BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Pseudomonas putida*, *P. fluorescens* and *P. aeruginosa* ISOLATES FROM *Oreochromis niloticus* (TILAPIA) AND *Clarias gariepinus* (AFRICAN CATFISH)

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ABSTRACT

Bacterial pathogens cause high economic losses in the fish farming industry and seriously threaten public health. This study described the phenotypic and the genomic characteristics of pathogenic *Pseudomonas* species in two finfishes (*Oreochromis niloticus* and *Clarias gariepinus*) from the Galadima fish market in Kano metropolis Nigeria. Twenty fish samples, including 10 Tilapia and 10 African catfish were randomly obtained and their liver, spleen, intestine and gills were collected. *Pseudomonas* spp. was screened via culturing and isolation techniques and biochemical tests. The molecular characterization of the isolates was carried out based on 16S rRNA gene sequence analysis. A total of 6 % *Pseudomonas* spp. isolates were confirmed as *P. putida* (5 %), *P. aeruginosa* (1.25 %), and *P. fluorescens* (1.25 %). This study confirms the presence of potential pathogenic *Pseudomonas* species in commercially important finfish species from the Galadima fish market in Kano, Nigeria. These findings highlight the need for regular monitoring and molecular surveillance of bacterial pathogens in aquaculture to mitigate economic losses and reduce potential public health risks associated with fish consumption.

Keywords: Tilapia, African catfish, Sequencing, Bacteria

Abbreviations:

PCR: Polymerase chain reaction,

NCBI: National Center for Biotechnology Information,

MR: Methyl red, VP: Voges Proskauer,

BLAST: Basic local alignment search tool**.**

INTRODUCTION

Aquaculture is currently the fastest-growing sector of food-animal globally Andleeb et al. (2020). The global production stood at 130.9×10^6 tons in 2022, contributing significantly to food security, especially in many of the food-insecure regions [1]. This supports the livelihood of millions of people worldwide [2]. The total fisheries and aquaculture harvested by non-high-income countries in recent decades (as per World Bank 2024 classification) has increased from about 33 % in the 1950s to 84 % in 2022, out of the total production, the upper-middle-income countries contributed 56 %, lower-middle-income countries contributed 26 %, high-income countries contributed 16 % and low-income countries contributed 2 % [2]. The total production of fish worldwide was estimated to reach approximately 196×10^6 tons in 2025, this is responsible for about 17.3 % of the total consumption of animal protein worldwide, about 6.8% of the total animal protein consumption in 2017, 20.3 kg was the estimated per capita consumption. This provides about 20 % of nearly 3.3 billion people's average per capita animal protein diet and 5.6 billion people with 10 % of such protein [3].

Finfishes (tilapia and catfishes) are inexpensive resources of animal protein, important sources of by-products [4] and serves as a source of income to low-income earners in rural (developing or undeveloped areas [5]. They are known generally to occupy various habitats (freshwater, brackish or marine) and take up different shapes, sizes and biological tendencies.

Infectious diseases have been reported to be a major setback to fish farming industry, causing great economic losses as a result of mortality, poor marketability, cost of treatment and sometimes spread of zoonotic diseases [6].

Bacteria especially *Pseudomonas* are gram-negative opportunistic pathogens, highly adaptable and can survive in various environmental conditions, including the aquaculture environment [7].

Numerous species of *Pseudomonas* have been pathogenic for Aquatic and other animals including humans [8]. Pseudomonadiasis is one of the most prevalent diseases affecting fish globally [9], causing severe economic losses and decreased fish farming efficiency [10]. It has been reported to be among the most common diseases of fish, causing almost 100 % mortality in some cases [11]. Although many species implicated were reported to be opportunistic pathogens: *P. putida, P. fluorescens, P. aeruginosa, P. anguilliseptica, P. baetica, P. chlororaphis, P. koreensis, P. luteola, P. plecoglossicida* and *P. pseudoalcaligenes* were reported as primary pathogens of various fish aquaculture [7, 8].

In this part of the world, lack of information on the exact pathogen causing fish disease limits preventative and control measures [6, 9]. Therefore *Pseudomonas* species-specific detection may be of help in establishing a more complete understanding of the pathological significance of these microorganisms [12]. Molecular detection could also guide researchers to gain a clear understanding of the ecological impact of the pathogen and also overcome the deficiencies of the traditional approaches.

To the best of the researchers' knowledge concerning the limited availability of literature and data on the molecular characterization of important microorganisms of fish and environmental health concerns from the study area, this study is the first of its kind. Therefore, this study describes the phenotypic and genomic characteristics of pathogenic *Pseudomonas* species in fish (*Oreochromis niloticus* and *Clarias gariepinus*) from the Galadima fish market in Kano metropolis Nigeria.

RESULTS

Pseudomonas species isolation rates

In this study, three *Pseudomonas* species (*P. fluorescens P. aeruginosa* and *P. putida*) reported to be fish pathogens were isolated and characterized. The overall isolation rate of pathogenic *Pseudomonas* from all the samples was 6 (7.5 %) after molecular characterization (5 % *P. putida*, 1.25 % *P. aeruginosa* and 1.25 % *P. fluorescens*) (Table 5).

Table 5: Summary of the total isolation rate after Molecular characterization

Isolate	*Clarias gariepinus*	*Oreochromis niloticus*	Total (%)
P. putida	3	1	4 (5)
P. fluorescens	1	0	1 (1.25)
P. aeruginosa	1	0	1 (1.25)
Total	5	1	7.5

Pseudomonas fish species-specific isolation rates

The isolation rate from the African catfish (*Clarias gariepinus*) was 5 (12.5 %) while the specific isolation rates from the gills, liver, spleen and intestine were 3 (40 %), 0 %, 0 (0 %), 2 (20 %) respectively (Table 4). The isolation rate from the Tilapia (*Oreochromis niloticus*) was 1 (2.5 %) while the specific isolation rate from the gills, liver, spleen and intestine were 0 %, 0 %, 1 (10) %, 0 % respectively (Table 4). Table 4 shows the isolation rate in different parts of *C. gariepinus* and *O. niloticus* from the market. There was significant difference ($p<0.05$) in the isolation rate between *C. gariepinus* and *O. niloticus*.

Table 4: The percentage of *Pseudomonas* isolation in fish species from Galadima market, Kano Nigeria at the level of Biochemical characterization

	Clarias gariepinus		*Oreochromis niloticus*	
Organs examined	**Isolation rate (out of 10)**	**%**	**Isolation rate (out of 10)**	**%**
Gills	4	40	1	10
Liver	0	0	0	0
Spleen	1	10	1	10
Intestine	2	20	0	0
Total (out of 40)	7	17.5	2	5

***Pseudomonas* isolation rates from different organs (fish body parts)**

There was also a significant difference ($p<0.05$) in the isolation rate between the various body parts, with a higher isolation rate (30 %) observed in the gills when compared with the 0 % from the liver and the spleen (Table 6). The gills and intestine are the only parts of the *C. gariepinus* contaminated, the isolation rate from the *C. gariepinus* was 30 % and 20 % respectively. For the spleen, the isolation rate from the *C. gariepinus* was 0 % and 10 % from the *O. niloticus*. No *Pseudomonas* species were isolated in all the liver samples from both *C. gariepinus* and *O. niloticus* (Table 6). The gills samples from *C. gariepinus* had a higher isolation rate (30 %) than the *O. niloticus* (0 %). Also, the intestine from *C. gariepinus* had a higher isolation rate (20 %) than the *O. niloticus* (10 %). However, the spleen samples from *O. niloticus* had a higher isolation rate (10 %) than the *C. gariepinus* (0 %) (Table 6).

Table 6: The percentage of *Pseudomonas* isolation in fish species from Galadima market, Kano Nigeria after Molecular characterization

	Clarias gariepinus		*Oreochromis niloticus*	
Organs examined	**Isolation rate**	**%**	**Isolation rate**	**%**
Gills	3	30	0	0
Liver	0	0	0	0
Spleen	0	0	1	10
Intestine	2	20	0	0
Total	5	12.5	1	2.5

DISCUSSION

Globally, fish and fishery products are essential for nutrition as well as international trade for manycountries to earn foreign exchange. They are highly perishable and easily prone to variations in quality because of differences in species, feeding habits and environmental habitats [14]. Consumption of raw or poorly processed fish products poses the greatest public health risk because they can serve as carriers of many infectious agents of health hazards [15]. It is therefore of utmost importance to monitor the quality in the production of fish and fishery products. *Pseudomonas spp* are recognized to be primary invasive or opportunistic pathogens of many animals including fish. The bacterium plays a great role in being a potential pathogen for humans and an indicator of food quality (spoilage organism) [8].

Masbouba [16] reported a higher isolation rate of 36.9 % *Pseudomonas fluorescens* and 29.1 % *Pseudomonas aureginosa* in diseased *Clarias gariepinus*. Perhaps the higher isolation rate was because the previous study was on clinically sick fish from an outbreak on a farm. Olayemi et al. [17] also isolated *Pseudomonas aerugenosa* from the gills and of *Clarias gariepinus* in Ile-Ife, Nigeria.

The occurrence of *Pseudomonas* in the spleen, intestine and gills of fish (*C. gariepinus* and *O. niloticus*) sampled from the Galadima fish market in Kano metropolis showed that some fishes are contaminated by bacteria. This calls for concern because *P. putida* and *P. fluorescens* are pathogens of fish, causing septicemia in fish, which may not be differentiated from motile Aeromonas septicemia of fish occurring in stocks exposed to stresses [18,19]. Also, as a source of proteins, this poses a serious health hazard to humans and may serve as a source of zoonosis to the consumers [20].

The 0 % isolation rate from the gills and intestines of the fish examined in this study differs considerably from the 63 % and 31 % reported from gills and intestines previously reported respectively from Khartoum [21]. The difference in the methodology for the isolation and characterization, locations of the studies, the nature of the hygienic practices and also the different sources of water might have contributed to the differing results. The higher isolation rate in the previous study might also be associated with clinical infections.

Fisheries are vital to food security, livelihoods, and economic development in Africa, including Nigeria [20, 22]. The findings of this study indicate that fish in the study area are prone to *Pseudomonas* infections. Such infections can compromise fish quality, stunt growth, increase mortality rates, and ultimately reduce the income of fish farmers. Additionally, managing these infections often requires antibiotic treatment, which raises production costs [23].

Pseudomonas species are particularly concerning due to their resistance to multiple antibiotics. In Nigeria, the regulation of antibiotic use in aquaculture is weak, increasing the risk of antimicrobial resistance (AMR) [24]. This not only threatens local aquaculture but also contributes to the global AMR crisis. The situation becomes even more alarming when *Pseudomonas* infections are transmitted to humans [25]. Notably, *Pseudomonas aeruginosa* is an opportunistic human

pathogen and can pose serious health risks, especially to immunocompromised individuals, through the handling or consumption of contaminated fish [26].

In conclusion, infection by *P. putida*, *P. aeruginosa* and *P. fluorescens* in fish from Kano, Nigeria do occur. Therefore, raising awareness and promoting understanding of pathogenic bacterial infections in fish farming is essential. Strict hygienic control, preventive measures, and biosecurity practices play a vital role in maintaining healthy aquaculture environments

Materials and methods

Study Area

Galadima market was the study area and is located at Fagge Local Government Area of Kano state (Latitude 12.0127° N and longitude 8.5344° E) (NPC, 2006). It is the largest fish market for fresh fish, farmers supply fresh fish for wholesale and retails from the wild and in captivity (aquaculture) for consumption.

Study design

The research was carried out to isolate *Pseudomonas* species of medical importance in major fish species freshly brought for sale at the Galadima fish market in the Kano metropolis. Eighty (80) samples of gills, intestine, liver and spleen were collected from 20 fish (10 *Clarias gariepinus* and 10 *Oreochromis niloticus*) and their morphometric measurements were recorded (Table 1). This was cultured on Nutrient agar and biochemical characterization of the isolates was carried out and later sequencing.

Culture and Bacterial isolation

Culture, isolation and identification were carried out based on the procedure described by [8]. Each sample was inoculated on Nutrient agar and incubated at 37ºC for 24h. The growths were subjected to Gram staining and biochemical test for further identification.

The inoculation Media was prepared based on the manufacturer's instructions. A sterilized spatula was used to cauterize the surface of the sample. The cauterized surface was then cut using sterilized scissors. A swab stick was then placed deeply into the sample tissue through the cut surface to make a primary smear. Using a sterilized wire loop a secondary and tertiary smear was then made, this was incubated at 37ºc for 24 hours. The plates were checked for growth after 24 hours, subcultured and incubated at 37ºc for another 24 hours. Colonial morphology was then studied (Figure I). All the isolates were sent to South Africa for sequencing.

Biochemical characterisation

After the assessment of colony morphology, the biochemical characterization (Figure 2) was carried out using conventional biochemical tests. Gram staining, urease, citrate, catalase activities and oxidase, Indole, Methyl red (MR), Voges Proskauer (VP) and motility tests [8]. The test tubes containing the test media were labeled and arranged properly in a test tube rack, each was inoculated with an inoculum of the isolates and incubated at 37ºc for 24 hours, after which Kovac's reagent, VP1 & VP2 were added to the incubated peptone water for both Indole and MRVP tests respectively, reaction recorded (Table 1) and DNA extracted for sequencing.

Table 2: Biochemical tests and reactions

S/N	Test	*Pseudomonas* spp
1.	Gram reaction	-
2.	Oxidase	+

3.	Indole	-
4.	Methyl red	-
4.	Voges-proskauer	-
5.	Citrate	+
6.	Catalase	+
7.	Urease	-
8.	Motility	+

Extraction of genomic DNA, amplification by PCR and Sequencing

The study isolates were sent to Inqaba Biotec™ (Pretoria, South Africa) where PCR, agarose gel electrophoresis and Sanger sequencing were carried out. The extraction of the isolates genomic DNA was carried out using a DNA kit (QIAamp) from Qiagen, Germany. All the DNA samples were then optimized by concentration and purity assessment before molecular analysis. Primer selection, PCR amplification, purification of the amplified products, DNA sequencing and the sequence analysis were performed as described by Duman *et al.,* 2021 [8].

The primer for the PCR amplication was universal primer 16S rRNA (small subunit ribosomal RNA) gene as shown in Table 3.

Table 3: Universal Primers

Universal primer set used for PCR amplification.	**Target**	**Sequence (5' to 3')**
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

.Identification at the species level was done by sequencing the DNA dependent RNA polymerase sub-units. The sequences obtained from the sequencing were analyzed and aligned. By BLAST the homologous searches were done from the results of the sequencing. The nucleotide sequence of the PCR product showed 99.29, 99.22, 94.81 % and 94.72 *Pseudomonas putida*, 96.52 % *Pseudomonas aeruginosa* and 94.72 % *Pseudomonas fluorescens* similarities to *Pseudomonas* species published on NCBI respectively. Figure 3 is a gel image showing position and base pairs for the different Pseudomonas species.

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Figure legends

Figure 1: Pseudomonas on nutrient agar

Figure 2: Biochemical identification of Pseudomonas species

Figure 3: Gel image showing position and base pairs for the different Pseudomonas species

Figure 1: Pseudomonas on Nutrient agar

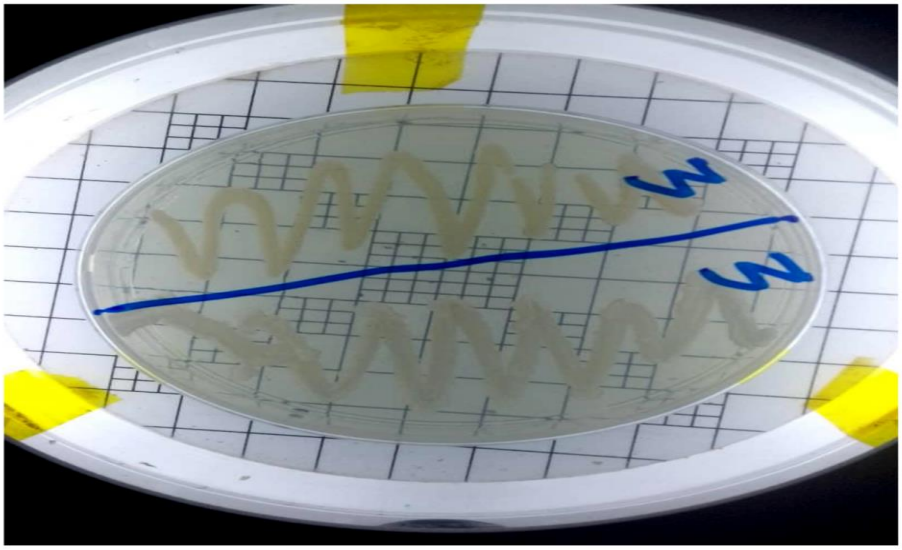


Figure 2: Biochemical identification of *Pseudomonas* species

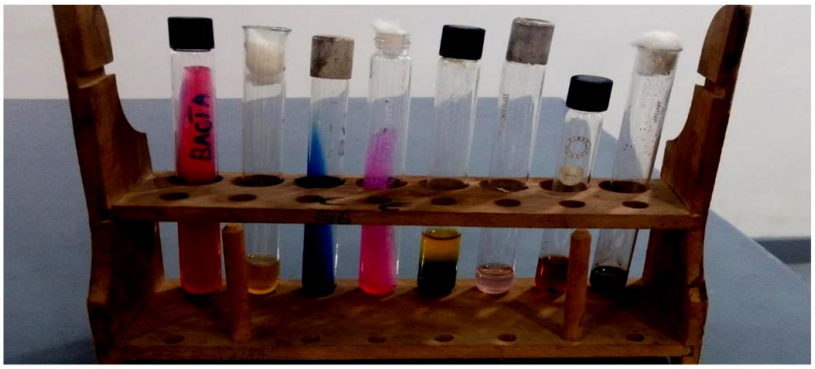
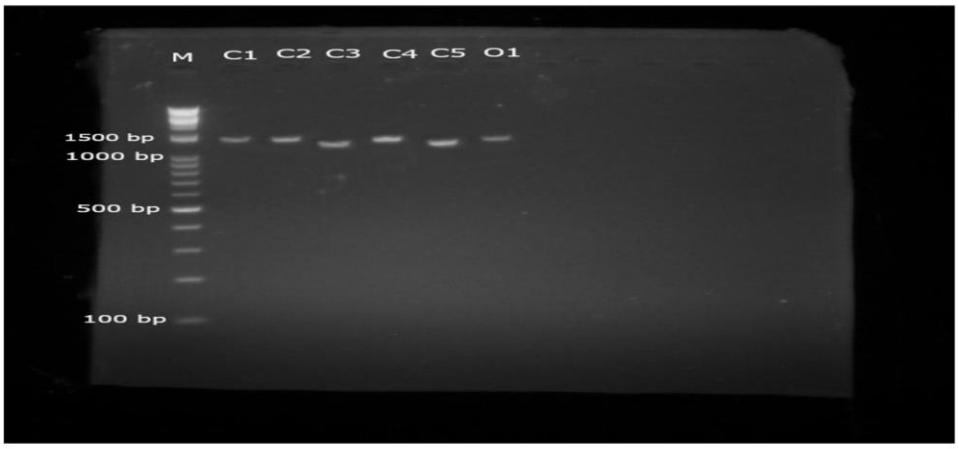


Figure 3: Gel image showing position and base pairs for the different *Pseudomonas* species



Keys: *M = Molecular ladder; C1= P. putida (around 1500 bp); C2= P. putida (around 1500 bp); C3= P. fluorescen (around 1430 bp); C4= P. putida (around 1500 bp); C5= P. aeruginosa (around 1450 bp); O1= P. putida (around 1500 bp); "C" is Claris gariepinus; "O" is Oreochromis niloticus*