Original Article

Diversity of Antibiotic-resistant of Tentative Motile *Aeromonas* Species Isolated From *Clarias gariepinus* (Burchell 1822) Cultured in Earthen Ponds

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ABSTRACT

Background: Aeromonas species is one of the most important causes of diseases in *Clarias gariepinus*, a public health threat with significant economic losses.

Objectives: In this research, the prevalence and variety of Aeromonas species isolated from *C. gariepinus* cultured in an earthen pond were investigated, as well as the antibiogram and multiple antibiotic resistance index

Methods: Aeromonas species were isolated by culture and biochemical test and confirmed using a Microbact 24E kit. The antibiotic susceptibility to 10 antibiotics was determined using the Kirby-Bauer disk diffusion method.

Results: Aeromonas species were isolated with a prevalence of 43.1% with 4 different phenospecies with the highest prevalence of 46(24 %) for *Aeromonas hydrophila* followed by 15(7.8%) for *Aeromonas caviae*, then 10(5.2%) for *Aeromonas veronii sobria*, and the least was observed for *Aeromonas veronii veronii* 6(3.1%). The *Aeromonas* species showed high resistance to amoxicillin, ampicillin, colistin sulfate, oxytetracycline, trimethoprim/sulfamethoxazole, and penicillin, with varying resistant patterns, and the multiple antibiotic resistance index values ranged between 0.20 and 0.80.

Conclusion: There was a diversity of Aeromonas species associated with multiple antibiotic-resistant leading to the wide spread of antimicrobial resistance. Therefore, there is a need to control the use of antibiotics and ensure the effective use of biosecurity and preventive management measures in fish farms.

Keywords: Antibiogram, Fish farm, Freshwater fish, Multiple antibiotic resistance, Zoonosis

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Introduction

Aquaculture offers a substitute method of producing fish for human use and fulfilling the expanding population's demand for protein, which has resulted in a rise in fish production levels (Boyd et al., 2022; Mirsadeghi et al., 2022). To close the fish supply and demand gap, an excellent choice for aquaculture in Africa, particularly Nigeria, is *Clarias gariepinus* because of its hardiness and widespread acceptance (Adeleke et al., 2021; Adah et al., 2022).

Fish farming activities must be intensified to keep up with the rising demand for fish for human consumption. This condition has resulted in challenges with water quality and increased stocking density, facilitating a higher incidence of disease outbreaks (Opiyo et al., 2018; Mzula et al., 2021).

The economic viability of fish production at any stage of fish culture is significantly impacted by the occurrence of various diseases, mainly caused by bacteria and of particular importance, including those of the genus *Aeromonas* (Chandrarathn et al., 2018; Fowoyo & Achimugu, 2019; Adah et al., 2021; Mzula et al., 2021).

Aeromonas species are ubiquitous Gram-negative rod, facultatively anaerobic, non-spore-forming motile bacilli that are pathogenic to fish and are responsible for varying clinical signs and mortality of cultured *C. gariepinus* leading to huge losses in fish farms globally (Fernández-Bravo & Figueras, 2020; Raj et al., 2021).

As a result, antibiotics are used to treat and prevent disease and promote growth (Mulyani et al., 2018; Okocha et al., 2018; Zdanowicz et al., 2020). More so, the emergence and spread of antibiotic-resistant bacteria have been linked to the marked increase in the utilization of antimicrobials in fish farming in many countries (Manyi-Loh et al., 2018; Pepi & Focardi, 2021; Okeke et al., 2022). This condition can ultimately decrease the effectiveness of antimicrobial agents used for treatment and encourage the growth and spread of resistant bacteria in aquaculture (Stratev & Odeyemi, 2016; Pepi & Focardi, 2021).

Additionally, the increased antibiotic resistance confers *Aeromonas* species with an additional virulent trait, which increases morbidity and mortality in fish farms (Scarano et al., 2018; Chen et al., 2019). Consequently, it is necessary to frequently assess and track antibiotic-resistant *Aeromonas* species from fish intended for human consumption

from various regions of the world to evaluate and detect the occurrence, trends, and changes in the resistance pattern toward antimicrobial drugs (Borella et al., 2020; Grilo et al., 2022). Therefore, this study aims to isolate and identify *Aeromonas* species from *C. gariepinus* cultured in earthen ponds from fish farms in Kwara State, Nigeria, and determine their antibiogram and resistance pattern in 10 commonly used antibiotics on the fish farms.

Materials and Methods

Study area and design

The research was carried out in Kwara State, North-central Nigeria. This state connects Nigeria's northern and southern regions. With geographic coordinates of longitude 5° 00'E, latitude 8° 30'N, and a total size of 13947.27 sq. miles. The state is in Nigeria's north-central geopolitical zone (35705 km^2). The state is bordered by the states of Oyo, Osun, and Ekiti to the south, Niger State to the north, Kogi State to the east, and the Benin Republic to the west (NBS, 2016; Adam et al., 2022). Based on the farms' availability and willingness to participate, a cross-sectional study involved a multistage random sampling of 24 operational grovw-out farms of *C. gariepinus* reared in an earthen pond.

Fish sample collection and examination

A total of 192 *C. gariepinus*, 8 fish per farm (length of 15 cm to 38 cm and weights of 352 g to 1000 g) were randomly selected and used from active operational farms within the research area. Between 6:00 AM and 8:00 AM, fish from the earthen pond were caught with a fishnet. To ensure the survival of the fish samples, the fish were placed in a plastic bucket with water from the ponds, with a perforated lid, and transported alive to the Fish Clinic Unit, Veterinary Teaching Hospital University of Ilorin, Kwara State, for examination. Samples were taken following the international standards for animal welfare and aquatic animal health surveillance and the guidelines for identifying fish diseases (Austin & Newaj- Fyzul, 2017; Abdulrahman, 2022).

Aeromonas species isolation and identification

Portions of the kidney and liver were aseptically sampled and seeded into separate labeled kryo bottles containing 20 mL of alkaline peptone water (Oxoid, UK) as the pre-enrichment broth and incubated at 370C for 24 hours. Growth in the selective enrichment cultures was transferred with a loop and inoculated into *Aeromonas* agar supplemented with ampicillin (10 mg/L) (Austin & Austin, 2016; Monir

et al., 2017). The dark green, opaque with dark centers colonies were picked from *Aeromonas* agar supplemented with ampicillin (10 mg/L) as presumptive *Aeromonas* species were streaked on MacConkey agar (Ahammed et al., 2016; Austin & Austin, 2016).

Aeromonas isolates were biochemically characterized using standard biochemical tests such as citrate test, hydrogen sulfide, indole test, methyl red test, motility test, sugar (glucose, inositol, and mannitol) urease test, Voges Proskauer test (Austin & Austin, 2016), and confirmed using Oxoid rapid microbat identification test kits for gram-negative bacteria, Microbact 24E (MB24E) (Oxoid Ltd, Basingstoke, England. United Kingdom). The kit contained 24 biochemical substrates, acid production from (arabinose, adonitol, arginine dihydrolase, lactose, inositol, raffinose, rhamnose, salicin, sorbitol, and sucrose) citrate, β-galactosidase, gelatin liquefaction, glucose, hydrogen sulfide, indole production, lysine decarboxylase, malonate utilization, mannitol, ornithine decarboxylase, tryptophan deaminase, urea hydrolysis, Voges Proskauer, and xylose (Khan et al., 2018).

Antibiotic susceptibility and multiple antibiotic resistance (MAR) index

The Clinical and Laboratory Standards Institute's recommendations for the standard disk diffusion method were utilized to test the isolated *Aeromonas* species' resistance to 10 commonly used antibiotics comprising of amoxicillin (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), colistin sulfate (10 µg), florfenicol (30 µg), gentamycin (10 µg), neomycin (30 µg), oxytetracycline (30 µg), penicillin (10 IU), and trimethoprim/sulfamethoxazole (SXT) (25 µg) (Oxoid, UK). By measuring the diameter of the zones of inhibition (in mm) around the disk, the antibiotics were interpreted as sensitive, resistant, and intermediate (CLSI, 2020). The multiple antibiotic resistance (MAR) index was calculated as the ratio of resistant phenotypes to the total number of antibiotics the strains were exposed (Dhanapala et al., 2021).

Statistical analysis

A Microsoft Excel 2016 spreadsheet was used to first enter the data gathered from this study. The SPSS software, version 20 for Windows was used to conduct the statistical analysis to determine the prevalence rates of the *Aeromonas* species. The percentage of *Aeromonas* species resistance was also determined for each antibiotic. The degree of each antibiotic of resistance from the earthen ponds was compared using the chi-squared test. Values of P<0.05 were considered significant.

Results

Inappetence, sluggish movements, and dead and dying fish were all seen during the spot inspection of fish on the farm. Physical examination revealed the following symptoms: skin discoloration, exophthalmia, erosions, and severe hemorrhages on the skin, eyes, and barbels. Oedema, petechiae hemorrhages on fin rot, and abdominal hyperemia postmortem examination revealed enlarged and necrotic foci on the liver, the gallbladder was distended, the intestines were hemorrhagic and fluid-filled, and the liver, kidney, and spleen were all enlarged and congested (Figure 1).

A total of 77 *Aeromonas* species with a prevalence of 40.1% were isolated with 4 different phenospecies with the highest prevalence of 46(24%) for *Aeromonas hydrophila* followed by 15(7.8%) for *Aeromonas caviae*, then 10(5.2%) *Aeromonas veronii sobria*, and the least 6(3.1%) for *A. veronii veronii* from *C. gariepinus* reared in earthen ponds in the study area. There was a significant difference (P=0.001) in the prevalence rates of *Aeromonas* species isolated in this study (Table 1).

Multiple variations of antimicrobial resistance to more than 8 antibiotics were recorded among the isolated *Aeromonas* species. The highest resistance for *A. caviae* was recorded for oxytetracycline and colistin (80%), followed by ampicillin (73.3%), amoxicillin, and penicillin (66.7%), then neomycin and trimethoprim/sulfamethoxazole (53.3%). The least resistance was observed for ciprofloxacin (20%), gentamycin (26.7%), and florfenicol (33.3%). More so, there was a higher significant resistance (P≤0.01) of *A. caviae* to the different antibiotics used (Figure 2). *A. hydrophila* displayed a high resistance level to all the antibiotics used, with colistin sulfate having the highest resistance (82.6%), followed by oxytetracycline (80.4%), then ampicillin (65.2%), penicillin (60.9%), amoxicillin and trimethoprim/sulfamethoxazole (58.7%). However, the least resistance was observed for ciprofloxacin (15.2%), followed by gentamycin (17.4%) florfenicol (30.4 %), and neomycin (32.6%). The resistance of *A. hydrophila* in this study differed significantly (P<0.01) (Figure 2). There was the highest resistance for *A. veronii sobria* recorded for amoxicillin, oxytetracycline, and colistin sulfate (80%), followed by ampicillin, neomycin, and penicillin (70%), then florfenicol (40%). The least resistance was recorded for ciprofloxacin (10%) and gentamycin (30%). In addition, the resistance of *A. veronii sobria* to the various antibiotics differed significantly (P<0.01) (Figure 2). In *A. veronii veronii*, the highest resistance was recorded for amoxicillin, trimethoprim/sulfamethoxazole, and

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Figure 1. Clinical signs observed on the fish sampled

a) Hemorrhages and pinpoint lesions on the fish, b) Erosions and abdominal dropsy in fish sampled, c) Postmortem lesion of enlarged, congested internal organs with necrotic lesions on the liver, d) Postmortem lesion of enlarged, congested internal organs with little or no abdominal fat

penicillin (83.3%), followed by ampicillin, neomycin, and colistin sulfate (66.7%). The least resistance was recorded for ciprofloxacin, oxytetracycline (16.7%), then gentamycin, and florfenicol (33.3%) (Figure 2). There was also a significant difference in this species.

Although there were similar susceptibility patterns within the *Aeromonas* species as they were susceptible to ciprofloxacin, gentamycin, and florfenicol, the susceptibility of *Aeromonas* species to the antibiotics used differed significantly (P<0.00). Multiple antibiotics resistance index (MAR), resistance pattern, and prevalence of specific patterns among different phenospecies

of *Aeromonas* from *C. gariepinus* sampled from the earthen ponds (Table 2). Regardless of the other species, *Aeromonas* showed MDR patterns of 3-8 antibiotics. The three antibiotic combinations with a MAR value of 0.3 had the highest prevalence of multidrug resistance patterns (36%), and the least was observed for 8 combinations of the antibiotics (4%). Our results indicated a tendency towards a higher number of resistances among *A. veronii sobria* and *A. veronii veronii* compared to the other 2 phenotypes. However, multidrug resistance patterns varied significantly (P<0.05; Table 2).

Discussion

Table 1. Prevalence of *Aeromonas* species isolated from *C. gariepinus* cultured in earthen ponds farms in Kwara State, Nigeria

Aeromonas Species	No. (%)	χ^2	P
A. caviae	15(7.8)		
A. hydrophila	46(24.0)		
A. veronii sobria	10(5.2)	68.9	<0.001#
A. veronii veronii	6(3.1)		
The overall prevalence of *Aeromonas* species	77(40.1)		

#P<0.001.

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Table 2. Multiple antibiotic resistant index, resistance pattern, and prevalence of specific pattern among different phenospecies of Aeromonas Isolated from fish cultured in earthen ponds

S/N	*Aeromonas* Species	Number of Antibiotics	Resistant Pattern	MAR Index	Prevalence of Specific pattern (%)	χ²	P
1	*A. hydrophila*		AMOX, CT, OXE	0.3	9 (36)		
2	*A. hydrophila*	3	AMP, CT, OXE	0.3			
3	*A. hydrophila*		AMOX, AMP, CT	0.3			
4	*A. hydrophila*		CT, OXE, P	0.3			
5	*A. veronii biovar sobria*		AMOX, OXE, P	0.3			
6	*A. veronii biovar sobria*		CT, OXE, SXT	0.3			
7	*A. veronii biovar veronii*		AMOX, P, OXE	0.3			
8	*A. veronii biovar veronii*		CT, OXE, P	0.3			
9	*A. veronii biovar veronii*		AMP, OXE, SXT	0.3			
10	A. caviae	4	AMOX, CIP, N, SXT	0.4	4(16)		
11	*A. veronii biovar sobria*		CT, FFC, N, OXE,	0.4			
12	*A. veronii biovar sobria*		AMOX, AMP, FFC, N	0.4			
13	*A. caviae*		CT, OXE, P, SXT	0.4		12.34	0.03#
14	*A. caviae*	5	AMP, CT, GEN, OXE, P	0.5	3(12)		
15	A. caviae		AMOX, AMP, CT, N, OXE	0.5			
16	*A. caviae*		AMP, CIP, OXE, P, SXT	0.5			
17	A. hydrophila	6	AMOX, CT, GEN, OXE, P, SXT	0.6	6(24)		
18	*A. hydrophila*		AMP, CIP, CT, OXE, N, SXT	0.6			
19	*A. caviae*		AMOX, AMP, FFC, GEN, O, P	0.6			
20	*A. veronii biovar veronii*		AMP, CIP, CT, GEN, N, OXE	0.6			
21	*A. veronii biovar veronii*		AMOX, CT, FFC, OXE, P SXT	0.6			
22	*A. veronii biovar veronii*		AMOX, GEN, SXT, OXE, FFC, P	0.6			
23	A. hydrophila	7	AMP, CT, FFC, N, OXE, P, SXT	0.7	2(8)		
24	*A. veronii biovar sobria*		AMOX, AMP, CT, GEN, OXE, P, SXT	0.7			
25	*A. veronii biovar sobria*	8	AMOX, AMP, CT, FFC, GEN, N, OXE, P	0.8	1(4)		

Abbreviations: MAR index: Multiple antibiotic resistant index; AMOX: Amoxicillin; AMP: Ampicillin; CIP: Ciprofloxacin; CT: Colistin sulfate; FFC: Florfenicol; N: Neomycin; CN: Gentamicin; OXE: Oxytetracycline; P: Penicillin; SXT: Trimethoprim/Sulfamethoxazole.
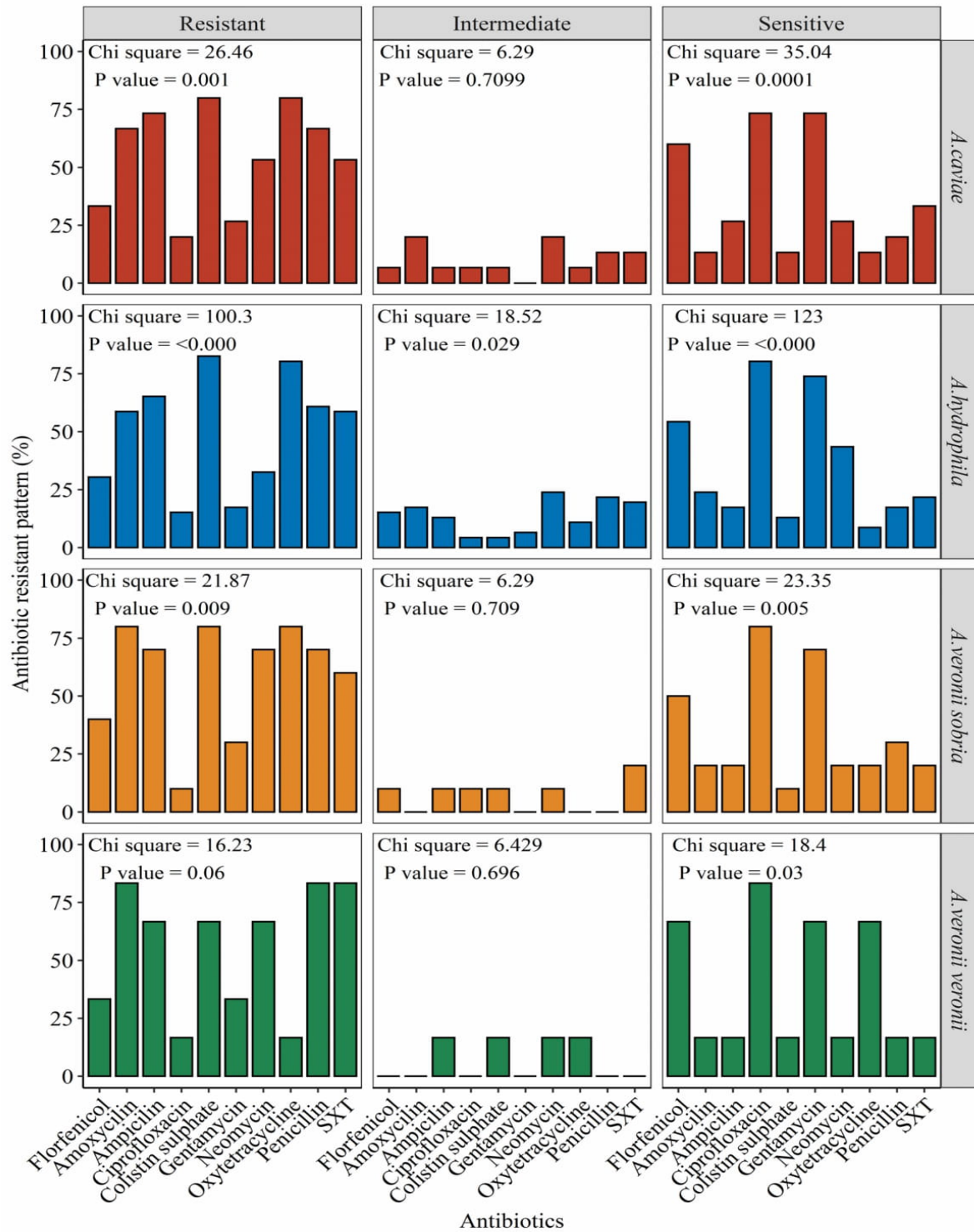
#P<0.05.

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Figure 2. Distribution of antibiotics susceptibility patterns of *Aeromonas* species isolates from *C. gariepinus* cultured in earthen ponds fish farms in Kwara State, Nigeria

SXT: Trimethoprim/Sulfamethoxazole.

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Outbreaks of *Aeromonas* disease are one of the most important drawbacks in fish farms. In this present study, the overall prevalence of *Aeromonas* species isolated from *C. gariepinus* reared in earthen ponds was 40.1%; this is, however, higher than the findings of Perretta et al. (2018), who isolated a prevalence of 35.5% from fish in Uruguay, El-Gohary et al. (2020) from Egypt with a prevalence of 33.3% and Adah et al. (2021), with a prevalence of 19.6% in Kaduna State. However, our findings were lower than the prevalence obtained by Kishk et al. (2020), who got a prevalence of 64% of *Aeromonas* species isolated from fish farms in Egypt. The variability in the prevalence of *Aeromonas* species observed may be due to the different fish species, holding facilities, sampling methods, geographic locations, and management practices.

This study isolated 4 *Aeromonas* species (*A. hydrophila, A. caviae, A. veronii sobria*, and *A. veronii veronii*), with *A. hydrophila* being the most dominant species. This finding is consistent with the findings of Perretta et al. (2018), Borella et al. (2020), Kishk et al. (2020), and Salem et al. (2020). However, it differs from the results of Ashiru et al. (2017) and Grilo et al. (2021), who opined differences in the most prevalent phenospecies of *Aeromonas* from the fish farm. This finding is most likely a result of the diverse *Aeromonas* species present and their ability to adapt to the aquatic environment, leading to their widespread distribution. It is worthy of note that these *Aeromonas* species isolated in this study are important pathogens of fish associated with varying diseases in fish farms and also of public health interest (El-Gohary et al., 2020; Borella et al., 2020, Adah et al., 2021).

Multiple variations of antimicrobial susceptibility have been recorded among *Aeromonas* species, hence the need for antibiotic susceptibility testing, which is crucial for determining the extent of antimicrobial resistance and choosing the right drugs to treat diseases in fish farms, thereby reducing the risk to human health. In this study, *Aeromonas* species isolated showed a high resistance level to the various antibiotic used regardless of the different phenospecies; high resistance was recorded for β-lactam antibiotics (amoxicillin, ampicillin, and penicillin). A similar high resistance to these β-lactamases has been recorded by Borella et al. (2020) and Salem et al. (2020), which could be due to the production of multiple, inducible, chromosomally encoded β-lactamases. Furthermore, the resistance of *Aeromonas* species to oxytetracycline, neomycin, sulfamethoxazole, and colistin sulfate has also been recorded (Sarder et al., 2016; Borella et al., 2020; El-Gohary et al., 2020; Dhanapala et

al., 2021). This could be attributed to the extensive use of these drugs as they are readily available over the counter, given either in feeds or baths (Adah et al., 2022).

The *Aeromonas* species were susceptible to gentamycin, ciprofloxacin, and florfenicol, similar to the findings of (Rahman et al., 2021; Woo et al., 2022). These findings could be attributed to the less frequent use of these drugs in aquaculture than other antibiotics. However, this is contrary to the reports of Ahmed et al. (2018), El-Gohary et al. (2020), and Lin et al. (2022), who reported resistance of *Aeromonas* species to ciprofloxacin, gentamycin, and florfenicol. The varying resistance patterns of *Aeromonas* species isolated among *C. gariepinus* may be due to variations in the frequency, duration, quantity, and usage of antimicrobial drugs in various fish farms sampled. Furthermore, diverse antibiotic resistance patterns may exist based on the environment and selective pressure; these patterns can quickly change.

The patterns displayed by various species indicate how complicated the understanding of antibiotic resistance is in the studied area. This result has also been previously reported by (Borella et al., 2020; Nhinh et al., 2021).

The MAR index of *Aeromonas* species of >0.2 recorded in this study is consistent with the findings of Salem et al. (2020) and Saleh et al. (2021), suggesting that the *Aeromonas* species from *C. gariepinus* from earthen ponds have been exposed to indiscriminate use of antibiotics during culture consequently has resulted in the development of antibiotic resistance as noted in this study which subsequently affects the outcome of therapy in the fish farms (Salem et al., 2020).

Conclusion

In conclusion, this study revealed several antibiotic-resistant *Aeromonas* species with different multiple resistance patterns. This finding suggests the indiscriminate use of antibiotics on the fish farm resulted in a MAR index greater than 0.02. This issue has led to a significant public health issue. Therefore, using available antibiotics in Nigeria's aquatic industry must be closely examined and periodically monitored to ascertain the growth and spread of bacterial resistance, necessitating biosecurity measures.

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Ethical Considerations

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