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Isolation and Identification of Antibiotic Resistant Bacteria among Members of *Enterobacteriaceae* Isolated from Select Fish Farms in Katsina Metropolis, Nigeria

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ABSTRACT

Background: Enterobacteriaceae family is a non-spore-forming, facultative anaerobes that include a wide range of gram-negative bacteria found in the gastrointestinal tracts of humans and warm blooded animals.
Objective: The focus of the study was to isolate and identify antibiotic resistance bacteria among the member of Enterobacteriaceae from fish farms within Katsina metropolis.
Methods*:* Total of 100 samples of *Clarias gariepinus* with an average weight of 78.23g were randomly collected weekly from the fish farms in each of the locations for a period of five weeks. The isolation of the bacteria was carry out through serial dilution method and the isolates identified through gram staining, and biochemical tests, Cefoxitin 30µg), Tetracycline (10µg), Ciprofloxacin (5µg), Erythromycin (15µg), Chloramphenicol (30µg) and Gentamycin (30µg) were used for screening the isolates and Vitek - 2-compact System was uses for identification multi-drug resistance isolates.
Results: Different bacterial species were found to be resistant to one or more antibiotic these include *Klebsiella Pneumoniae, E.coli, and Proteus mirabilis*. A high percentage of resistance to Erythromycin and Chloramphenicol was shown for 96% and 49% respectively of the total isolates.
Conclusion: Conclusively the research highlighted different multi-drug resistance enterobacteriaceae associated with *Clarias gariepinus* in the fish farms within Katsina metropolis, which of great concern to consumers and public health. This raises the growing concern in searching for alternative treatment.
Unique Contribution: The study highlighted some members of enterobacteriaceae family with anti-biotic resistance against some common antibiotic use in treatment of bacterial diseases in fish farm.
Key Recommendation: Molecular identification will reveal specific resistance mechanism, this help to predicts trend in resistance development and offering data that can inform better management practices.

Key word: *Enterobacteriaceae, Clarias gariepinus, Serial dilution, Multi-drug resistance,*

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INTRODUCTION

Freshwater fish farming represents a substantial proportion in the aquaculture industry, particularly in developing countries. They provide food and livelihood, and contribute to economic development (Davis *et al.,* 2009). A great diversity of freshwater aquatic organisms has been cultured, of which, tilapia, carp, and catfish are the major species in global aquaculture production (Davis *et al.,* 2009; Eissa et al., 2009). In Africa, fish constitutes about 17% of animal protein consumed (Allison *et al.,* 2009). Fish has high nutritional values such as low saturated fat and good source of essential fatty acids, the omega-3 fatty acids which cannot be synthesized by the human body. Fish are known to contain low fat and low cholesterol and to be highly digestible making them suitable to the infants, children, and elderly.

Recently, the farming systems of these species have been shifted from simple traditional to intensive culture methods (Davis *et al.,* 2009; Mzula *et al.,* 2020). In the intensive practices, fishes are confined in densely stocked and often high organic load conditions which trigger the development and spread of pathogenic bacteria *(*Heuer et al., 2009; Tavares 2017). The problems and economic losses related to bacterial diseases have, therefore, become increasingly frequent. The growing popularity of fish and fishery products in Nigeria has led to the construction of several fish ponds, with positive impact upon fish farming (Njoku *et al.,* 2015).

Enterobacteriaceae family is non-spore-forming, facultative anaerobes that include a wide range of Gram-negative bacteria found in the gastrointestinal tracts of humans and warm blooded animals (Rawash *et al.,* 2019). Enterobacteriaceae are opportunistic pathogens that cause common food and water-borne bacterial illnesses in fishes (Newaj *et l.,* 2008). Kousar *et al.,* (2020) reported fish ailments including saprolegniasis, lernaeasis, bacterial hemorrhagic septicemia, and anoxia. The prevalence of Enterobacteriaceae in fish and fish ponds has been linked to water-borne infections in humans, including typhoid fever, cholera, food poisoning, gastroenteritis, dysentery, and salmonellosis (Fakorede *et al.,* 2020; Kousar *et al.,* 2020. Aondover *et al.,* 2024)

The surveillance of fish disease is under estimated in West Africa and the risk of dissemination is very high, as countries with limited resources do not have veterinary clinics providing clinical and biological diagnostics to monitor the use of antibiotics in animals (Ouedraogo *et al.,* 2017). In West Africa, antibiotic resistance induces the emergence of resistant enterobacteria (Pitout and Laupland, 2008). Several studies have shown that 90% of bacterial strains in marine environment are resistant to more than one antibiotic and 20% are resistant to at least five antibiotics (Kouadio *et al.,* 2017; Benie *et al.,* 2017; Dib *et al.,* 2018; Adibe-Nwafor, 2023; Onyejelem, & Aondover, 2024).

To prevent and treat bacterial infections in aquaculture, antibiotics are commonly used via medicated feed or direct addition to the culture water (Rico *et al.,* 2013). These administration methods often result in heavy use of antibiotics, broader aquatic areas exposed and a wide range of bacteria impacted to the drugs in comparison to the use of antibiotics in terrestrial animal production. Antibiotics play an essential role in the treatment and prevention of bacterial fish diseases; however, indiscriminate use of these antibiotics in aquaculture have contributed

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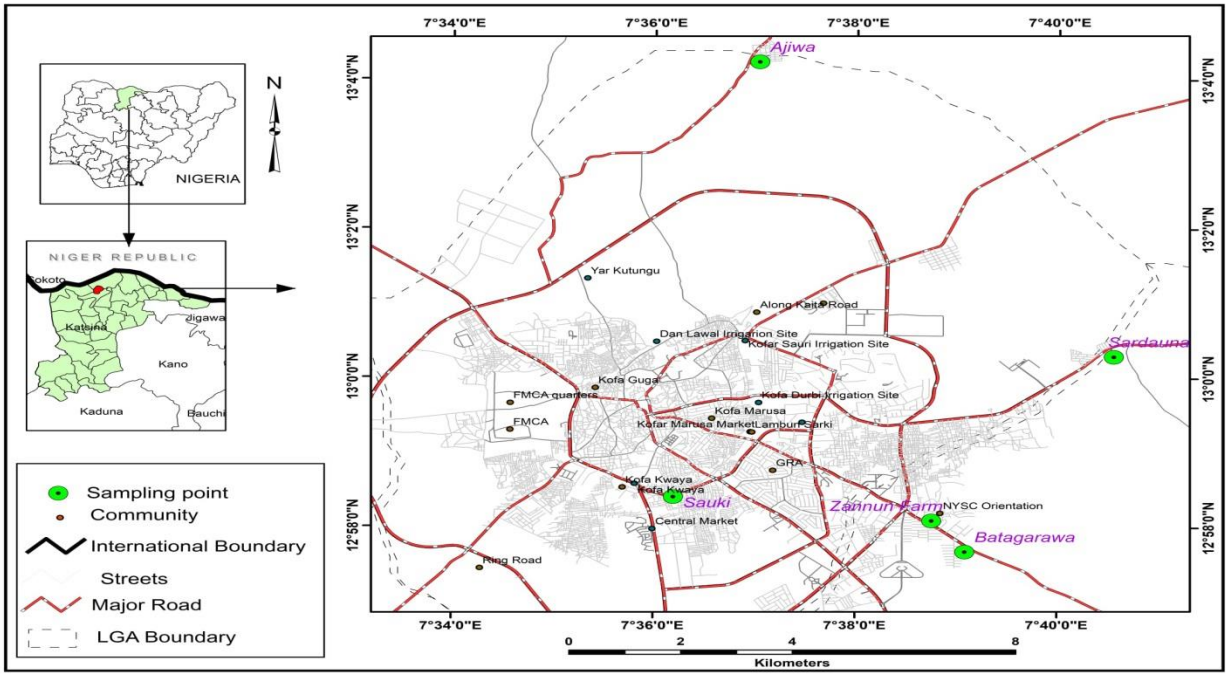
immensely to the surge in bacterial resistance to drugs leading to enhanced capacity of the bacteria virulence features.

Multiple antibiotic resistance (MAR) is a worldwide problem caused by the abuse of antibiotics (Odeyemi *et al.*, 2012; Sharma *et al*., 2015). Moreover, the increased antibiotic resistance confers bacterial pathogens an additional virulent feature, which generates increased mortality of fish in commercial farms (Daood, 2012). This study aim to isolate, and characterize antibiotic resistant gene among member of *Enterobacteriaceae* from fish farms in Katsina state, Nigeria.

MATERIALS AND METHODS

Questionnaire Administration: The questionnaire was developed to gain insights into fish farming practices, the selected farmers, those including the use of antibiotics, types of antibiotics used, history of disease outbreaks and type of feed used at the fish farms. The questionnaire form has been provided in the supplementary materials section.

Study Location: The study was conducted in five selected fish farm in Katsina municipal, Katsina State, Nigeria. Katsina State is situated in the North -west region of Nigeria and shares common borders with Zamfara, Kaduna, Kano and jigawa. The global location of the State is 12°59′20N 07°36′03”E.



A= Zannun
B= Sauki
C= Sardauna
D= Batagarawa
E= Ajiwa

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Figure 3.1**:** Map of Katsina metropolis showing sample locations of selected fish farms within Katsina Metropolis

Sample Collection: Five farms were selected based were choose for this study in Katsina municipal, Katsina central zone and denoted with the alphabets A, B, C, D and E. Twenty (20) samples of *Clarias gariepinus* with an average weight of 78.23 ± 21.2 g were randomly collected weekly from the selected farms in each of the locations for five weeks through the fish farmers' assistance. The total numbers of fish collected per week were twenty (*n*=20), and at the end of the sampling period (May-June), a total of one hundred fish (*n*=100) samples were collected. The fishes were sampled from the ponds using a scoop net as described by Adeshina *et al.* (2021) and immediately placed in a polythene bag and transported in aseptic condition to avoid external contact to department of microbiology, federal university Dutsin-ma for bacteriological analysis as described by Kim *et al*. (2007).

Media Preparation: The culture media used for the bacteriological analysis were prepared according to the manufacturer's instructions (Biomark Ind. and Oxoid UK). MacConkey (MCA), nutrient agar (NA) and Muller Hinton agar were sterilized after preparations by autoclaving at 121^0c for 15 minutes.

Serial Dilution and Isolation of Enterobacteria: For the determination of bacteria, the surface of the skin was disinfected with 70% ethanol, later using sterile swap stick, the intestine of each fish were swapped and serially diluted to reduce bacterial load. Each swap was placed in a sterile test tube containing 10ml of distilled water (Winokur *et al.,* 2000). Thereafter, dilutions were made using tenfold to 10^5 dilution factors. To which, an aliquot of 0.1 ml from 10^2 and 10^3 dilution factors was pipetted onto MacConkey (MCA, using spread plate method and incubated at 37°C for 24hours. Following bacterial growth, colonies were counted after incubation period using colony counter and each colony that appeared on the plate was regarded as one colony forming unit (CFU/ml), The plates with 30-300 colonies were used for the determination of bacterial population, The average was taken, and the number obtained was divided by amount of ml used multiply by dilution factor used, this provided the colony forming unit per ml of a sample in (cfu/ml) (Prakash and Karmagam, 2013). The colonies on each plate were characterized, and then purified by sub culturing on nutrient agar slant and kept at 4°C for subsequent use.

Identification of bacterial isolates

Gram Staining: Concisely, gram staining was carried out to a 24h culture of the isolates, after which a smear of each was made on a grease-free slide and heat-fixed properly. Crystal violet which is the primary stain was added to each slide and allowed to stay for 60 s after which it was washed off using clean running water. Ligous iodine were added as mordent and after 60 s washed off. The slides were decolorized using 95% ethanol to remove excess stains and rinse with water. The slides were counterstained using a secondary stain called Safranin and rinsed with clean running water. The slides were air-dried and observed under a microscope (X100 objective, oil immersion) for Gram reaction, morphological characteristics and cellular arrangements as described by Nishant (2023).

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Catalase Test: Catalase test was carried out using hydrogen peroxide (H_2O_2), 24 hour colony was picked using inoculation loop and placed on a clean glass slide, and then hydrogen peroxide was dropped on the glass slide containing colony and then observed for bubbling of gas (Sapkota, 2020).

Oxidase Test: An oxidase test was performed were filter paper soak in tetramethyl*p*-phenylenediamine-dihydrochloride substrate. Sterile distilled water was apply to moisten the paper, and the colony was picked using a sterile platinum loop and observed for any color change from deep blue or purple between 10 and 30 s (Shield and Cathcart, 2010; Nwafor, 2023).

Sugar Fermentation Test: For the fermentation of sugar, an inoculation needle was used to touch the top of each of the isolated colonies; the triple sugar iron agar (TSI) was inoculated at 37 °C in ambient air for 24 h, and the reaction was observed for color change (Cappuccino and Sherman, 2008).

Indole Test: For the Indole test, the inoculum was placed in a sterile test tube with prepared 4 mL tryptophan broth and incubated for 24 h, followed by introducing 0.5 mL of Kovac's reagent in the culture broth and observed color change (MacFaddin, 2000).

Antibiotic Susceptibility Testing and Selection of MDR isolates: Kirby Bauer disk diffusion method was used; pure bacterial isolates were subjected to antimicrobial susceptibility testing (AST). Cefoxitin (30µg), tetracycline (10µg), ciprofloxacin (5µg), erythromycin (15µg), chloramphenicol (30µg), and gentamycin (30µg) were the antibiotic disks (Bioanalyse TUR.) used for the isolates AST. Every isolate was streaked for 24 hours on brand-new, sterile Mueller Hinson agar plates. Each antibiotic disc was then added, and the plates were then incubated for 18 to 24 hours at 37 °C. Zones of inhibition were then measured using a meter rule and expressed in millimeters. The Clinical and Laboratory Standards Institute breakpoints methods (CLSI, 2020) were utilized to interpret the diameters of inhibition. Multi-drug resistant (MDR) bacteria were identified and chosen from isolates that exhibited resistance to three or more distinct classes of antibiotics.

Identification and Characterization of Selected Multi-drug Resistant Bacteria (MDR) Using Vitek - 2-compact System: Vitek 2 compact system was used to characterized MDR selected isolates after AST,–Using an inoculation loop, each overnight-grown selected MDR bacterial isolate was suspended into a polystyrene test tube filled with 3 milliliters of normal saline to reach 0.5 to 0.60 McFarland standard using a DensiCHEK Plus instrument (Biomerieux, USA). During the characterization, an identification card for gram-negative bacteria was used. After that, the gram-negative bacteria identification card tube was inserted into the polystyrene test tube that had three milliliters of ready-made bacterial suspensions on the cassette. The test tube was, thereafter, inserted into the Vitek machine, where the card wells were automatically filled. The filled and sealed cards were inserted into the Vitek 2 reader-incubation chamber and incubated at 37^0C for 18-24hrs. After the incubation period the identities of the isolates were read from the software on the monitor and printed (Biomerieux, USA).

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RESULTS

Mean bacterial colony count: Mean bacterial count for each selected plate from all the five 5 selected fish farm was shown in Table 1. The farm E had the highest (2.06 x 10^5 cfu/ml) mean bacterial count, followed by farm C (1.63x10^5cfu/ml), while farm A had the lowest (9.6 x 10^4cfu/ml) mean bacterial count.

Table .1 Mean bacterial counts (cfu/ml) of *clarius grapienus* (cat fish) from five fish farm within Katsina state.

Sample code	**Number of colonies** (cfu/ml)
A	9.6 X 10^4
B	1.27 X 10^5
C	1.63 X 10^5
D	1.4 X 10^5
E	2.06 X 10^5

Keys:
A: Zannun
B: Sauki
C: Sardauna
D: Batagarawa
E: Ajiwa

Antibiotics resistant phenotypes of the bacterial isolates from *Clarius grapienus* collected from five fish farms in Katsina state, Nigeria

Antibiotics resistant phenotypes of all the tested bacterial isolates were shown on Table 2. The AST revealed significant variability in resistance patterns among the bacterial isolates. Among the bacteria isolates, highest (95.9%) antibiotic resistance was observed to erythromycin, followed chloramphenicol (48.9%) while the least (2%) was to gentamycin.

Table 2. Antibiotics resistant phenotypes of the bacterial isolates from *Clarius grapienus* collected from five fish farms in Katsina state, Nigeria

Antibiotics (con. in µg)	**Resistance No (%)**	**Intermediate**	**Susceptible**
No (%)			
TE (10)	7(14.3)	11(22.4)	31(63.3)
FOX (30)	18(36.7)	9(18.4)	22(44.9)
CIP (5)	4(8.2)	8(16.3)	37(75.5)
E (15)	47(95.9)	2(4)	0(0)
CN (30)	1(2)	1(2)	47(95.9)
C (30)	24(48.9)	3(6.1)	22(44.9)

CODE: FOX: Cefoxitin, CN: Gentamycin, E: Erythromycin, CIP: Ciprofloxacin, C: Chloramphenicol: TE: Tetracycline.

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Multidrug resistant patterns among bacteria isolates from *Clarius grapienus* collected from five fish farms in Katsina, Katsina State, Nigeria

Multidrug resistant patterns of bacteria isolates from this study were shown on Table 3. The results showed that seven (14.3%) isolates were multidrug resistant (MDR) bacteria among the total (49) isolates from this study. All the MDR bacteria showed resistance to cefoxitin while the most common resistant phenotypes are resistance to Cefoxitin, Chloramphenicol, Erythromycin, and Tetracycline and to Cefoxitin, Ciprofloxacin, Chloramphenicol Erythromycin.

Table 3: Multidrug resistant patterns among bacteria isolates from *Clarius grapienus* collected from five fish farms in Katsina, Katsina State, Nigeria

No. of Antibiotics	Resistance phenotype	No of isolate
3	FOX, C, E	1
4	FOX, C, E, TE	2
4	FOX, CIP, C, E	2
3	FOX, E, CN	1
4	FOX, CIP, C, TE	1

CODE: FOX: Cefoxitin, CN: Gentamycin, E: Erythromycin, CIP: Ciprofloxacin, C: Chloramphenicol: TE: Tetracycline.

Bacterial isolates in *Clarius grapienus* from selected fish farms within Katsina metropolis as identified by Vitek 2 compact system and their percentage identification

Identified multidrug resistant isolates using Vitek 2 compact system from this study were listed on Table 4. The table showed the MDR bacterial isolates that were identified using Vitek 2 compact system and their percentage identification. The isolates with the highest frequencies of appearance are *Proteus mirabilis* (37; 57.14%) and *Spingomonas paucimobilis* (2/7; 28.57). Whereas *Escherichia coli* and *Klebsiella Pneumoniae ozaenae* appears only once (1/7; 14.28).

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Table **4. Bacterial isolates from fish farms with Katsina metropolis that were identified using Vitek 2 compact system and their percentage identification**

Sample Locations	Bacterial specie identified	%identity	Resistant phenotypes
A	*Klebsiella Pneumoniae ozaenae*	91%	FOX, E, CN
A	*Escherichia coli*	99%	FOX, E, C, TE
A	*Proteus mirabilis*	98%	FOX, E, C, TE
B	*Spingomonas paucimobilis*	97%	FOX, E, CIP, C
D	*Proteus mirabilis*	95%	FOX, E, CIP,C
C	*Proteus mirabilis*	99%	FOX, E, C
E	*Spingomonas paucimobilis*	98%	FOX, E, C, TE

DISCUSSION

Antibiotic resistance has been shown to be significantly exacerbated by the widespread use of antibiotics in fish farming, both for therapeutic and prophylactic purposes (Watts *et al.*, 2017). Moreover, information gathered from farm managers in this study via the interviews it is indicated that antibiotics were used for either therapeutic or preventive purposes in majority of the sampled farm. This may have also resulted to the isolation of MDR bacteria from this study as suggested by Watts *et al.*, (2017).

Ajiwa farm had the highest mean bacterial count (2.06 x 10^5 cfu/ml) (Table 1). Compared to Zannun farm, the mean bacterial count in Zannun farm is 9.6 x 10^4cfu/ml, which is still a high. Numerous reasons, that include, the type of fish-holding facility, the source of water utilized for the fish's culture, and inadequate sanitary practices on the premises, could be responsible for the high bacterial count of samples from Ajiwa farm. However, the comparatively lower amount of bacterial load seen in other farms may indicate that farm managements have implemented hygienic waste disposal techniques and other stringent sanitation measures (Boyd and Massaut, 1999). The findings also aligned with the research conducted by Cynthia *et al.* (2022) on the identification of molecular drivers of antibiotic resistance in some fish farms. The authors found that certain farms had a significantly higher bacterial load than others. This authors reported could be caused by a variety of reasons, such as the type of fish-holding facility, the supply of water utilized for fish culture, and inadequate hygienic practices on the farm.

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All farm managers, with the exception of farm B (Sauki Farm), acknowledged the use of antibiotics. All microorganisms isolated from these farms exhibited varying degrees of antibiotic resistance. This may be as a result of previous exposure of these bacteria to similar antibiotics. The use of antibiotics like tetracycline, cefoxitin and chloramphenicol in fish feed was the reason for the development of resistant bacteria, according to a study carried out at certain fish farms in Ghana's Ashanti area (Agoba *et al.,* 2017). Since antibiotics are widely available over-the-counter in Nigeria, misuse and inappropriate disposal of these drugs are frequent (Donkor *et al.,* 2012). According to some research, there are varied amounts of antibiotic residues in waste water and landfills (Azanu *et al.,* 2018), which has been connected to the potential introduction of antibiotics into aquatic habitats. This may also be the reason for the isolation of antibiotics resistant bacteria from fish samples from these farms, residue of antibiotics from nearby farmlands may have been washed into the fish ponds. These antibiotic-resistant bacteria may have emerged in the environment due to the country's widespread use of these drugs.

In line with the findings of other authors (Badger-Emeka *et al.,* 2018; Sanders *et al.,* 2001; Michalik, 2017; Spanu *et al.,* 2006), VITEK 2 identified the Gram-negative isolates in our investigation. Table 4. lists the seven (7) Gram-negative isolates that were found. Based on the identification percentage identity of between 91 and 99% were observed among the isolates. The fish's strong host defense response likely prevented the bacterial species reported in this study from killing the fish, but the species are both pathogenic and opportunistic, meaning they may contribute to fish disease (Efuntoye *et al.,* 2012). This result is in line with a study conducted in 2022 by Nmema *et al.* on identification; antimicrobial susceptibility screening and ESBL status of gram-negative bacteria from healthy fish by vitek-2 automated system and found *E. coli, proteus, Klebsiella* and *salmonella* to be majority of Enterobacteriaceae to be identified.

Furthermore, it is possible that these organisms contribute to the spread of diseases to humans. Human food-borne bacterial illnesses have been linked to fish and fish products (Novotyn *et al.,* 2004, Hastein *et al.,* 2006, Efuntoye *et al.,* 2012). According to (Emikpe *et al.,* 2011), the infections may have entered the ponds through handling by human healthy carriers or during the production process. The fact that the isolates came from fish that seemed to be in good health suggests that the bacteria did not pose a significant risk to the fish in the environmental investigations. These microorganisms do, however, have implications for public health. In Ago – Iwoye, Ibadan, Oyo State, Nigeria, some of the bacteria species obtained in this study were also identified from healthy *Clarias gariepinus* (Efuntoye *et al.,* 2012). Furthermore, some researchers have documented the presence of harmful bacteria in freshwater aquaculture environments in *Clarias gariepinus* (Ajayi, 2012; Emikpe *et al.,* 2011; Udeze *et al.,* 2012 and Adedeji *et al.,* 2012). Because these bacteria have been linked to a variety of illnesses, their presence in fish puts fish consumers at risk. Urinary tract infections have been linked to strains of *Escherichia coli* and *Proteus mirabilis* (Adesoji *et al.,* 2017).

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CONCLUSION

This study highlights the significant prevalence of antibiotic-resistant bacteria (ARB) in fish farms in Katsina State, Nigeria. The significant number of total bacterial populations and the discovery of multiple antibiotic-resistant strains, particularly within the Enterobacteriaceae family, raise serious concerns regarding aquaculture operations and the uncontrolled use of antibiotics. Therefore, the presence of these antibiotic-resistant bacteria in all of the fish farms that were collected for this study—particularly Zannun farm—showed that these farms could serve as a reservoir for consumers to contract these antibiotic-resistant bacteria, which is a serious public health problem. Thus, our study highlights how important it is to closely monitor and regulate the use of antibiotics in aquaculture in Katsina State.

RECOMMENDATIONS

1. Molecular identification will reveal specific resistance mechanism, this helps to predict trends in resistance development and offering data that can inform better management practices.
2. Searching for alternative treatment such as bacteriophage therapy will be a crucial in combating anti-biotic resistance.
3. Good sanitation practice and understanding the environmental parameter that favor the growth and development of resistance will guide in decelerating the resistance mechanism

Ethical clearance

Ethical consent was sought and obtained from the participants used in this study. They were made to understand that the exercise was purely for academic purposes, and their participation was voluntary.

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The study was not funded.

Conflict of Interest

The authors declare no conflict of interest

Authors' Contributions.

Abdurrahman Y. planned this study. Abdurrahman Y. performed the experiment under the guidance of Ayodele T. Adesoji and Ado Aminu. Abdurrahman Y. and Naziru Abdullahi wrote the initial manuscript, Muhammad A. Said and Mustapha Sani Yauri proof read the paper, Abba Amiru Musa assisted in collecting and interpreting of data. All authors read and approved the final paper.

Availability of data and materials.

The datasets on which conclusions were made for this study are available on reasonable request.

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International Journal of Sub-Saharan African Research (IJSSAR)
Vol. 2, Issue 4, pp. 1-13, December 2024, ISSN: 3043-4467 (Online), 3043-4459 (Print)
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