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Occurrence of *Aeromonas spp.* Resistant to Some Selected Antibiotics Isolated from Farmed *Clarias gariepinus*

Resistensi *Aeromonas spp.* yang Diisolasi dari *Clarias gariepinus* yang Dibudidayakan terhadap Beberapa Jenis Antibiotik

Debora Arimie Adah[1*], Lawal Sa'idu[2], Sonnie Joshua Oniye[3], Moshood Abiola Raji[4], Adakola Sylvanus Adah[5]

[*1]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ilorin, Ilorin-Nigeria
[2]Veterinary Teaching Hospital, Ahmadu Bello University, Zaria-Nigeria
[3]Department of Zoology, Ahmadu Bello University, Zaria-Nigeria
[4]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin-Nigeria
[5]Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Ilorin, Ilorin-Nigeria

ABSTRACT

Background: One of the major causes of disease in *Clarias gariepinus* is *Aeromonas spp.* which has been linked to significant economic losses and poses a risk to public health. **Purpose:** The prevalence and heterogeneity of Aeromonas species isolated from *Clarias gariepinus* cultured in concrete tanks, as well as the antibiogram and multiple antibiotic resistance index, were examined. **Methods:** In this study, using culture, biochemical characterization, and Microbact 24E kit, *Aeromonas spp.* isolates were confirmed. The Kirby Bauer disc diffusion method assessed the antibiotic susceptibility to 10 different antibiotics. **Results:** Four different species of *Aeromonas* were identified, with an overall prevalence of 55 (28.6%). *Aeromonas hydrophila* had the highest prevalence of 35 (18.6%), followed by 10 (5.2%) for *Aeromonas caviae*, then 5 (2.6 %) for *Aeromonas veronii* biovar. sobria, and *Aeromonas veronii* biovar. veronii, respectively. The Aeromonas species exhibited high resistance to amoxicillin, ampicillin, colistin sulphate, oxytetracycline, trimethoprim/ sulphamethoxazole, and penicillin, with varied patterns of resistance, and the multiple antibiotic resistance (MAR) index values ranged between 0.10 and 0.80. **Conclusion:** Several antibiotic resistant Aeromonas species were linked to the widespread emergence of antimicrobial resistance. As a result, it is crucial to regulate the use of antibiotics in fish farms and to ensure that biosecurity and preventive management strategies are applied effectively.

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***Correspondence:**
Debora Arimie Adah
E-mail: adah.ad@unilorin.edu.ng

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ABSTRAK

Latar Belakang: Salah satu penyebab utama penyakit pada *Clarias gariepinus* adalah *Aeromonas spp.* Yang dikaitkan dengan kerugian ekonomi yang besar dan menimbulkan risiko bagi kesehatan masyarakat. **Tujuan:** Untuk mengetahui prevalensi dan heterogenitas spesies Aeromonas yang diisolasi dari Clarias gariepinus yang dikultur dalam tangki beton, serta memeriksa antibiogram dan indeks resistensi antibiotik ganda. **Metode:** Dalam penelitian ini menggunakan kultur, karakterisasi biokimia, dan kit Microbact 24E, isolat spesies Aeromonas yang terkonfirmasi. Metode difusi cakram *Kirby Bauer* digunakan untuk menilai kerentanan antibiotik terhadap 10 antibiotik yang berbeda. **Hasil:** Empat spesies Aeromonas yang berbeda diidentifikasi, dengan prevalensi keseluruhan 55 (28,6%). *Aeromonas hydrophila* memiliki prevalensi tertinggi 35 (18,6%) diikuti oleh 10 (5,2%) untuk *Aeromonas caviae*, kemudian 5 (2,6%) untuk *Aeromonas veronii* biovar. sobria, dan *Aeromonas veronii* biovar. veronii. Spesies Aeromonas menunjukkan resistensi tinggi terhadap amoksisilin, ampisilin, colistin sulfat, oxytetracycline, trimetoprim / sulphamethoxazole, dan penisilin, dengan pola resistensi yang bervariasi, dan nilai indeks resistensi antibiotik ganda (MAR) berkisar antara 0,10 dan 0,80. **Kesimpulan:** Beberapa spesies Aeromonas yang kebal antibiotik dikaitkan dengan meluasnya kemunculan resistensi antimikroba. Oleh karena itu, penting untuk mengatur penggunaan antibiotik di peternakan ikan dan untuk memastikan bahwa strategi biosekuriti dan manajemen pencegahan diterapkan secara efektif.

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Kata kunci: Akuatik Zoonosis, Ikan air tawar, Resistensi Antibiotik, MAR Index

INTRODUCTION

Fish continues to be the world's cheapest source of protein obtained from aquaculture which continues to be the fastest-growing industry for generating animal food (Subasinghe *et al.* 2021). As fish production has expanded, fish are reared in different culture facilities such as in concrete tanks thereby promoting intensive management of the fish to increase yield and profit (Adah *et al.* 2022). Despite the various potential of pisciculture in Nigeria, its production is nevertheless challenged by a number of issues, such as increased changes in water quality, and stress leading to high morbidity and mortality (Nwakuche *et al.* 2019). One of the most often cultivated fish species in Nigeria is *Clarias gariepinus*, which serves as the primary seed for aquaculture production has been affected by fish bacteria and is among the most significant issues in the aquaculture industry (Dauda *et al.* 2018). In consequence, disease outbreaks can have detrimental effects on the quantity and quality of production, with fish mortality being the worst (Adah *et al.* 2022, Ndashe *et al.* 2023)

Aeromonas species, belonging to the family Aeromonadaceae are Gram-negative bacteria having a worldwide distribution, have been associated with many bacterial diseases in fish farms (Fernández-Bravo and Figueras 2020). Increased stressful conditions associated with high stocking density, handling, fluctuations in water quality and other unsuitable aquaculture activities observed in Intensive management practices of fish farms are linked to the development of clinical manifestations of disease in fish (Gilani *et al.* 2021), Consequently, due to the outbreaks of disease on fish farms, there has been a greater reliance on antimicrobials for disease prevention and treatment (Ferri *et al.* 2022). It is not surprising that antibiotics are frequently used in aquaculture at therapeutic doses to treat diseases and at subtherapeutic doses as preventative measures to boost feed efficiency and improve growth rates, which results in the widespread occurrence and spread of antibiotic-resistant bacteria (Ibrahim *et al.* 2020).

Due to the decreased effectiveness of antimicrobial treatments, antibiotic-resistant bacteria are linked to higher rates of morbidity and mortality from disease and transmission of numerous antibiotic-resistant bacteria to humans through the food chain (Prestinaci *et al.* 2015). It is important to regularly assess, monitor the trend, and diversity of the resistance pattern towards antimicrobial medications, from fish for human consumption from different regions of the world (Chowdhury *et al.* 2022). Hence the aim of this study is to isolate and identify Aeromonas species from *Clarias gariepinus* cultured in concrete tanks from fish farms in Kwara State, Nigeria, as well as to ascertain their antibiogram and resistance pattern to regularly used antibiotics

MATERIAL and METHOD

Study Area and Design

The research was carried out in Kwara State, situated in Nigeria's North Central Geopolitical Zone which is bordered to the south by Oyo, Osun, and Ekiti, to the north by Kogi and Niger, to the east by Kogi, and to the west by the Republic of Benin. Its geographical coordinates are 5° 00'E and 8° 30'N (Adam *et al.* 2022). Based on the farmers' availability and willingness to take part in the study, a cross-sectional study was carried out using a multistage random sampling of 24 functional grow-out farms of *Clarias gariepinus* reared in concrete tanks .

Fish Sample Collection and Examination

Eight fish from each farm, totaling 192 *Clarias gariepinus* (*C. gariepinus*), with lengths ranging from 17 to 42 cm and weights ranging from 370 to 1100 g, were randomly selected. Fish from the concrete tanks were caught with a fishnet between the hours of 6:00 and 8:0 and then transferred to a plastic bucket filled with pond water and secured with a perforated lid before being brought alive to the Fish Clinic Unit of the Veterinary Teaching Hospital University of Ilorin, Kwara State, for analysis. The recommendations for the diagnosis of fish diseases, as well as the international standards for animal welfare and aquatic animal health surveillance, were followed when collecting samples (Li *et al.* 2022).

Aeromonas Species Isolation and Identification

The fish were dissected and portions of the kidney and liver were aseptically sampled and seeded into separate labelled kryo bottles containing 20 ml of pre-enrichment broth alkaline peptone water (Oxoid, Uk) and incubated at 37^{0}C for 24 hours. Aeromonas agar supplemented with ampicillin (10 mg/L) (Oxoid, Uk) was used to inoculate the growth from the selective enrichment broth. The dark green colonies from Aeromonas agar treated with ampicillin (10 mg/L) (Oxoid, Uk) were selected as presumptive Aeromonas species were streaked on Mac Conkey agar (Austin and Austin, 2016). Aeromonas isolates were biochemically characterized using standard biochemical tests such as citrate test, hydrogen sulphide, indole test, methyl red test, motility test, sugar (glucose, inositol, and mannitol) urease test, Voges Proskauer test (Austin and Austin, 2016), and confirmed using Oxoid rapid microbact identification test kits for Gram-negative bacteria, Microbact 24E (MB24E) (Oxoid Ltd, Basingstoke, England. United Kingdom) (Mailafia *et al.* 2021).

Antibiotic Susceptibility and Multiple Antibiotic Resistance (MAR) index

The Clinical and Laboratory Standards Institute's recommendations for the standard disc diffusion method were utilized to test the isolated Aeromonas species resistance to 10 widely used antibiotics comprising of amoxicillin (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), colistin sulphate (10 µg), florfenicol (30 µg), gentamycin (10 µg), neomycin (30 µg) oxytetracycline (30 µg), penicillin (10 IU) and trimethoprim/sulphamethoxazole (SXT) (25 µg) (Oxoid, UK) and by measuring the diameter of the zones of inhibition (in mm) around the disc, antibiotics were interpreted as sensitive, resistant and intermediate (CLSI, 2020). The multiple Antibiotic Resistance (MAR) index was calculated as the ratio of the number of resistant phenotypes to the total number of antibiotics to which the strains were exposed

Statistical Analysis

A Microsoft Excel 2016 spreadsheet was used to first enter all of the data gathered from this study. The Statistical Package for the Social Sciences for Windows version 20.0 was used to conduct the statistical analysis to determine the prevalence rates of the Aeromonas species. Additionally, the prevalence of multiple resistant Aeromonas strains was he degree of each antibiotic of resistance from the concrete tanks was compared using the chi-squared. Values of $p < 0.05$ were considered significant.

RESULT

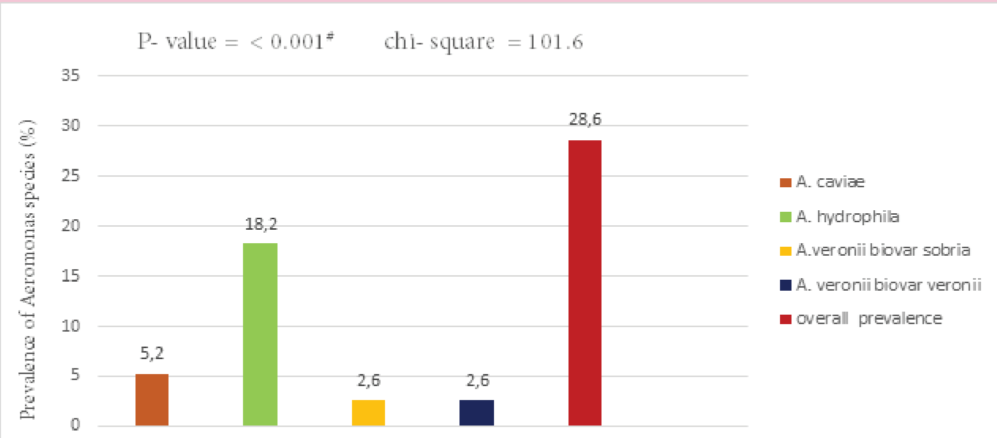
The biochemical characterization revealed that each of the fifty-five isolates had characteristic biochemical activity to be Aeromonas species. Of the 55 Aeromonas species four different Aeromonas complexes were identified with a prevalence of (28.6%). The most prevalent Aeromonas species was *Aeromonas hydrophila* 35 (18.2%), followed by *Aeromonas caviae*, 10 (5.2%) and then 5 (2.6%) each of *Aeromonas veronii* biovar sobria and *Aeromonas veronii* biovar veronii were isolated from *Clarias gariepinus* reared in concrete tanks in the study area. The prevalence rates of Aeromonas species isolated in this study differed significantly (p=0.001) (figure 1).

Multiple variations were recorded for the isolated Aeromonas species to 8 antibiotics used. The highest resistance for *Aeromonas caviae* was recorded for oxytetracycline (90%) followed by ampicillin, florfenicol, and penicillin (70.00%) respectively, then amoxicillin, and trimethoprim/sulphamethoxazole and colistin sulphate (60.%), and then neomycin (50%). The least resistance was recorded for ciprofloxacin (10%) and gentamycin (20%). The resistance differed significantly to the different antibiotics used ($p \leq 0.01$) (Table 1). *Aeromonas hydrophila* displayed a high resistance level to eight of the ten antibiotics used with oxytetracycline having the highest resistance (88.57%), followed by trimethoprim/-sulphamethoxazole and penicillin (80%) then ampicillin and colistin sulphate (71.43%), penicillin (60.9%), amoxicillin and trimethoprim/sulphamethoxazole (58.7%). However, the least resistance was observed for ciprofloxacin (2.86) and gentamycin (22.86%). The resistance of *Aeromonas hydrophila* in this study differed significantly ($p < 0.01$) (table 1). There was the highest resistance for *Aeromonas veronii* biovar sobria recorded for amoxicillin, oxytetracycline, and florfenicol (80%) followed by ampicillin, neomycin, colistin sulphate and penicillin (60%) respectively. The least resistance was recorded for ciprofloxacin and gentamycin (20 %) respectively. There was no significant difference in the resistance recorded ($p < 0.4$) (table 1). In *Aeromonas veronii* biovar veronii, the highest resistance was recorded for ampicillin (100%) followed by neomycin, oxytetracycline, colistin sulphate and penicillin (80%, the amoxicillin, florfenicol and trimethoprim/sulphamethoxazole, (60%) the least resistance was recorded for ciprofloxacin (0%), then gentamycin, (20%). There was also a significant difference in this specie $p < 0.04$ (table 1).

Although there were similar susceptibility patterns within the Aeromonas species as they were susceptible to ciprofloxacin and gentamycin. The susceptibility of Aeromonas species to the antibiotics used differed significantly. Table 2 shows the multiple resistance index (MAR), resistance pattern, and prevalence of specific patterns among different phenospecies of Aeromonas from *C. gariepinus* sampled from the concrete tanks. Varying multiple antibiotics resistance index (MAR), resistance pattern, and prevalence of specific patterns of the different Aeromonas species isolated from *C. gariepinus* sampled from the concrete tank was recorded in this study (table 2). The MDR patterns of Aeromonas ranged from 1 to 8 antibiotics, regardless of the species. The multidrug resistance patterns were most prevalent in three antibiotic combinations with a MAR value of 0.3 (28 %).

DISCUSSION

In this present study, the overall prevalence of Aeromonas species isolated from *Clarias gariepinus* reared in concrete tank was 28.6 %, which is similar to the findings of (Jafari-Sales and Shadi-Dizaji 2019). This overall prevalence is however higher than the findings of (El-Gamal *et al.* 2017), who recorded a prevalence of 25.9% and (Adah *et al.* 2021) who obtained a prevalence of 19.6% from *Clarias gariepinus* in Kaduna State. It was however lesser than the prevalence of 39.3 % obtained by (Morshdy *et al.* 2022) from Nile tilapia and Mugil cephalus in Egypt. The various fish species, holding



Picture 1. Prevalence of isolated Aeromonas species isolated from *Clarias gariepinus* raised in concrete tank farms in Kwara State

Tabel 1.

Occurrence of multiple resistant Aeromonas species isolated from catfish cultured in concrete tanks fish farm

Antibiotics Used	*Aeromonas caviae* (10)			*Aeromonas hydrophila* (35)			*Aeromonas veronii sobria* (5)			*Aeromonas veronii veronii* (5)		
	R	I	S	R	I	S	R	I	S	R	I	S
Amoxicillin	6 (60.00)	1 (10.00)	3 (30.00)	20 (57.14)	5 (14.29)	10 (28.57)	3 (60.00)	1 (20.00)	1 (20.00)	3 (60.00)	1 (20.00)	1 (20.00)
Ampicillin	7 (70.00)	1 (10.00)	2 (20.00)	25 (71.43)	3 (8.57)	7 (20.00)	4 (80.00)	0 (0.00)	1 (20.00)	5 (100.00)	0 (0.00)	0 (0.00)
Florfenicol	7 (70.00)	1 (10.00)	2 (0.00)	24 (68.57)	4 (1.43)	8 (22.86)	4 (80.00)	0 (0.00)	1 (20.00)	3 (60.00)	1 (20.00)	1 (20.00)
Neomycin	5 (50.00)	3 (30.00)	2 (20.00)	17 (48.57)	10 (28.57)	8 (22.86)	3 (60.00)	1 (20.00)	1 (20.00)	4 (80.00)	0 (0.00)	1 (20.00)
Oxytetracycline	9 (90.00)	0 (0.00)	1 (10.00)	31 (88.57)	2 (5.71)	3 (8.57)	4 (80.00)	1 (20.00)	0 (0.00)	4 (80.00)	0 (0.00)	1 (0.00)
Gentamycin	2 (20.00)	1 (10.00)	7 (70.00)	8 (22.86)	2 (5.71)	25 (71.43)	1(20.00)	0 (0.00)	4 (80.00)	1 (20.00)	1 (20.00)	3 (60.00)
Ciprofloxacin	1 (10.00)	0 (0.00)	9 (90.00)	1 (2.86)	1 (2.86)	33 (94.29)	1 (20.00)	0 (0.00)	4 (80.00)	0 (0.00)	0 (0.00)	5 (100.00)
SXT	6 (60.00)	2 (20.00)	2 (20.00)	28 (80.00)	4 (11.43)	3 (8.57)	2 (40.00)	2 (40.00)	1 (20.00)	3 (60.00)	1 (20.00)	1 (20.00)
Colistin sulphate	6 (60.00)	2 (20.00)	2 (20.00)	25 (71.43)	5 (14.29)	5 (14.29)	3 (60.00)	1 (20.00)	1(20.00)	4 (80.00)	0 (0.00)	1 (20.00)
Penicillin	7 (70.00)	1 (10.00)	2 (20.00)	28 (80.00)	2 (5.71)	5 (14.29)	3 (60.00)	2 (40.00)	0 (0.00)	4 (80.00)	0 (0.00)	1(20.00)
Chi-square	21.27	7.2	28.31	102.4	17.6	123	9.42	8.33	18.25	17.74	6.52	9.03
P-value	0.001	0.62	0.001	< 0.00	0.04	<0.00	0.4	0.5	0.03	0.04	0.67	0.43

Note. R : Resistant; I: Intermediate ; S: Sensitive ; SXT: Trimethoprim/Sulphamethoxazole

Tabel 2.

Multiple antibiotic resistance patterns and MAR index of the different Aeromonas species isolated from

Aeromonas species	Resistant pattern	MAR INDEX	Prevalence of Specific pattern (%)
A. veronii biovar sobria	P	0.1	1 (4.00)
A. hydrophila	P, AMP	0.2	2 (8.00)
A. hydrophila	CT, OXE	0.2	
A. hydrophila	SXT, CT, OXE	0.3	
A. caviae	AMOX, P, AMP	0.3	
A. veronii biovar sobria	CT, OXE, AMP	0.3	7 (28.00)
A. veronii biovar sobria	CT, OXE, AMP	0.3	
A. veronii biovar sobria	CN, CT, N,	0.3	
A. veronii biovar veronii	AMOX, SXT, CT	0.3	
A. veronii biovar veronii	SXT, CT, OXE	0.3	
A. hydrophila	AMOX, CT, OXE, P	0.4	2 (8.00)
A. caviae	SXT, CT, OXE, AMP	0.4	
A. hydrophila	CT, N, OXE, P, AMP	0.5	
A. hydrophila	AMOX, SXT, CT, OXE, AMP	0.5	2 (8.00)
A. hydrophila	AMOX, SXT, CT, OXE, P, AMP	0.6	
A. caviae	SXT, CT, OXE, FFC, P, AMP	0.6	4 (16.00)
A. veronii biovar sobria	AMOX, CN, SXT, OXE, FFC, P,	0.6	
A. veronii biovar veronii	AMOX, SXT, CT, OXE, P, AMP	0.6	
A. caviae	AMOX, SXT, CT, N, OXE, FFC, AMP	0.7	
A. caviae	AMOX, FFC, SXT, CT, OXE, P, AMP	0.7	4 (16.00)
A. veronii biovar sobria	AMOX, SXT, CT, OXE, FFC, P, AMP	0.7	
A. veronii biovar veronii	AMOX, SXT, CT, N, OXE, P, AMP	0.7	
A. caviae	AMOX, CN, CT, N, OXE, FFC, P, AMP	0.8	
A. veronii biovar veronii	AMOX,FFC, SXT, CT, N, OXE, P, AMP	0.8	3(12.00)
A. veronii biovar veronii	AMOX, CN, SXT, CT,N, OXE,P,AMP	0.8	

Note. Multiple Antibiotic Resistant Index (MAR index)= AMOX: Amoxycillin; AMP: Ampicillin; CIP: Ciprofloxacin; CT: Colistin sulphate; FFC: Florfenicol; N: Neomycin; CN: Gentamicin; OXE: Oxytetracycline; P: Penicillin; SXT: Trimethoprim/sulphamethoxazole. Multiple antibiotics resistance (MAR), χ^2 = Chi Square # = Significant at P < 0.05

facilities, sample techniques, geographical regions, and management practices could all play a role in the variation in the observed prevalence of Aeromonas species.

Our findings revealed that *Clarias gariepinus* were infected with four different Aeromonas species (*A. hydrophila, A. caviae, A. veronii* biovar sobria, and *A. veronii* biovar veronii) with *A. hydrophila* being the most dominant species this is consistent with the findings of (El-Gamal *et al.* 2017) and (Borella *et al.* 2020) however, it is different from the results of (Khor *et al.* 2015, Ebeed *et al.* 2017) and (El-Tawab *et al.* 2018) who opined that *A. caviae* and *A. veronii* biovar sobria, were the most prevalent specie from the fish farm. This finding is most likely the result of the presence of the different Aeromonas species, which have a wide distribution due to their successful adaptation to aquatic environments. It is worthy of note that these Aeromonas species isolate in this study are important pathogens of fish associated with varying diseases in fish farms and also of public health interest (Borella *et al.* 2020, Adah *et al.* 2021) Fish farmers regularly use antibiotics frequently for prophylactic or therapeutic use without expert help, in order to prevent great financial losses thereby leading to resistance (Borella *et al.* 2020). This has therefore necessitated the need of antibiotic susceptibility testing, which is essential for identifying the level of antimicrobial resistance and for selecting the appropriate medications to treat disease in fish farming and lowering the risk to human health (Nhinh *et al.* 2021). In this study, Aeromonas species isolated showed a high level of resistance to the various antibiotic used regardless of the different phenospecies. High resistance was recorded for β-lactam antibiotics, (amoxicillin, ampicillin, and penicillin), which is similar to the reports of (Nhinh *et al.* 2021) and (Borella *et al.* 2020). This could be due to the production of multiple, inducible, chromosomally encoded beta-lactamases. Furthermore, the resistance of Aeromonas species to oxytetracycline, neomycin, sulfamethoxazole, and colistin sulphate has also been recorded by (Borella *et al.* 2020) and (Dhanapala *et al.* 2021) this could be attributed to the extensive use of these drugs as they readily available over-the-counter given either in feeds or baths (Adah *et al.* 2022). It is noteworthy that the Aeromonas species were susceptible to gentamycin and ciprofloxacin, which was similar to the findings of (Rahman *et al.* 2021) and (Woo *et al.* 2022) these findings could be attributed to the less frequent use of these drugs in aquaculture compared to the other antibiotics. However this is contrary to the reports of (Li *et al.* 2022), who reported resistance of Aeromonas species to ciprofloxacin and gentamycin. It's possible that variations in the frequency, length, amount, and usage of antimicrobial medications in the different fish farms studied are responsible for the different resistance patterns of Aeromonas species found among *Clarias gariepinus*. A variety of antibiotic resistance patterns may also occur, depending on the environment and selective pressure, these patterns can easily change (Borella *et al.* 2020, Nhinh *et al.* 2021).

The MAR index of Aeromonas species of > 0.2 recorded in this study are consistent with the findings of (Salem *et al.* 2020) and (Saleh *et al.* 2021) suggesting that the Aeromonas species from 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

Four different Aeromonas species with the prevalence of 28.6% was recorded 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 has led to a significant public health issue. Thus, the usage of available antibiotics in Nigeria's aquatic industry must be carefully scrutinized and periodically monitored to ascertain the establishment and spread of bacterial resistance, necessitating the deployment of biosecurity measures.

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CONFLICT of INTEREST

The author declares no conflict of interest.

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ETHICAL APPROVAL

This study was approved by the University of Ilorin animal care and use committee (UILCAUC) with reference number UREC/FVM/1130.

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