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

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Running Title: Diversity of antibiotic resistance from fish farm

Abstract

Background: *Aeromonas* species has been considered one of the most important aetiology of diseases in *Clarias gariepinus* leading to significant economic losses and is also a public health threat.

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

Methods: The isolation of *Aeromonas* species was done by culture, and biochemical test and was confirmed using a Microbact 24E kit. The antibiotics susceptibility to ten antibiotics was determined using the Kirby-Bauer disc diffusion method.

Results: *Aeromonas* species were isolated with a prevalence of 43.1 % with four 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 sulphate, oxytetracycline, trimethoprim/sulphamethoxazole and penicillin, with varying resistant patterns and the multiple antibiotic resistance (MAR) index values ranged between 0.20 and 0.80.

Conclusions: 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

Introduction

Aquaculture offers a substitute method of producing fish for human use and to fulfil the expanding population's demand for protein, this 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 have had to be intensified in order to keep up with the rising demand for fish for human consumption. This 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 and 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 *Clarias gariepinus* leading to huge losses in fish farms globally (Fernández-Bravo and Figueras, 2020; Raj *et al*., 2021).

As a result of this, antibiotics are been used to treat and prevent disease, as well as to 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 and Focardi, 2021; Okeke, *et al*., 2022). This can ultimately decrease the effectiveness of

antimicrobial agents used for treatment and encourage the growth and spread of resistant bacteria in aquaculture (Stratev and Odeyemi, 2016; Pepi and Focardi, 2021).

Additionally, the increased antibiotic resistance confers *Aeromonas* species with an additional virulent trait, which results in increased 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 in order to assess 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 *Clarias gariepinus* cultured in earthen ponds from fish farms in Kwara State, Nigeria, as well as to determine their antibiogram and resistance pattern to 10 commonly used antibiotics on the fish farms.

Materials and Methods

Study Area and Design

The research was carried out in Kwara State, Northcentral Nigeria. Nigeria's northern and southern regions are connected by Kwara State With geographic coordinates of longitude 5° 00'E and latitude 8° 30'N and a total size of 13,947.27 sq. miles, the state is situated in Nigeria's North Central geopolitical zone (35,705 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 availability and willingness of the farms in participating in the study, a cross-sectional study was conducted that involved a multistage random sampling of 24 operational grow out farms of *Clarias gariepinus* reared in earthen pond

Fish Sample Collection and Examination

A total of one hundred and ninety-two (192) *Clarias gariepinus (C. gariepinus), eight fish* per farm that had a total length of 15cm to 38 cm and weights of 352 g to 1000g were randomly selected and used from active operational farms within the research area. Between the hours of 6:00 and 8:00, fish from the earthen pond were caught with a fishnet. To assure the survival of the fish samples, the fish were then 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, as well as the guidelines for the identification of fish diseases (Austin and Newaj- Fyzul, 2017; Abdulrahman, 2022).

Aeromonas Species Isolation and Identification

Portions of the kidney and liver were aseptically sampled and seeded into separate labelled kryo bottles containing 20 ml of alkaline peptone water (Oxoid, Uk) as the pre-enrichment broth and incubated at 37^0 C for 24 hours. Growth in the selective enrichment cultures was transferred with a loop and inoculated into *Aeromonas* agar supplemented with ampicillin (10mg/L) (Austin and Austin, 2016; Monir, *et al*., 2017). The dark green, opaque with dark centres colonies were picked from *Aeromonas* agar supplemented with ampicillin (10mg/L) as presumptive *Aeromonas* species were streaked on MacConkey agar (Ahammed, *et al*., 2016; 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 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 sulphide, 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 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 (30ug), 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 (Dhanapala *et al*., 2021).

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 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. Values of $P < 0.05$ were considered significant.

Results

Inappetence, sluggish movements, and the presence of dead and moribund fish were all seen during the spot inspection fish of the farm. Physical examination revealed the following symptoms: skin discolouration, exophthalmia, erosions, and severe haemorrhages on the skin, eyes, and barbels. oedema, petechiae haemorrhages on fin rot, and abdominal hyperemia postmortem examination revealed enlarged and necrotic foci on the liver, the gallbladder was distended, the intestines were haemorrhagic and fluid-filled, and the liver, kidney, and spleen were all enlarged and congested. (figure1).

A total of seventy-seven *Aeromonas* species with a prevalence of (40.1%) were isolated with four 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 was observed for *Aeromonas veronii veronii* 6 (3.1%) 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 eight (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/sulphamethoxazole** (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 ($P \leq 0.01$) of *Aeromonas caviae* to the different antibiotics used (figure 2). *Aeromonas hydrophila* displayed a high resistance level to all the antibiotics used with colistin sulphate having the highest resistance (82.6%), followed by

157 oxytetracycline (80.4%) then ampicillin (65.2%), penicillin (60.9%), amoxicillin and
158 **trimethoprim/sulphamethoxazole** (58.7%). However, the least resistance was observed for
159 ciprofloxacin (15.2%), followed by gentamycin (17.4%,) florfenicol (30.4 %), and Neomycin
160 (32.6%). The resistance of *A. hydrophila* in this study differed significantly ($P < 0.01$) (figure 2).
161 There was the highest resistance for *A. veronii sobria* recorded for amoxicillin, oxytetracycline,
162 and colistin sulphate (80%) followed by ampicillin, neomycin, and penicillin (70%) respectively
163 then florfenicol (40%). The least resistance was recorded for ciprofloxacin (10%) and
164 gentamycin (30%). In addition, the resistance of *A. veronii sobria* to the various antibiotics
165 differed significantly ($P < 0.01$) (figure 2). In *A. veronii veronii*, the highest resistance was
166 recorded for amoxicillin, **trimethoprim/sulphamethoxazole**, and penicillin (83.3%) followed
167 by ampicillin, neomycin, and colistin sulphate (66.7%) the least resistance was recorded for
168 ciprofloxacin, oxytetracycline (16.7%) then gentamycin, and florfenicol (33.3%) respectively
169 (figure 2). There was also a significant difference in this specie.
170 Although there were similar susceptibility patterns within the *Aeromonas* species as they were
171 susceptible to ciprofloxacin, gentamycin, and florfenicol, the susceptibility of *Aeromonas*
172 species to the antibiotics used differed significantly ($P < 0.00$). Multiple antibiotics resistance
173 index (MAR), resistance pattern, and prevalence of specific patterns among different
174 phenospecies of *Aeromonas* from *C. gariepinus* sampled from the earthen ponds (table 2).
175 Regardless of the different species of *Aeromonas* showed MDR patterns of 3 - 8 antibiotics. The
176 three antibiotic combinations with a MAR value of 0.3 had the highest prevalence of multidrug
177 resistance patterns (36%) and the least was observed for eight combinations of the
178 antibiotics (4%). Our results indicated that there was a tendency towards a higher number of

resistances among A. *veronii sobria* and *A.* veronii *veronii* compared to the other two phenotypes but the patterns of multidrug resistance varied significantly at $P < 0.05$ (table 2).

Discussion

Outbreaks of *Aeromonas* disease are one of the most important limitations in fish production. 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 lesser 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 species of fish, holding facilities, sampling methods, geographic locations, and management practices.

In this study, four different *Aeromonas* species (*A. hydrophila*, *A. caviae*, *A. veronii sobria*, and *A. veronii veronii*) were isolated with *A. hydrophila* being the most dominant species this 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 is different from the results of Ashiru *et al*.(2017) and Grilo *et al*.(2021) who opined differences in the most prevalent of the 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 successfully leading to their widespread distribution. 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 (El-Gohary *et al*., 2020; Borella *et al*., 2020, Adah *et al*., 2021).

Multiple variations of antimicrobial susceptibility had been recorded among *Aeromonas* species, hence the need for antibiotic susceptibility testing which is crucial for determining the extent of antimicrobial resistance and for choosing the right drugs for the treatment of diseases in fish farms thereby reducing the risk to human health. 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). A similar high resistance to these β-lactamases has been recorded by Borella *et al*. (2020) and Salem *et al*. (2020) and 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 (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, which was 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 compared to the 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 patterns of antibiotic resistance may exist based on the environment and selective pressure these patterns can change very quickly.

The various patterns displayed by various species indicate how complicated the understanding of antibiotic resistance is in the studied area. This 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)

In conclusion, this study revealed several antibiotic-resistant *Aeromonas* species that have 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. Therefore, the use of available antibiotics in Nigeria's aquatic industry must be closely examined and periodically monitored to ascertain the growth and spread of bacterial resistance, necessitating the implementation of biosecurity measures.

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Ethical Statement

All procedures were performed based on the ethical approval of the ethical committee of the University of Ilorin, Nigeria

Conflict of Interest

The authors declared no conflict of interest.

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442 Table 1: Prevalence of *Aeromonas* species isolated from *Clarias gariepinus* cultured in earthen
443 ponds farms in Kwara State, Nigeria

***Aeromonas* Species**	**N %**	**χ^2 value**	**P-value**
Aeromonas caviae	15 (7.8)		
Aeromonas hydrophila	46 (24.0)		
Aeromonas veronii sobria	10 (5.2)	68.9	< 0.001[#]
Aeromonas veronii veronii	6 (3.1)		
Overall prevalence of *Aeromonas* species	77 (40.1)		

444 N = number of isolates; %= percentage; [#] = significant; χ2 = Chi-square;

445

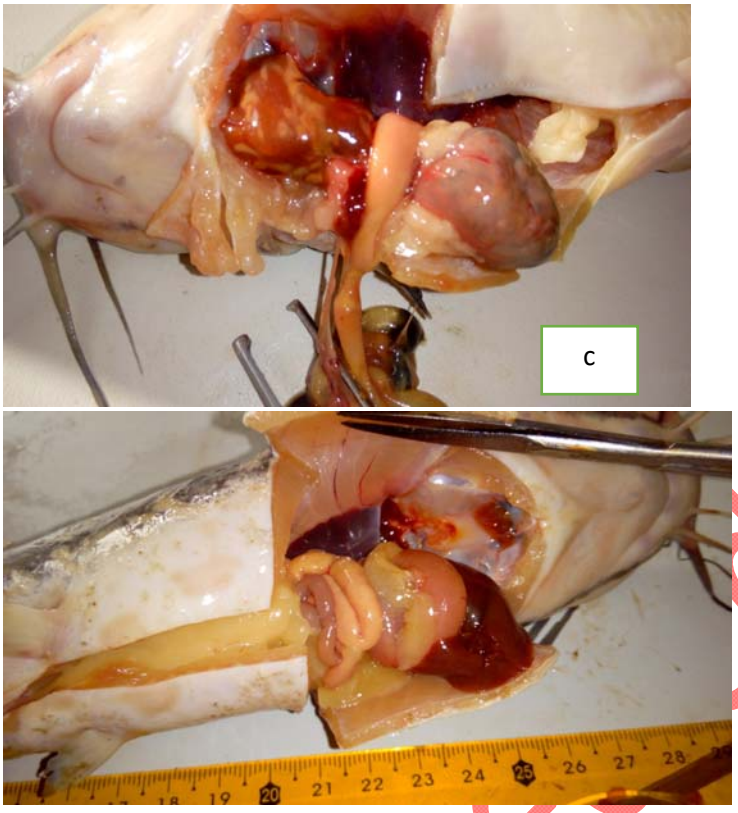
Table 2: Multiple antibiotic resistant index, resistance pattern and prevalence of specific pattern among different phenospecies of *Aeromonas* isolated from fish cultured in earthen ponds

s/n	*Aeromonas* species	Number of antibiotics	Resistant pattern	MAR INDEX	Prevalence of Specific pattern (%)	X
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. cav*iae	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. cav*iae		CT, OXE, P, SXT	0.4		
14	*A. cav*iae	5	AMP, CT, GEN, OXE, P	0.5	3(12)	
15	*A. cav*iae		AMOX, AMP, CT, N, OXE	0.5		
16	*A. cav*iae		AMP, CIP, OXE, P, SXT	0.5		1
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. cavi*ae		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)

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 $P < 0.05$.

465 a b



C d

Figure 1(a-d): Showing clinical signs observed on the fish sampled a) Haemorrhages 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

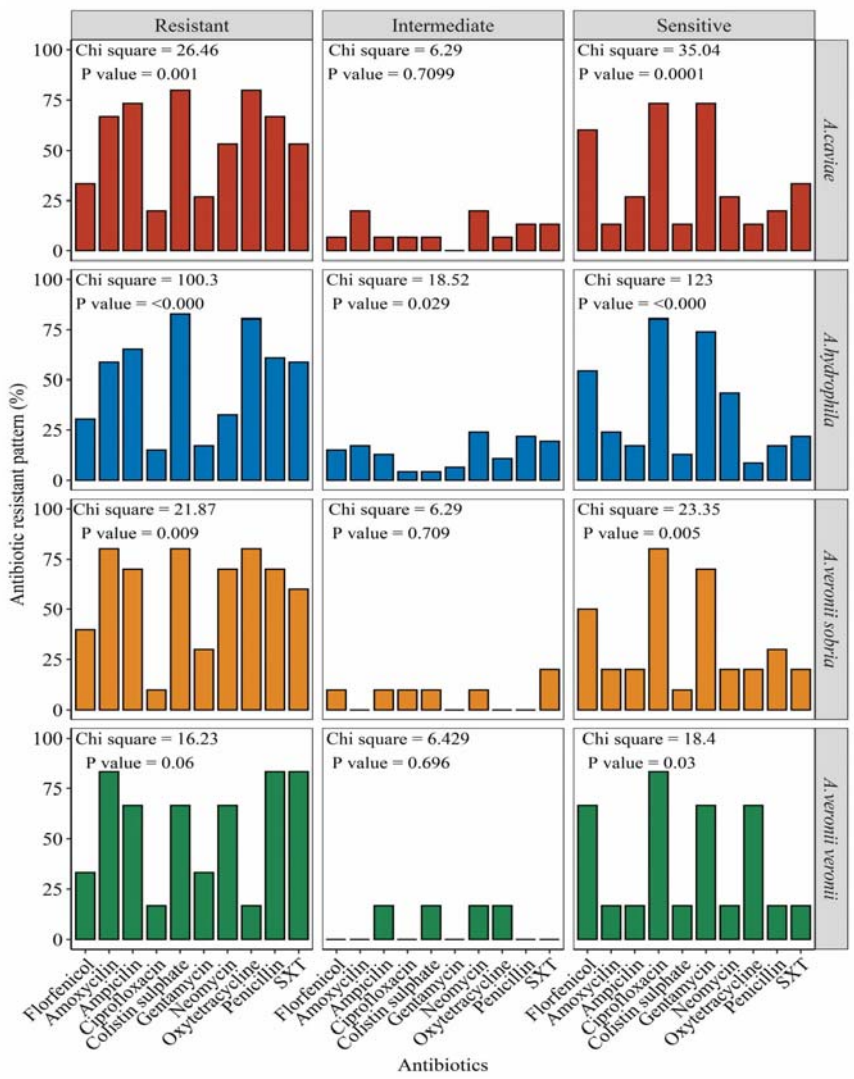

Resistant
Intermediate
Sensitive
A.caviae
A.hydrophila
A.veronii sobria
A.veronii veronii
Chi square = 26.46
P value = 0.001
Chi square = 6.29
P value = 0.7099
Chi square = 35.04
P value = 0.0001
Chi square = 100.3
P value = <0.000
Chi square = 18.52
P value = 0.029
Chi square = 123
P value = <0.000
Chi square = 21.87
P value = 0.009
Chi square = 6.29
P value = 0.709
Chi square = 23.35
P value = 0.005
Chi square = 16.23
P value = 0.06
Chi square = 6.429
P value = 0.696
Chi square = 18.4
P value = 0.03
Antibiotic resistant pattern (%)
0
25
50
75
100
Florfenicol
Amoxycilin
Ampicilin
Ciprofloxacin
Colistin sulphate
Gentamycin
Neomycin
Oxytetracycline
Penicillin
SXT
Antibiotics

Figure 2: Distribution of antibiotics susceptibility patterns of *Aeromonas* species isolates from *Clarias gariepinus* cultured in earthen pond fish farms in Kwara State, Nigeria

SXT: