disc dispenser. The plates were incubated overnight at 37 °C.

The following 17 antibiotics representing various classes of antimicrobials were tested: gentamicin (CN: 10 µg), norfloxacin (NOR: 10 µg), imipenem (IPM: 10 µg), chloramphenicol (C: 30 µg), penicillin G (P: 10 µg), ampicillin (AMP: 10 µg), erythromycin (E: 30 µg), streptomycin (S: 10 µg), ofloxacin (OFX: 5 µg), kanamycin (K: 30 µg), enrofloxacin (ENR: 5 µg), nalidixic acid (NA: 30 µg), clindamycin (DA: 2 µg), amoxycillin/clavulanic acid (AMC: 20/10 µg), cephalothin (KF: 30 µg), trimethroprim (W: 5 µg), doxycycline (DO: 30 µg).

After incubation, inhibition zones were recorded in millimetres as the diameter of the clear zones (including the diameter of the disc) and interpreted as susceptible, intermediate and resistant according to the antimicrobial susceptibility interpretation chart.

C. Molecular Identification

The identity of the isolate was confirmed by amplifying and sequencing their 16S ribosomal RNA gene. DNA was extracted from a pure culture by freeze-thaw method. Briefly, a single colony was resuspended in 30 µL sterile TE buffer pH 8.0 in a 1.5 mL centrifuge. The solution was freeze-thawed about 5 times, then heated at 95 °C for 10 min and allowed to cool at room temperature. The solution was used as a template for PCR. The amplification was done using generic primers Bac8uf (5′-AGAGTTTGATNHTGGYTCAG-3′) and Univ1492r (5′-GGNTCCTTGTTACGACTT-3′) as described by authors [21]. Briefly, in a final volume of 50 µL, 2 µL template with 0.5 µL of DreamTaq Polymerase 5U, 5 µL DreamTaq Buffer 10X, 0.25 µL of 10 mM sterile dNTPs and 0.25 µL of 100 µM of each primer and 41.75 µL of sterile ddH_2O were used. The cycling conditions were as follows: 5 min at 95 °C, 40 cycles of 1 min at 95 °C, 30 seconds at 52 °C and 2 min at 72 °C, final elongation step for 10 min at 72 °C. Successful gene amplification of about 1500 bp amplicon was investigated by electrophoresis on a 1.5% agarose gel stained with Stain-G and visualized on a UV source. The amplicons were purified using GeneJet Gel extraction kit (ThermoFisher) according to manufacturer instructions and sent for sequencing to a commercial company (Seqlab, Göttingen, Germany).

D. Phylogeny

The sequence was quality checked using Geneious Pro Version 5.5.9 and then subjected to BLAST search at the National Center for Biotechnology Information (NCBI) database. Related 16S rRNA gene sequences were aligned using Muscle alignment tool of MEGA X [22] software that was also used for phylogenetic analysis. Phylogenetic trees were constructed using the Maximum Likelihood method. The first tree was based on 16S rRNA gene sequences of *P. aeruginosa* isolated over the world, while the second one was based the isolates from Nigeria. Bootstrap analysis [23] based on 700 replications was used to estimate the confidence level of tree topologies.

III. Results

A total of 25 flies arrived alive in the laboratory and were dissected and their gut were inoculated in culture media. Nineteen were female (76%) and six were male (24%). All the flies were non-teneral.

From the first culture agar plates (Nutrient agar), a variety of colonies were observed. Eighteen samples (72%) showed the presumptive presence of *Pseudomonas sp*. (pigment production). Among them were 12 cultures from female flies and six from male flies. On the cetrimide agar, only 14 out of 18 suspects were able to grow and produce the greenish pigmentation. All the 14 suspected isolates grew on MacConkey agar but did not ferment the lactose. On Mueller-Hinton agar, the suspected isolates produced a characteristic greenish pigment while on blood agar, one colony produced a gamma-haemolysis, one produced an alpha-haemolysis and only 12 produced a beta-haemolysis (Fig. 1). The Gram staining showed the suspected colonies to having a rod shape.

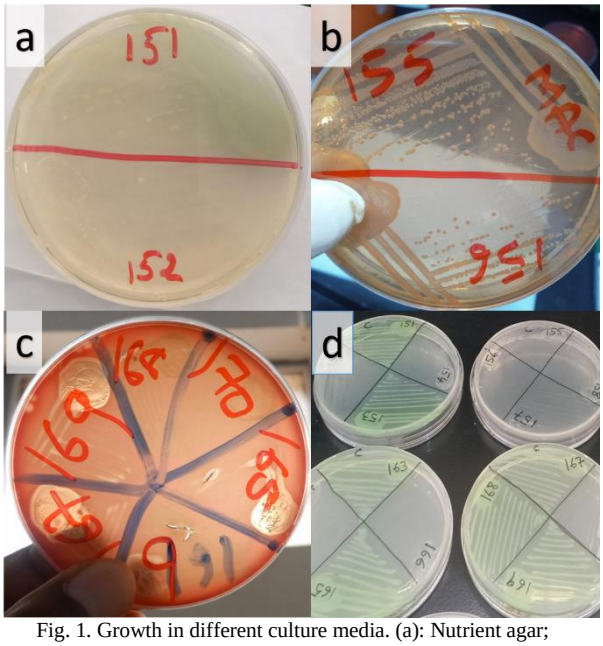

Fig. 1. Growth in different culture media. (a): Nutrient agar; (b.): MacConkey Agar; (c): Blood Agar; (d): Cetrimide agar.

The results of the biochemical test vary among some of the 12 suspected colonies (Table I). But all these isolates were positive for oxidase, catalase, and motility, and negative for indole, sulphur urease, and then Alkaline/Alkaline for TSI test (Fig. 2).

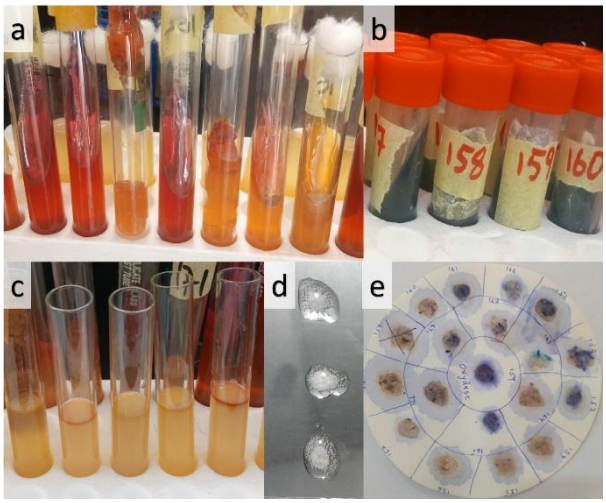

Fig. 2. Biochemical tests: (a): Triple Sugar Ion test; (b): Citrate test; (c): MR-VP test; (d): Catalase test; (e): Oxidase test.

TABLE I: BIOCHEMICAL PROFILE OF *P. AERUGINOSA*

Tests	Results
TSI	Alkaline/Alkaline: 12/12
Gas production	No: 10/12
H_2S production	Negative:12/12
Indole	Negative: 12/12
Motility	Positive: 12/12
Citrate	Positive:7/12
MR	Negative:7/8
Urease	Negative: 12/12
Oxidase	Positive:11/12
Catalase	Positive: 12/12

Among these suspected isolates, eight colonies that all the biochemical test results were in accordance with known characteristics of *P. aeruginosa* were selected.

A. Antimicrobial Susceptibility Patterns

Antimicrobial susceptibility patterns of five isolates of *P. aeruginosa* (151, 161, 162, 171, 173) to 17 antimicrobials commonly used was examined (Fig. 3). The results showed that this *P. aeruginosa* strains were susceptible to norfloxacin and ofloxacin. Surprisingly, they were resistant to gentamicin and imipenem (Table II).

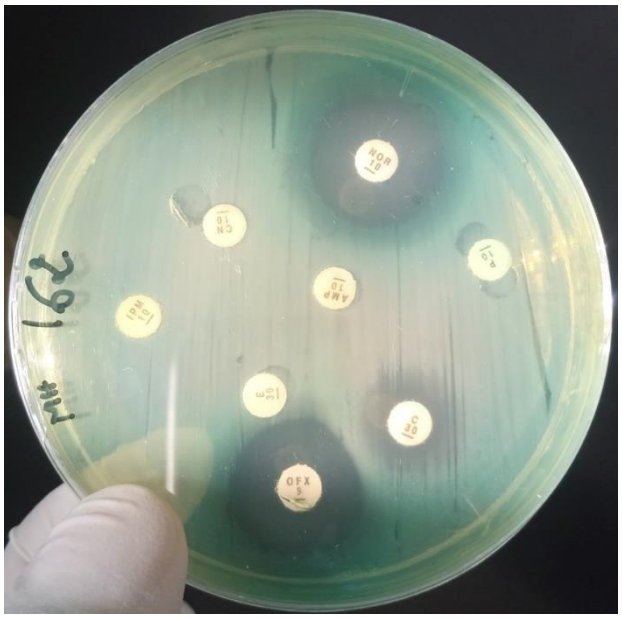

Fig. 3. Antibiotic susceptibility test. Test performed by Kirby-Bauer disc diffusion technique on Mueller-Hinton Agar.

TABLE II: ANTIMICROBIAL SUSCEPTIBILITY PROFILE

Classes	Antimicrobial agent (code)	Potency (µg)	Conclusion
Aminoglycoside	Gentamicin	10	Resistant
	Kanamycin	30	Resistant
	Streptomycin	10	Resistant
Carbapenem	Imipenem	10	Resistant
Fluoroquinolone	Norfloxacin	10	*Susceptible*
	Ofloxacin	5	*Susceptible*
	Enrofloxacin	5	Resistant
Quinolone	Nalixic acid	30	Resistant
Macrolide	Erythromycin	30	Resistant
	Clindamycin	2	Resistant
Penicillin	Ampicillin	10	Resistant
	Penicillin	10	Resistant
	Amoxi/Clavunic acid	20/10	Resistant
	Cephalothin	30	Resistant
Chloramphenicol	Chloramphenicol	30	*Intermediate*
Sulfonamide	Trimethroprim	5	Resistant
Tetracycline	Doxycycline	30	Resistant

A. Phylogenetic Analysis

The BLAST search with the partial 16S rRNA gene of our isolate showed a high identity with other *Pseudomonas aeruginosa* strains. The phylogenetic tree based on 16S rRNA gene sequences of *P. aeruginosa* isolated over the world (Fig. 4) showed its close relatedness with the strain Rlim (OP522351) from Malaysia. Fig. 5 shows the phylogenetic relationship with clinical isolates obtained in Nigeria (OP209787, ON738240, ON721331, MN519592, MT661526, MZ379421).

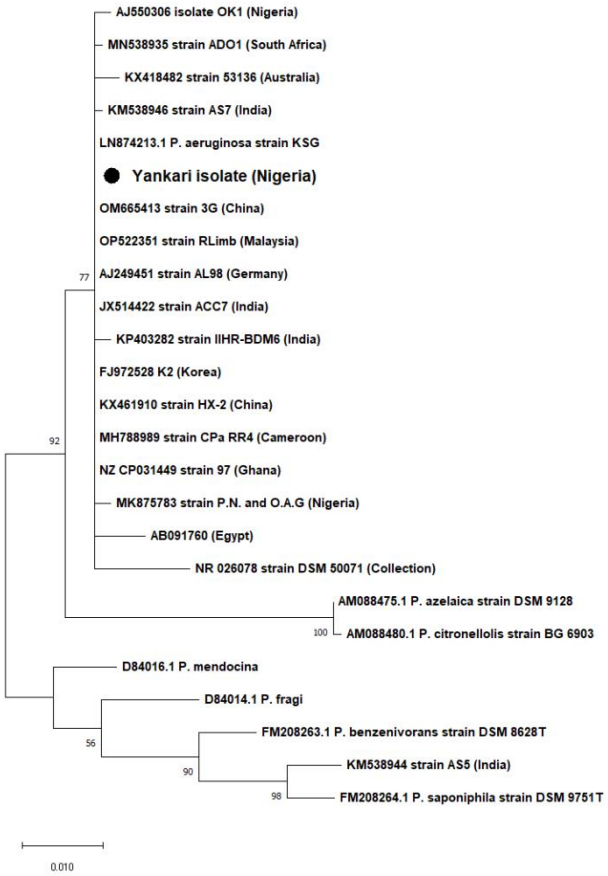

Fig. 4. Phylogenetic tree analysis of 16S rRNA of Yankari's isolate (marked by a black circle) with other related strains in the world. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model assuming gamma distribution on MEGA X. Bootstrap probability values based on 700 replicates are indicated at branch-points.

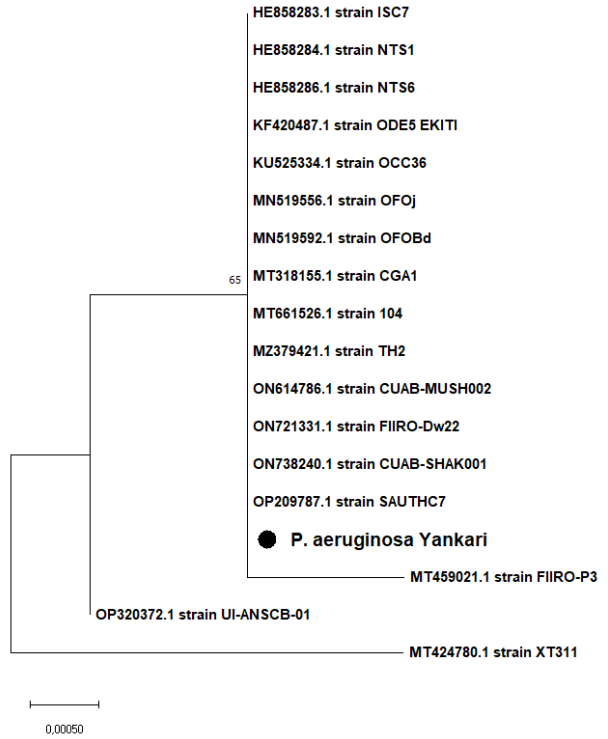

Fig. 5. Phylogenetic tree analysis of 16S rRNA of Yankari's isolate (marked by a black circle) with other related strains in Nigeria. The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model on MEGA X. Bootstrap probability values based on 700 replicates are indicated at branch-points.

The 16S rRNA gene sequence generated in this study have been deposited into GenBank under accession number OQ437998.

IV. Discussion

Pseudomonas aeruginosa is one of the most common bacteria isolated in hospital environments, and a leading cause of nosocomial infections. Its emergence as a major opportunistic human pathogen may be in part a consequence of its resistance to many antibiotics and disinfectants that eliminate other environmental bacteria. *P. aeruginosa* is a significant source of bacteraemia in burn victims, urinary tract infections in catheterized patients, nosocomial pneumonia in patients on respirators, and also the predominant cause of morbidity and mortality in cystic fibrosis patients [24].

From the 25 dissected guts, eight (32%) were confirmed harbouring *Pseudomonas aeruginosa* by biochemical test and growth characteristics. Nevertheless, some non-selected colonies in other flies may also belong to *Pseudomonas sp*., since the result of biochemical test may vary from one strain to another due to the genetic diversity of various populations of *P. aeruginosa* strains and its high rate of genetic recombination [15].

The presence of the genus *Pseudomonas* has been shown in other studies [7], [9], [25]. Their role, in the gut of tsetse, is not yet understood. They may participate in gut metabolism or the defence system indirectly by activating or enhancing the host immune system through their excretory and secretory products. The secondary metabolites of this bacterium could modulate the fly vector's competence, as has already been reported in other insect parasite vectors. It is crucial to investigate whether it could modulate the fly vector's competence. So it can be used in the design of tsetse vector control strategies using paratransgenic technique which prevents the survival of trypanosomes within the tsetse fly.

From the antimicrobial susceptibility test, our isolate showed resistance to many antibiotics confirming the multi-drug resistance of *P. aeruginosa*. Among the panel of 17 antimicrobials, the isolate was susceptible to only norfloxacin and ofloxacin of fluoroquinolone class. However, cases of resistance to these antibiotics have been reported [10], [12], [26]. Multidrug resistance of *P. aeruginosa* particularly the hospital strains, has been reported in different parts of the world. The augmentation of resistance to antipseudomonal drugs is a serious threat to the management of diseases due to this organism.

Gentamicin is a known frontline antibiotic in the treatment of bacterial infection by gram-negative bacteria including *P. aeruginosa*. However, our isolate showed resistance to gentamicin as well as imipenem which is also known as a strong antipseudomonal drug. Other reports also showed an increased prevalence of resistance to these drugs [12], [26].

This strain is isolated from tsetse fly in a remote area, and that can be considered as a wild strain. The phylogenetic trees showed this strain in the same clade as most of the Nigerian clinical samples. Its resistance to multiple antimicrobials should call for attention.

V. Conclusion

A multidrug-resistant *Pseudomonas aeruginosa* was isolated from the midgut of tsetse flies in Nigeria. The presence of *P. aeruginosa* in tsetse gut contributes to the repertoire of cultivable tsetse gut bacteria. Its potential in trypanosomiasis control needs more investigations as a component in the paratransgenic approach.

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Competing Interests

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

Data Availability

The 16S rRNA partial gene sequence generated in this study has been deposited in the NCBI database with accession number: OQ437998.

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