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RESEARCH PAPER

Molecular Characterization and Antibiotic Resistance Profiling of Bacteria Associated with *Clarias gariepinus* from selected fish farms in Ota

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Abstract

Antibiotic resistance of genetically diverse bacteria is of high implication for fish farming and human consumption. The present study evaluates the genetic diversity and antibiotic resistance profile of bacteria pathogens isolated from *Clarias gariepinus* in aquaculture farms. Bacteria were isolated from *C. gariepinus* (n=15), and identified using biochemical methods, and antimicrobial susceptibility test was performed. Multiple antibacterial resistance index (MARI) was determined and isolates with multidrug resistance (MDR) pattern were genotyped and sequenced for 16sRNA and further determined for genetic diversity. Of the fifty bacteria, *Vibrio* species (30%), *Escherichia coli* (14%), *S. aureus* (12%), *Proteus* spp (12%), *Salmonella* and *Shigella* spp (8%), *Micrococcus luteus* (6%), *Bacillus* spp (4%), *Klebsiella aerogenes* (4%), and *P. aeruginosa* (2%) were observed. All the bacteria isolates showed resistance were generally resistant to several other antibiotics: Impenem, Cefuroxim, Ofloxacin, Augmentin, Cefexime, Cefotaxin, Ciprofloxacin, Ampiclox, Nitrofuranton, and Nalidixic acid while only few bacteria showed intermediate sensitivity. Genetically, diverse multidrug resistance bacteria is a worrisome concern in aquaculture industry and regular surveillance and regulation of antibiotics would ensure sustainable fish production, and public health assurance.

Introduction

Fish production is a vital constituents of global food security, aquatic ecosystem management and a key agricultural sector of food production globally. Fish is a good source of high quality protein, Vitamin D and Omega 3 fatty acid (Ava, 2020). However, fish diseases have been a serious problem to both aquaculture, and public health. It is responsible for huge economic losses, both in Nigeria and on a global scale (Ogunji and Wuertz, 2023).

Various organisms are responsible for fish diseases which also determine the type of disease in fish, these includes: bacteria, fungi, viruses, and protozoa. These pathogens can damage their host during interaction, either through toxins or virulence factor**.** The indiscriminate use of veterinary drugs and chemical substances in aquaculture management has become worrisome especially in the developing country, simply because antibiotics can easily be purchased over the counter, and are mostly used without prescription (Durojaiye *et al*., 2019). This excessive and improper use of antimicrobials in aquaculture, has become a major concern due to the rise of antimicrobial resistance (AMR), which significantly threatens fish health and food safety. When antibiotics are used inappropriately either to treat or prevent fish disease, resistant bacteria may develop in the aquatic environment. They can easily enter the human food chain when consumed fish contaminated with AMR bacteria or antibiotic residues, and consequently reduces the effectiveness of antibiotics used to treat human infections.

Some of the clinical sign of bacterial diseases in fish includes: hemorrhagic septicaemia which is characterized by redness and loss of blood through peritoneum, body wall, and viscera, and it is usually caused by *Aeromonas* sp; Columnaris disease of warm water fish usually caused by *Flavobacterium columnaris*, chronic mycobacterial diseases in fish, and several others (Petty *et al*., 2022**).** Antibiotics such as sulfamethoxazole and oxytetracycline can adversely affect gastrointestinal health of fish even when used at the permissible dose, and administered for a long-term. In addition, irreversible alterations in the microbiota due to antibiotic exposure were discovered in *Gambusia affinis* (Carlson *et al*., 2017), which leads to compromised immune function, altered disease resistance, and the emergence of antibiotic-resistant strains. Such disruptions in microbial communities highlight the risks involved with the unguided use of antibiotics in aquaculture and thus calls for sustainable practices in order to prevent these adverse outcomes. A study by Dang *et al*. (2011) showed that antibiotics used for growth promotion can lead to the development of antimicrobial-resistant bacteria in the aquatic environment (Marti *et al*., 2014). Furthermore, sub-therapeutic use of antibiotics may add to the accumulation of residues in fish organs. These resistant bacteria can then be transmitted to humans when consumed contaminated fish, leading to a serious infection that may defy treatment (Zounkova *et al*., 2011).

However, lack of rapid and accurate identification of fish pathogens is one of the main set back for detection, treatment and management of fish diseases. The use of genomic for the evaluation of fish pathogens and the use of molecular methods have aided timely identification and diverse fish bacteria pathogens (Zhang et al, 2021).

This study focuses on identification of the resistant bacteria associated with *C. gariepinus* from major fish farms within Ota, and also evaluate their genetic diversity. It also aims at addressing the sustainable development goal 2 of the United Nations Organization on food security and safety.

Materials and Methods

Sample locations and collection

Fresh fish samples (n=5) was collected from each of the three farms located at Ado-Odo/Ota Local Government Area, Ogun State, Nigeria. The farms are located at Ewupe area (Farm A) at Latitude 6^0 42ˡ N and Longitude $3^0$11ˡ E; Kajola area (Farm B), lies at Latitude 6^0 39ˡ N and Longitude 3^0 8ˡ E; and at Iju area (Farm C) lying at Latitude 6^0 41ˡ N and Longitude 3^0 0ˡ E.

Five fresh fish (*C. gariepinus*) were collected from each location (Fish farms) to give a total of fifteen fish and were transported in sterile polythene bag filled with pond water for microbial analysis.

Assessment of Morphometric Parameters of the fish samples

Morphometric data were collected in duplicates according to the method described by Olugbojo and Ayoola (2015). Parameters such as standard length, head length, gill length and buccal depth of the fish samples were measured using graduated measuring rule while weight was obtained with the use of weighing balance (A&D Ltd, Model No- R001, Korea).

Evaluation of Water quality of the pond water

Water quality parameters such as dissolved oxygen was carried out using Dissolved oxygen meter with inbuilt thermometer (Smart Sensor AR 8210 instrument), temperature, pH (hydrogen ion concentration) using pH meter (Hanna Instrument) and conductivity using Conductivity meter (Hanna instrument H186303). This was carried out to determine the quality status of the water samples in the study area/fish sample location. (Tower *et al*, 2014)

Preparation of Samples and Bacterial culture

Skin, gill, and gut of each fresh fish were removed in aseptic condition and 1.0 g of each samples were homogenized in sterile normal saline. Serial dilution was used as previously described by Ben-David and Davidson (2014). Serial dilutions were placed on separate selective media including Salmonella Shigella Agar (SSA) and Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS) and were incubated at 37^0C for 24 hours.

Bacterial Isolation and characterization

Isolation was carried out using serial dilution technique. Colonial, morphological and biochemical characteristics were determined to establish the probable organisms according to Fawole and Oso (2004) and colonies were estimated using Colony Counter (Model no- J.P Selecta 4905000, Made in Spain) as described by the American Public Health Association method (APHA, 1999). Each bacteria isolates was examined based on colonial characteristic and cellular morphology using Gram Stain technique, followed by biochemical characterization (Fawole and Oso, 2004; Bergey and Holt, 2000; Olutiola *et al*., 2000; and Chessbrough, 2010)

Antibiotic Susceptibility test

Antibiotic Susceptibility test (AST) was performed on all the bacteria isolates obtained from the fish using Kirby-Bauer disc diffusion method (Nassar, *et al.,* 2019). An overnight culture was prepared to a turbidity equivalent to 0.5 McFarland's standard and was spread on Mueller-Hinton agar plates with the aid of sterile swabs for even distribution. Antibiotic discs including Impenem (10µg), Cefuroxin (30µg), Ofloxacin (5µg), Levofloxacin (5µg), Gentamicin (10µg), Augmentin (30µg), Cefexime (5µg), Cefotaxin (25µg), Ciprofloxacin (5µg), Azithromicin (15µg), Erythromycin (15µg), Cefriaxone sulbactam (45µg), Ampiclox (10µg), Nitrofuranton (300µg), and Nalidixic acid (30µg) were placed on seeded plates and incubated for 24 hours at 37^0C. After incubation, the diameter of the inhibition zones, was measured and interpreted as 'resistant (R)', 'intermediate sensitive (I)' or 'sensitive (S)' according to Clinical and Laboratory Standards Institute (CLSI, 2020). MARI (Multiple Antibiotic Resistance Index) was calculated as the number of antibiotics that each bacteria resist was divided by the number of antibiotics used. If the value obtained is greater than 0.2 mm, it shows that the bacteria has high-level resistance, but when the value is less than 0.2 mm, it implies that the bacteria is less susceptible (CLSI, 2020)

Molecular identification of the Bacteria Isolates by 16S rRNA Gene Amplification

Bacteria isolates with high resistance were selected for molecular characterization and genetic diversity. DNA was extracted from the resistant strains using Genelute DNA extraction kit (Sigma-Aldrich, USA) following manufacturer description. The quality of the obtained DNA samples were examined on 1% agarose gel electrophoresis.

PCR analysis was run with a universal primer for bacteria, 16sRNA. The PCR mix comprises of 2.5µl of 10X PCR buffer, 1.0µl of 50 Mm $MgCl_2$, 2.0 µL of 2.5 Mm dNTPs (Deoxynucleotides Triphosphates), 0.5µl 5p Mol Forward primer [27F: AGAGTTTGATCMTGGCTCAG], 0.5µl of 5pMol, reverse primer [1525R: AAGGAGGTGWTCCARCCGCA], 0.1µl of 5 units/µlTaq with 3µl of 25 ng/µl

template DNA and 13.4µp of distilled water to make up 25 µl reaction mix. The PCR followed initial denaturation temperature of 94^0C for 5 minutes, followed by 40 cycles of 94^0C for 1 minute, 56^0C for 40 seconds, 72^0C for 1 minute, and final extension temperature of 72^0C for 7 minutes. The PCR amplicons were purified before and followed with sequencing (PacBio Sequel II)

Genetic diversity

Nucleotide sequences were aligned using ClustalW and further run through NCBI database. The homology sequences obtained using Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (NCBI) were used to determine the bacterial identity. Also, NTSYS (Numerical Taxonomy System) software was used to produce phylogenetic tree.

Data analysis

Numerical data obtained on morphometric and water quality parameters were subjected to Analysis of Variance (ANOVA) using SPSS statistical package. Duncan Multiple Range Test was used to separate the means at P<0.05.

Results and Discussion

Morphometric Characteristics of Clarias gariepinus

The result of the morphometric parameter is shown in Table 1. There is a significant difference in the fish body weight in farm A (358.50^a±4.04 g), B (412.50^b±14.43 g) and C (440.00^c±11.54 g) across the farms respectively (P<0.05). Also, the standard length: Farm A (32.65^a±0.40 cm), Farm B (35.80^c±0.69 cm), and Farm C (35.10^b±0.12 cm). Head length: Farm A (11.55^a±0.05 cm), Farm B (13.15^c±0.06 cm) and Farm C (12.05^b±0.05), gill length: Farm A (10.15^b±0.17 cm), Farm B (11.00^c±0.23 cm) and Farm C (10.10^a±0.11 cm) and Buccal depth: Farm A (10.90^a±0.12 cm), Farm B (12.60^c±0.23 cm) and Farm C (11.55^b±0.05 cm). They all depicted significant difference (P<0.05). Whereas the Total length and Fork length showed no significant difference between farm B and C in each case except in Farm A: Total length Farm A (40.10^a±0.46 cm), Farm B (43.00^b±0.23 cm) and Farm C (43.05^b±0.29 cm). Fork length: Farm A (7.45^a±0.05 cm), Farm B (7.90^b±0.12 cm) and Farm C (7.95^b±0.17 cm) (Table 1).

Table 1. Morphometric Characteristics of *C. gariepinus*

Parameters	Farm A	Farm B	Farm C
Body Weight (g)	358.50^a±4.04	412.50^b±14.43	440.00^c±11.54
Standard Length(cm)	32.65^a±0.40	35.80^c±0.69	35.10^b±0.12
Total length (cm)	40.10^a±0.46	43.00^b±0.23	43.05^b±0.29
Head length (cm)	11.55^a±0.05	13.15^c±0.06	12.05^b±0.05
Gill length (cm)	10.15^b±0.17	11.00^c±0.23	10.10^a±0.11
Buccal depth (cm)	10.90^a±0.12	12.60^c±0.23	11.55^b±0.05
Fork length (cm)	7.45^a±0.05	7.90^b±0.12	7.95^b±0.17

Foot note: Valu**e** = Mean ± SD

Along the rows, superscript of the same alphabet either a, b, or c, depicts no significant difference (P>0.05) while superscript of different alphabet shows that there was significant difference (P<0.05)

Water quality parameter of Farm A, B and C

The values of Dissolved Oxygen (DO) measured in each of the study areas ranged from 6.86 - 6.91mg/L, with no significant difference between Farm B and C (P>0.05) except Farm A (Table 2).

The pH value ranged between 6.68 - 6.93 (p<0.05), and water temperature ranged between 28.70-30.00^0C. The difference in the value recorded is highly significant. (P<0.05). Water conductivity ranged between 130.50 – 147.50

µS/cm, and there is a high significant difference in the Conductivity values across the study areas.

Table 2. Water quality parameter of Farm A, B and C

Parameter	Farm A	Farm B	Farm C
DO (Mg/L)	6.91^{a}±0.02	6.88^{b}±0.01	6.86^{b}±0.01
pH	6.68^{c}±0.01	6.93^{a}±0.01	6.90^{b}±0.01
Temperature (^{0}C)	30.00^{a}±0.01	28.70^{c}±0.01	29.50^{b}±0.01
Conductivity (µS/cm)	147.50^{a}±0.71	136.50^{b}±0.71	130.50^{c}±0.70

Foot note: Valu**e** = Mean ± SD
Superscript of the same alphabet along the row, for each separate season shows no significant difference (P>0.05)
Superscript of different alphabet along the row for each separate season shows that there was significant difference (P<0.05)
Legend DO- Dissolved oxygen, pH- Hydrogen ion concentration

Distribution and Percentage frequency of bacteria isolates found in C. gariepinus

The highest bacteria occurrence was found in *Vibrio parahaemolyticus* (16%) across the study locations and body parts, followed by *E. coli* (14%), *Proteus spp* (12%), *Staphylococcus aureus* (12%), *V. cholera* (10%), *Shigella* spp (8%), *Salmonella* spp (8%), *Micrococcus luteus* (6%), *Klebsiella aerogene* ((4%), *Aeromonas* spp (4(%), *Bacillus* spp (4%) and *Pseudomonas aeruginosa* (2%) (Fig 1)

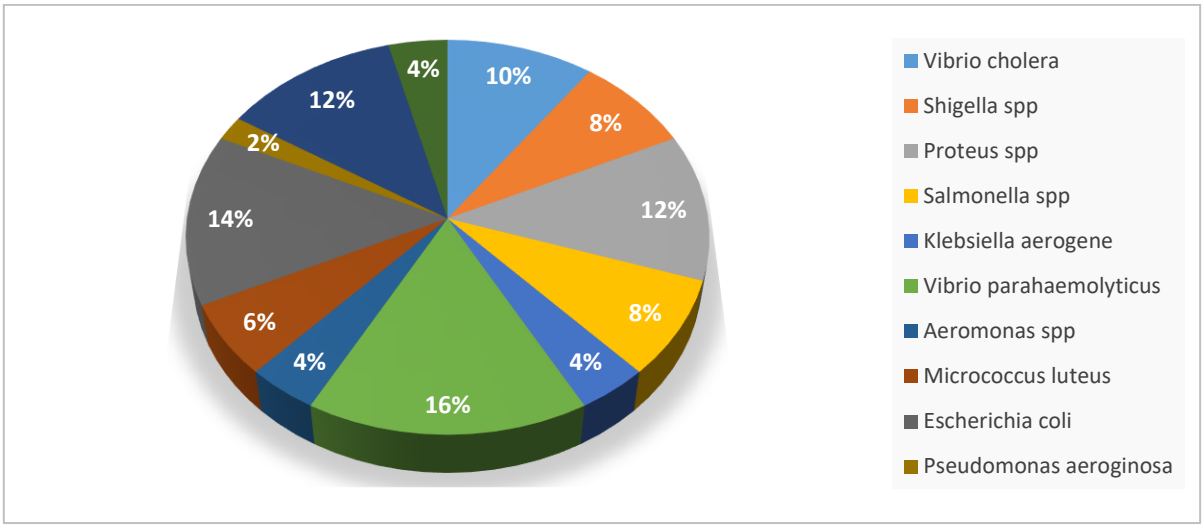

Figure. 1. Percentage frequency of bacteria isolates found in *C. gariepinus*

Distribution of bacteria load of fish samples isolated from Farm A, B and C

The gut harboured more bacterial population, followed by the gill and least in the skin (Table 3). It also depicted a significant difference (P<0.05), Farm A (5.32 x 10^{5} ± 3. 55^{a} cfu/g), Farm B (3.60 x 10^{6} ± 0.45^{b} cfu/g) and Farm C (1.77 x 10^{7} ± 0.12^{c} cfu/g) in the TAC of skin, gill and gut, however, there is no significant difference in the TAC of the bacterial population across the column (Table 3), when compare skin with skin, gill with gill and gut with gut in each of the study location. Also, the population of bacteria in the water sample showed that there were more bacteria in farm B, followed by farm A and least in farm C. There is no significant difference (P>0.05) in the result obtained in farm A and B, but the difference in that of C, compared with A and B is highly significant. Farm A (3.50 x10^{4} ± 0.37^{a} cfu/ml), Farm B (5.28 x 10^{4}± 0.39 ^{a} cfu/ml) and Farm C (2.55 x 10^{4} ± 0.36^{b} cfu/ml). Table 4 also showed bacteria detected in different body parts and study areas where the fish samples were obtained. There are variations in the bacteria detected in different body parts and study areas, but all bacteria detected in all the body parts were found in the water samples.

Table 3. Total aerobic count of fish samples isolated in Farm A, B and C (cfu/g±SD)

Study locations	Skin	Gill	Gut	Water samples
Farm A	$5.32 \times 10^5 \pm 3.55^a$	$3.60 \times 10^6 \pm 0.45^b$	$1.77 \times 10^7 \pm 0.12^c$	$3.50 \times 10^4 \pm 0.37^a$
Farm B	$3.34 \times 10^5 \pm 0.42^a$	$3.79 \times 10^6 \pm 0.27^b$	$3.59 \times 10^8 \pm 0.28^c$	$5.28 \times 10^4 \pm 0.39^a$
Farm C	$3.98 \times 10^5 \pm 0.37^a$	$4.00 \times 10^6 \pm 0.34^b$	$3.47 \times 10^7 \pm 0.48^c$	$2.55 \times 10^4 \pm 0.36^b$

Foot note: Valu**e** = Mean ± SD
Superscript of the same alphabet along the row, or column shows no significant difference (P>0.05)
Superscript of different alphabet along the row or column shows that there was significant difference (P<0.05)

Table 4. Distribution of bacterial isolates found in different organs of *C. gariepinus*

	FARM A				FARM B				FARM C			
Bacteria types	**Skin**	**Gill**	**Gut**	**Water**	**Skin**	**Gill**	**Gut**	**Water**	**Skin**	**Gill**	**Gut**	**water**
Bacillus spp	ND	ND	DE	DE	ND	ND	ND	DE	ND	ND	DE	DE
Aeromonas spp	ND	ND	ND	DE	DE	DE	DE	DE	ND	ND	ND	DE
***Proteus* spp**	DE	DE^2	ND	DE	ND	ND	ND	DE	DE^2	ND	ND	DE
Klebsiella aerogene	DE	ND	ND	DE	ND	ND	ND	DE	ND	DE	ND	DE
Vibrio Cholerae	ND	ND	DE	DE	DE	DE	ND	DE	DE	ND	DE	DE
Shigella spp	DE	ND	ND	DE	DE^2	ND	ND	DE	ND	DE	ND	DE
***Salmonella* spp**	DE	DE	ND	DE	ND	ND	ND	DE	DE^2	ND	ND	DE
***Micrococcus* spp**	ND	ND	ND	DE	DE	ND	DE	DE	ND	ND	DE	DE
P. aerugunosa	ND	ND	ND	DE	ND	ND	DE	DE	ND	ND	ND	DE
S. aureus	ND	ND	DE^2	DE	ND	DE^3	DE	DE	ND	ND	ND	DE
V. parahaemolyticus	DE^2	ND	DE^2	DE	ND	ND	ND	DE	DE^2	DE^2	DE^2	DE
E. coli	DE	ND	ND	DE	DE	DE	ND	DE	DE^2	DE^2	ND	DE

Legend: DE – Detected, ND – Not Detected, DE^2 – Detected along with another strain, DE^3 – Detected with two more strains.

Colonial, Morphological and Biochemical characteristics of bacteria isolated in Clarias gariepinus from the three locations

Cultural, Morphological, and Biochemical characteristics of bacteria isolated from the three research locations are shown in the Supplementary Table 1. It showed the edges, colour, elevations, shape and arrangement of various bacteria species present in the fish. This table also depicted the results of various identification procedures by which these bacteria species were identified.

Antibiotics susceptibility pattern of the bacterial isolates obtained from C. gariepinus from the three study locations

The antibiotic susceptibility profile of the bacterial isolates are shown in Table 5 and bar chart in Figure 2. The result shows their different degrees of resistance and sensitivity to clinically important antibiotics. Fifteen different antibiotics (Discs) were used in the experiment for the fifty gram positive and gram negative bacteria isolates. However, only those antibiotics discs that are applicable to each organisms were used, for each of the gram positive and gram negative bacteria; showing that not all antibacterial sensitivity discs were used for each of the fifty bacteria, therefore, the percentage sensitivity was based on the numbers of the antibacterial sensitivity disc used and the numbers of antibiotics each bacteria was sensitive to. These were well represented in the component bar chart (Figure 2). The first eight (8) bars depicted those antibiotics on which all the 50 bacteria were tested. The result showed that all the 50 bacteria isolates were sensitive to Ofloxacin while none were resistant or shown intermediate reaction. 49 and 35 of the 50 bacteria were sensitive to levofloxacin, and gentamycin respectively (Figure 2), while the rest few bacteria shared between resistance and intermediate reaction in each case. Likewise, 38, 33, 41, and 28 among the fifty bacteria were highly resistant to impenem, Cefuroxin, Augmentin, and Cefexin respectively while the rest few were shared between intermediate and sensitive.

However, some discs were used for selected bacteria which were not applicable to others bacteria as earlier mentioned, these are found in bars 9 to 15 (Figure 2); for instance, Ciprofloxacin (31 were sensitive out of 32 bacteria while only 1 was resistant), Azithromicin (26 were sensitive out of 28 bacteria used while the rest 2 were resistant), Erythromycin (22 were sensitive out of 27 bacteria used while 4 were resistant and 1 shown intermediate sensitivity), and ceftriaxone sulbactam (30 were sensitive out of 46 bacteria used, 14 were resistant while 2 shown intermediate sensitivity). Those antibiotics on which good numbers of these bacteria were highly resistant to, includes Cefotaxin (38 were resistant out of 49, 10 were sensitive while 1 shown intermediate sensitivity), Ampiclox (20 were resistant, while 2 were sensitive), Nitrofuranton (16 were resistant, 5 were sensitive and 2 shown intermediate sensitivity) and Nalidixic Acid (13 were resistant, 7 were sensitive and 2 depicted intermediate result). In all the results, only few bacteria showed intermediate sensitivity to each of the antibiotics used (Figure 2).

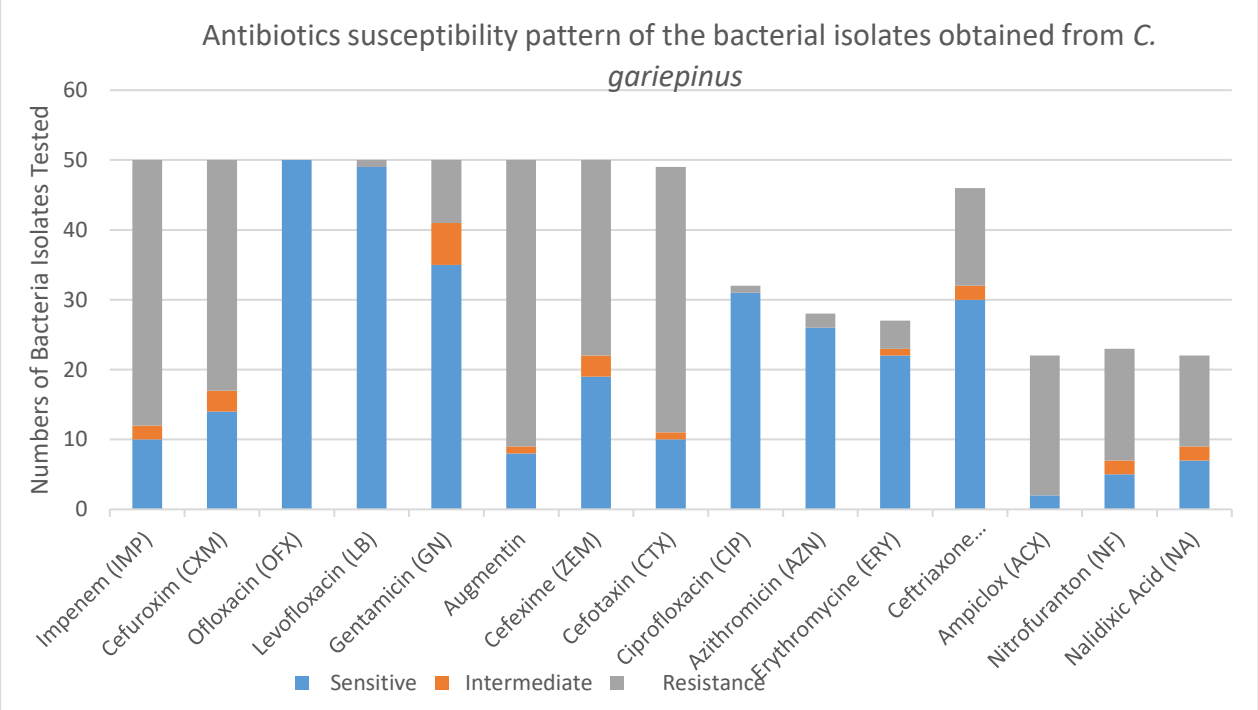

Figure 2. Percentage antibiotics susceptibility pattern of the bacterial isolates obtained from *C. gariepinus*

Table 5. Antibiotics Susceptibility Pattern of the Bacteria Isolates from *C. gariepinus* in farm A, B and C

S/N	Organism	MARI	IMP	CXM	OFX	ERY	GEN	AZN	ZEM	LBC	CIP	AUG	CTM	CRO	ACX	NF	NA
1	*A. hydrophila*	0.75	R	R	S	-	S	-	R	S	-	R	R	R	R	R	R
2	*Proteus sp*	0.58	R	R	S	-	S	R	S	S	-	R	R	S	S	S	S
3	*V. cholera*	0.78	R	R	S	-	S	-	R	S	-	R	R	R	R	R	R
4	*Micrococcus luteus*	0.75	R	R	S	-	S	-	R	S	-	R	R	R	R	R	R
5	*Salmonella spp*	0.00	S	S	S	-	S	-	S	S	-	S	S	S	S	S	S
6	*V. cholera*	0.58	R	R	S	-	S	-	S	S	-	R	R	R	R	R	S
7	*A hydrophyla*	0.66	R	R	S	-	S	-	R	S	-	R	R	S	R	R	R
8	*E. coli*	0.67	R	R	S	-	R	-	S	S	-	R	R	R	R	R	S
9	*V. parahaemolyticus*	0.33	R	S	S	-	S	-	S	S	-	R	R	S	R	I	S
10	*V. parahaemolyticus*	0.42	R	R	S	-	S	-	S	S	-	R	R	S	R	S	I

11	*V. parahaemolyticus*	0.25	S	S	S	-	S	-	S	S	-	S	S	S	R	R	R
12	*Klebsiella*	0.75	R	R	S	-	R	-	I	S	-	R	R	R	R	R	R
13	*Bacillus sp*	0.58	R	R	S	R	S	S	R	S	-	R	R	R	-	-	-
14	*Salmonella sp*	0.08	S	I	S	S	S	S	S	S	-	S	R	S	-	-	-
15	*V. parahaemolyticus*	0.42	R	R	S	S	S	S	R	S	-	R	R	S	-	-	-
16	*Proteus sp*	0.00	S	S	S	S	S	S	S	S	-	S	S	S	-	-	-
17	*S. aureus*	0.42	R	R	S	S	S	S	R	S	-	R	R	S	-	-	-
18	*E. coli*	0.17	S	S	S	S	S	S	R	S	-	S	R	S	-	-	-
19	*Shigella sp*	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
20	*Proteus* sp	0.42	R	R	S	S	I	S	R	S	S	R	R	I	-	-	-
21	*Proteus* sp	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
22	*S. aureus*	0.00	S	S	S	S	S	S	S	S	S	S	S	S	-	-	-
23	*Micrococcus luteus*	0.00	S	S	S	S	S	S	S	S	S	S	S	S	-	-	-
24	*V. parahaemolyticus*	0.50	R	R	S	S	S	S	R	S	S	R	R	R	-	-	-
25	*Shigella sp*	0.67	R	R	S	-	R	-	S	S	-	R	R	R	R	R	S
26	*Proteus sp*	0.42	R	S	S	R	I	S	R	S	-	R	R	S	-	-	-
27	*Proteus sp*	0.58	R	R	S	-	S	-	I	S	-	R	S	R	R	R	R
28	*V.*	0.25	R	S	S	S	S	S	R	S	S	R	S	S	-	-	-
29	*Salmonella sp*	0.00	S	S	S	S	S	S	S	S	S	R	S	S	-	-	-
30	*Klebsiella*	0.75	R	R	S	-	R	-	R	S	-	R	R	S	R	R	R
31	*S. aureus*	0.58	R	R	S	-	I	-	R	S	-	R	R	R	R	I	-
32	*Bacillus sp*	0.48	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
33	*S. aureus*	0.33	R	S	S	S	I	S	R	S	S	R	R	S	-	-	-
34	*V. parahaemolyticus*	0.17	I	S	S	-	S	-	S	S	-	R	R	S	-	S	I
35	*S. aureus*	0.67	R	R	S	S	R	S	R	R	R	R	R	I	-	-	-
36	*Shigella sp*	0.67	R	R	S	-	R	-	R	S	-	R	I	-	R	R	R
37	*E. coli*	0.83	R	R	S	-	R	-	R	S	-	R	R	R	R	R	R
38	*E. coli*	0.33	R	I	S	R	I	R	I	S	S	I	S	R	-	-	-
39	*Shigella sp*	0.25	S	S	S	R	S	S	S	S	S	R	R	S	-	-	-
40	*V. cholera*	0.50	R	R	S	-	S	-	S	S	-	R	R	S	R	R	S
41	*V. cholera*	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
42	*Pseudomonas sp*	0.25	S	S	S	-	R	-	S	S	-	S	S	-	R	S	R
43	*E. coli*	0.42	R	I	S	I	S	S	R	S	S	R	R	R	-	-	-
44	*Salmonella sp*	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
45	*V. cholera*	0.33	R	R	S	S	I	S	R	S	S	R	-	S	-	-	-
46	*S. aureus*	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
47	*Micrococcus sp*	0.42	R	R	S	S	S	S	R	S	S	R	R	S	-	-	-
48	*E coli*	0.50	I	R	S	-	S	-	R	S	-	R	R	-	R	R	R
49	*E coli*	0.67	R	R	S	-	R	-	S	S	-	R	R	-	R	R	R
50	*V. parahaemolyticus*	0.33	R	R	S	S	S	S	S	S	S	R	R	S	-	-	-

AUG – Augmentin	GN- Gentamicin	ZEM- Zefexime	LBC- Levofloxacin Ofloxacin	OFX -
CTM- Cefotaxime-	NF- Nitrofurantoin	CRO- Ceftriaxone sulbactam	CIP- Ciprofloxacin Azithromycin	AZN -
IMP- Impenem/Clastatin	CXM- Cefuroxime	NA- Nalidixic Acid	ACX- Ampiclox Erythromycine	ERY -

Source: Olugbojo (2020)

Molecular characterization

The blast results of the bacterial isolates are shown in Table 6. The strain descriptions are Aeromonas schubertii strain IAB I, Klebsiella pneumonia strain S 36A, Bacillus Subtilis strain xym4, Bacillus cereus strain CIFRI. S -17, Escherichia coli strain C214 and Vibrio cholerae strain RC3. Their percentage similarities are 80.00 %, 77.00 %, 100.00 %, 70.00 %, 97.95 % and 89.20 %. Their nucleotide length are 1221, 1105, 1013, 1158, 690 and 692. Their accession numbers are MF457901.1, EU931562.1, MG607370.1, OL477341.1, CP081151.1 and KF056928.1. Figure 3 and 4 showed the DNA bands of the various pathogenic strains of the bacterial isolates. Four in Figure 3 and three in Figure 4.

Table 6. Genotypic bacteria isolates from *Clarias gariepinus* (Blast Results)

Isolation site	Genotypic identification	Percentage similarity (%)	Nucleotide length	Ascension number
Gill	*Aeromonas schubertii* strain IAB 1	80	1221	MF457901.1
Gut	*Klebsiella pneumonia* strain S 36A	77	1105	EU931562.1
Skin	*Bacillus Subtilis* strain xym4	100	1013	MG607370.1
Gut	*Bacillus cereus* strain CIFRI. S-17	70	1158	OL477341.1
Gut	*Vibrio cholera* strain RC3.	89.20	690	KF056928.1
Skin	*E. coli* strain C214	97.95	692	CP081151.1

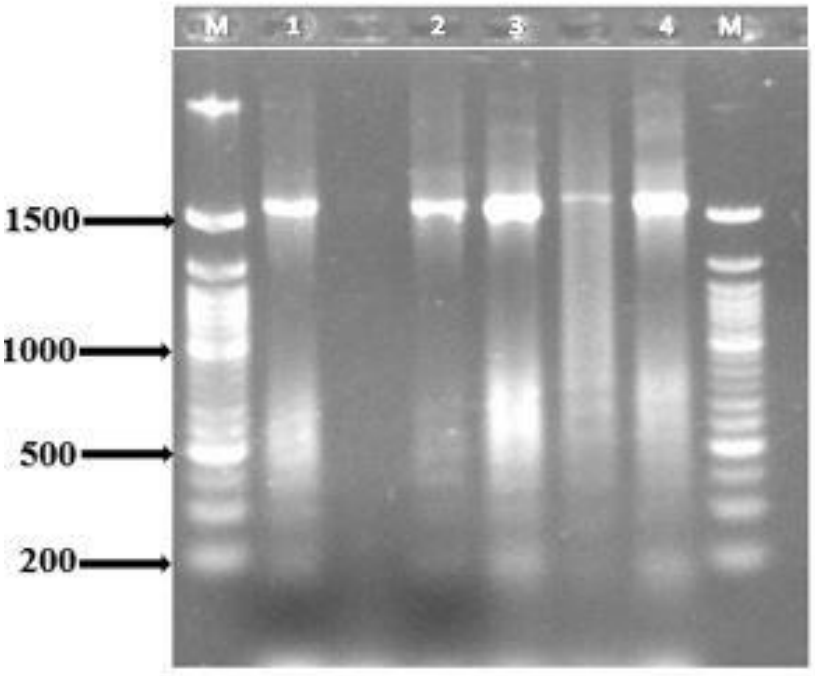

Figure 3. DNA bands showing four Pathogenic strains: M – DNA marker, 1 – Aeromonas spp, 2- Klebsiella aerogene, 3- Staphylococcus aureus, 4- Vibrio parahaemolyticus

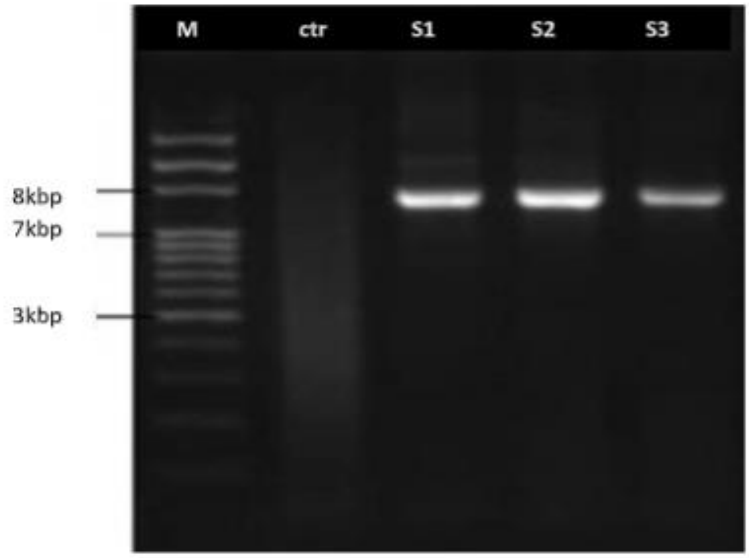

Figure 4. DNA Bands showing the three Pathogenic strains. M is the standard DNA marker. Ctrl is the control, S1 – E. coli, S2-Vibrio sp., S3 –Staphylococcus aureus

Phylogenetic diversity of the bacteria pathogens

The phylogenetic trees (Dendogram) of the resistant bacteria pathogens indicated Aeromonas schubertii strain IAB1, genetically related to Aeromonas schubertii ARB06; while Klebsiella pneumonia strain S36A, clonally related to Klebsiella pneumonia strain MAKM-5490. Bacillus subtilis strain xym4, clustered with Bacillus subtilis strain KTB9, and Bacillus cereus strain CIFRI.S-17, and Bacillus cereus strain CIFRI.N-8 according to Figure 5. Figure 6 also shows E. coli strain C 214 clustering with E. coli strain MMG 03. Likewise, Figure 7 depicted a circular phylogenetic tree of Vibrio spp. It revealed V. cholera strain RC3, grouped together with V. harvey strain CAPL B06, V. brasillensis strain HM-X-13/06, and V. aqumaris strain ED-3-6.

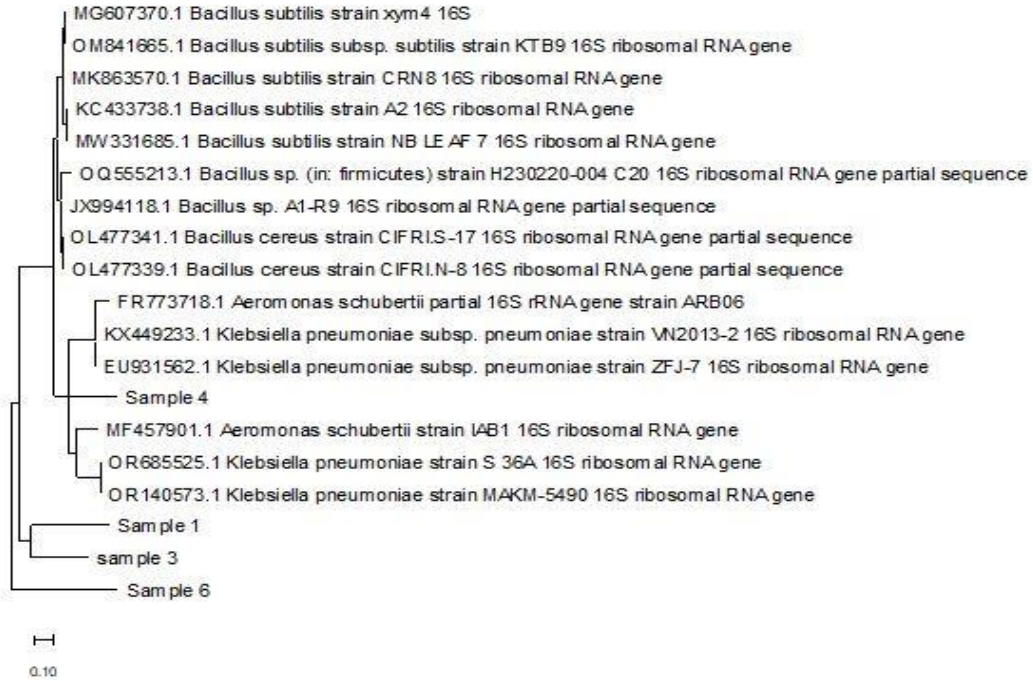

Figure 5. Phylogenetic tree of *A. schubertii, K. pneumonia, B. subtilis and B. cereus*.

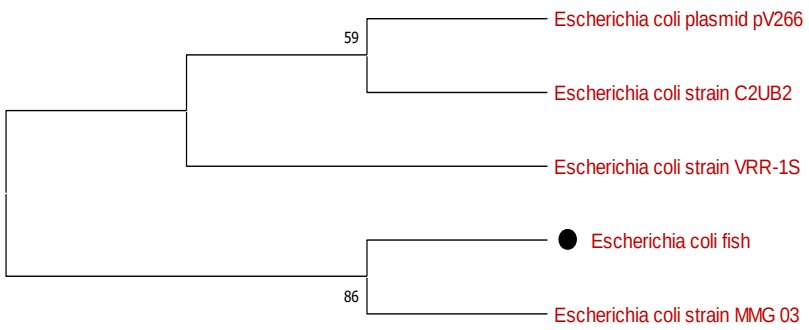

Figure 6. Phylogenetic tree of *E. coli*

Figure 7. Phylogenetic tree of *Vibrio cholera*

This study was undertaken to assess the antibacterial resistance profile and molecular diversity of the bacteria associated with Clarias gariepinus isolated from three major fish farms located in Ado-Odo Ota LGA of ogun state.

The fifty bacteria isolates found across the seasons and study locations (Table 4) which belong to eleven different genera were depicted in the pie chart in Figure 1, with their percentage frequency of occurrence. The result showed that there were more pathogens in Farm B than Farm A and C, especially Aeromonas and Vibrio spp. This actually depicted a possible fish health and food security risk, because Aeromonas spp have been found to be responsible for various diseases in cultured fish such as Aeromonas septicemia normally caused by Aeromonas hydrophila as reported by Chukwuma et al. (2020) and several types of Aeromonasis (diseases caused by Aeromonas spp). Likewise Vibrio spp are responsible for all kind of vibriosis such as hepathopancreatic necrosis diseases normally caused by Vibrio paraheamolyticus (Haenen, 2017), and several other vibriosis.

From the twelve different bacteria species isolated, six different species with high multiple antibacterial resistance index (MARI) were selected for molecular characterization. However, the size of the amplified band using 16S universal primer was 1.5 Kb for the 6 samples. This is similar to Doyle, 2007 and Akinyemi and Oyelakin (2014), who conducted a similar research on catfish from different body parts, with amplified band size of 1600 bp (1.6 Kb) for the bacteria isolates, contrary to Tubagus et al. (2009), whose amplified band was 500 bp. The blast search showed that the selected pathogens belong to both Gram positive and gram negative bacteria which is similar to what Oyelakin et al. (2019) also obtained.

The blast search conducted on the nucleotides showed specific bacterial strains such as: Aeromonas schubertii strain IAB I, Klebsiella pneumonia strain S 36A, Bacillus subtilis strain xym4, Bacillus cereus strain CIFRI. S-17, Escherichia coli strain C214 and Vibrio cholerae strain RC3. However, the result confirmed the biochemical test results to some extent. Aeromonas sp which was identified at genera level showed that it was actually Aeromonas, and the species was confirmed as A. schubertii. Klebsiella whose species was identified as aerogene was discovered to be Pneumonea through molecular analysis. However, those that were identified as Staphylococcus aureus and Vibrio parahaemolyticus through biochemical test were discovered to be Bacillus subtilis and Bacillus cereus respectively through molecular characterization. This result showed that, the most accurate approach for effective identification of bacteria is through16S rRNA molecular analysis. This is support by the previous finding (Lopez-Aladid et al., 2023).

So far, Ofloxacin was discovered to be the most effective antibiotics because none of the fifty bacteria isolate was resistant to it. Next to this are Levofloxacin with 49 bacteria showing sensitivity, and Gentamicin with 35 bacteria showing sensitivity. Others includes: Ciprofloxacin, Azithromicin, Erythromycin and Ceftriaxone Sulbactam, showing sensitivity based on the specific numbers of bacteria tested on them. For others antibiotics, the bacterial isolates showed higher resistance as depicted in the bar chart, while few showed intermediate sensitivity to them. This result is in tandem with Thai et al (2023), Zhai et al. (2023), and Croom and Goa (2003). This results also revealed that bacteria associated with the experimental fish (C. gariepinus) showed more resistance to commonly used clinical antibiotics except Levofloxacin Ofloxacin Gentamicin Ciprofloxacin, Azithromycin, and Erythromycin. This result is in tandem with Oladipo et al. (2013) who reported 58.3% resistance and 41.7% susceptibility.

Moreover, the occurence of the pathogenic bacterial isolates such as E. coli and Klebsiella spp, in C. gariepinus from the three study areas in Ota also showed a high level of pollution owing to the discharge of effluent from the neighbouring industries, hospitals and several other establishments who dispose wastes directly in to the water body, from where fish farmers channel the water they use to raise their fish, which are in turn sold to the people.

Most of this highly pathogenic strains that were selected for molecular analysis (Table 6) were found in the gut. This is similar to the previous

study (Talwar et al, 2018) in which fish gut was reported to be a site of high bacteria population than other body parts. Conversely, Table 3 showed that highest bacterial genera were generally found in the skin, this was confirmed by the previous report (Chatrema et al., 2020), nevertheless, both gill and gut had equal numbers of bacteria genera.

Conclusion

This studies provide insights into the bacterial pathogens bedeviling aquaculture industry and highlight the importance of surveillance and necessary antibiotic resistance management to ensure sustainable fish production, and public health assurance. The study clearly depicted variation in bacterial pathogens in different body parts of C. gariepinus harvested from three major fish farms in Ota. Although, more bacteria genera were found in the skin than in the gut, the bacterial population through total aerobic count showed that there were more bacterial population in the gut than found in the skin and gill. The results also revealed that Vibrio spp (Both V. cholera and V. parahaemolyticus) are the most prominent bacteria found in C. gariepinus, with a few strains of Shigella spp, Klebsiella spp, Aeromonas spp, Micrococus spp, E. coli, Pseudomonas spp, Staphyloccocus aureus and Bacillus spp. with high antibiotic resistance. It was also observed that most of the bacterial isolated from C. gariepinus across the study locations showed high rate of resistant pattern, which showed that they are highly pathogenic and could cause diseases in fish. They could also be transmitted to man if consumed fish infected with these pathogens. In several occasion, Fish and fishery products have been reported as a means of food-borne bacterial infections in man, therefore, the use of 16S rRNA analysis of bacteria showed that this method could be successfully used to genotype bacteria associated with C. gariepinus with accurate result.

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Ethical approval

The author declares that this study complies with research and publication ethics.

Informed consent

Not available.

Conflicts of interest

There is no conflict of interests for publishing of this study.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Authors' contribution

Olugbojo Joseph Abiodun and Adeolu Akanji Akinyemi contributed to actual design of the study. Olugbojo Joseph Abiodun, Akinyemi Adeolu Akannji, Obasa Samuel Olubodun , Dare Enoch Olugbenga and Akinduti Paul Sunday contributed significantly to the investigation and Project Administration. Olugbojo Joseph Abiodun drafted the manuscript, Adeolu Akanji Akinyemi, Obasa Samuel Olubodun, Dare Enock Olugbenga, and Akinduti Paul Sunday helped with editing and revising. Akinyemi Adeolu Akannji, Obasa Samuel Olubodun and Dare Enoch Olugbenga contributed to the supervision and validation of the study

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