SVU-IJVS-7(4): 50 - 60 Print ISSN: 2535-1826 Online ISSN: 2535-1877

Research Article Open Access

ISOLATION, PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF MULTI-DRUG RESISTANT ENTEROBACTER CLOACAE FROM DISEASED AFRICAN CATFISH IN LAGOS STATE

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https://doi.org/10.21608/svu.2024.328893.1348

Submit Date: 2024-10-27 Revise Date: 2024-11-21 Accept Date: 2024-11-23

ABSTRACT

Disease outbreaks cause enormous financial losses and pose significant challenges to the aquaculture industry globally. This may lead to increased mortality rates, slower growth and productivity, greater production costs, and fewer revenue streams. The purpose of this study was to ascertain the prevalence and pattern of antibiotic susceptibility of Enterobacter cloacae isolated from diseased African catfish (Clarias gariepinus) in Lagos State, Nigeria. Aquaculture farms in three local government districts in Lagos State were the subject of this cross-sectional study. In Lagos State, 13 commercial farms located in three local governments (Surulere, Ikorodu, and Alimosho) provided a purposeful sample of 120 diseased African catfish (Clarias gariepinus). The 16S rRNA gene characterization, biochemical testing, and morphological traits were used to identify the isolates. Using the agar disc diffusion method, the antimicrobial susceptibility pattern of Enterobacter cloacae was investigated. In infected Clarias gariepinus from Lagos State, the incidence of Enterobacter cloacae isolates was 60% (12/20) in Surulere, 76.7% (46/60) in Ikorodu, and 65% (26/40) in Alimosho. When comparing Ikorodu to other local governments, a noticeably greater prevalence was noted. Malaysian, Vietnamese, and Pakistani isolates of Enterobacter cloacae were closely related to the strains of OP595768.1 and OP595746.1 that were isolated. With a MAR value of 0.72, the isolates of Enterobacter cloacae were multidrug resistant. A study conducted in Lagos State revealed that Enterobacter cloacae, which was isolated from infected Clarias gariepinus, had evolutionary ancestry with harmful NCBI reference strains from various nations.

Keywords: 16S rRNA, gene, characterization, MAR, prevalence

INTRODUCTION

Aquaculture is fish growing in a confined and fairly shallow water body where all its life processes may be observed or regulated. It contributes directly to the nation's food security: by providing fish for consumption, employment creation, conserving resources, and generating foreign exchange through the export of fish and fish products. The world's fastest-growing food production industry is aquaculture (Edwards et al., 2019). As a profitable area of the nation's agriculture, aquaculture in Nigeria is only becoming more and more popular due to the growing need for fish, one of the main protein sources in the country (Adewumi, 2015). Due to the species' adaptability to various temperatures, low oxygen and salinity levels, and other environmental conditions, African catfish (Clarias gariepinus) farming has become a well-known agricultural industry (Anifowose et al., 2022). The world's largest producer of African catfish, Nigeria produces 2.6 billion USD worth of fish annually, which provides a livelihood for millions of people (Dauda et al., 2018). According to Remilekun et al., (2021) report, disease outbreaks lead to significant financial losses, which pose a significant challenge to the global aquaculture industry. The increased mortality leads to decreased income, revenue generation, joblessness, a decline in growth and productivity, and an increase in production expenses (Kaleem and Sabi, 2021).

In fish farming, high-rearing densities currently in use facilitate the infection and dissemination of pathogenic bacteria. Aquatic farmed species are subjected to extreme pressure from industrial agriculture practices, which weakens the ability of their natural immune systems to fight off bacterial infections that cause disease. Fish gastrointestinal tracts are typical locations for gram-negative, facultative aerobics-positive bacteria like Enterobacter cloacae, an enteric bacterium that is a member of the Enterobacteriaceae family. These bacteria are also positive for catalase and non-glucose fermenters (Davin-Regli and Pagès, 2015). Strong adhesion and invasion abilities of Enterobacter cloacae aid the capacity of the pathogen to infect the host.

A lack of prompt detection and treatment may lead to the mortality of numerous aquatic animals on farms (Gao et al., 2019). Several reports on fish infection-caused Enterobacter species have been reported, including Tilapia (Abdel-LatiF and Sedeek, 2017), Etroplus maculatus (Nair et al., 2021), Murgul cephalus (Thillai-Sekar et al., 2008), Claritas gariepinus, and Oreochromis niloticus (Matter et al., 2018). According to the study on Macrobrachium rosenbergii, Enterobacter cloacae has evolved a way of survival to flourish in unfavorable aquatic environments, including pH fluctuations, oxidative stress, a lack of nutrients, and so forth. As a result, by modifying key gene expressions regulated by sigma

factors, E. cloacae has managed to survive in these hostile aquatic environments (Gao et al., 2021). Hence, the present study is aimed at determining the incidence and antimicrobial susceptibility of Enterobacter cloacae from clinically diseased African catfish in Lagos.

MATERIAL AND METHODS

Study Area

Latitudes and longitudes of Lagos State are (6o22'N) and (20o42'E and 3o22'E). The state is surrounded by the Atlantic Ocean to the south, the Republic of Benin to the west and Ogun State to the northeast. Water bodies consist of 30% land area in Lagos State. The availability of water surrounding the state is used for aquatic and fishing sports. Annual rainfall is between 1.312 to 1.726 millimeters, meanwhile, two seasonal rainstorms occur between April and November.

Samples Collection

The sample collection method was a cross-sectional analysis that involved three local governments in the state. The samples were collected between January 2022 and September 2022. Diseased African catfish (n=120) was sampled from 13 commercial farms (Table 1). The samples were transported using an ice pack to the Aquatic Animal and Wildlife Medicine Laboratory located at the University of Ibadan

Isolation and Phenotypic Identification of Bacterial Isolates

The organs such liver, skin, and kidney were collected from diseased fish showing clinical signs including reddish lesions near the pectoral and ventral fin areas. The diseased fish were excised and swabbed using sterile cotton swabs and inoculated in 10 ml of buffered peptone water. The inoculum was cultured on MacConkey and nutrient agar (HiMEDIA®, USA), and incubated for 24 hours at 30°C. Lactose fermenter isolates were sub-cultured on Blood and Eosin Methylene Blue agar. Enterobacter cloacae isolates were characterised according to sugar fermentation, and various biochemical and morphological tests. The tests include gram staining, indole, Simons' citrate agar, catalase, oxidase, urease, triple sugar iron, Voges Proskauer, and hydrogen sulfide production (Anifowose *et al*., 2024)

Molecular Characterization of Bacterial Isolates

Table 1: Sample collection from different local government areas in the selected States

Indices/LGA	Surulere	Ikorodu	Alim
Number of Farms	3	6	4
Number of Samples	20	60	40

LGA – Local government area

An overnight pure culture of E. Cloacae was used, and the isolates were pelletised at 6000 rpm for 10 minutes. DNA extraction buffer (DEB) of about 1 ml which contained proteinase K of 0.05 mg/ml was added to the pelletised isolates. The mixture was liquified in a sterile mortar and kept in an Eppendorf tube of 1.5 ml. One hundred microlitters of 7.5 M potassium acetate and fifty microlitters of 20% Sodium Dodecyl Sulphate (SDS) were mixed with each isolate. Thereafter, the mixture was incubated for 30 minutes at 65°C, centrifugation took 10 minutes at 13,000 rpm, and the supernatant was streamed into autoclaved tubes.

The mixture was incubated at -20°C for 60 minutes and centrifugation took 10 minutes at 13,000 rpm. Decantation was carried out using three hundred microlitters of 70% ethanol to wash the supernatant. The pellets were centrifuged for 5 minutes at 13,000 rpm, incubated at 37°C for 15 minutes, and ethanol was extracted. The pellets were immersed in sterile distilled water of 30 µl and retained stock solution at 4°C. The forward and reverse primer used for 16S rRNA gene amplification AGAGTRTGATCMTYGCTWAC-3' and the reverse primer 5'-CGYTAMCTTWTTACGRCT-3'.

The PCR reaction combination (iTRON, Korea), PCR, and PCR results were assessed using Gel electrophoresis based on Anifowose *et al*., (2024). The removal of ethanol purification was carried out and amplified gene segments were sequenced from purified products in both directions.

Bioinformatic analysis

A distinct NCBI GenBank database for 16 rRNA gene sequences was employed using BLAST for analysis and identification of associated bacterial 16S rRNA gene sequences (Johnson *et al*., 2008). An accession number was generated from the NCBI database for each E. cloacae isolate. Ten good quality 16S rRNA gene sequences were sourced from the database of the NCBI GenBank. The Bioedit software with the interface of the Clustal W algorithm was used for multiple alignment of the sequences and a phylogenetic tree was created using software known as MEGA 11 (Larkin *et al*., 2007; Anifowose *et al*., 2024).

Antibiotic Susceptibility Testing

The antibiotic susceptibility was conducted using 12 isolates of *E. cloacae*. The agar-disc diffusion method was carried out and CLSI standards Mo2 was carefully followed (CLSI, 2020).

The resistance and sensitivity of *E.cloacae* isolates to antibiotics were carried using 12 antibiotics belonging to 8 different classes: fluoroquinolone (ciprofloxacin 5 µg (CIP), Enrofloxacin 5 µg (ENR)), polymyxin (Colistin sulphate 10 µg (CS)), aminoglycoside (streptomycin 10 µg (S), gentamicin 10 µg (G)), chloramphenicol 30 µg (CH), metronidazole 5 µg (M), tetracycline (doxycycline 30 µg (D),

tetracycline 30 µg (T), cephalosporin (third-generation) ceftriaxone 30 µg (CEF), and penicillin (ampicillin 10 µg (AP), ampicillin cloxacillin 20 µg (AM)).

Preparation of Antibiotic Disk

Each antibiotic was infused into sterile Whatman filter paper disks based on the CLSI Mo2 disk preparation guide. A maximum of six 6-mm disks were placed on an isolate-streaked sterile Mueller-Hinton agar plate (CLSI, 2020).

Preparation of Inoculums

The suspicion of *E. cloacae* was made from a pure overnight culture of the isolates on Tryptic Soy agar (HiMedia®). The density was adjusted to McFarland 0.5 turbidity standard using normal saline. Then, the suspended *E. cloacae* colonies were streaked over Muller-Hinton agar (HiMedia®). The *Staphylococcus aureus* strain was used for quality control (CLSI, 2020).

Inoculation of agar plates

The Antibiotic disks were picked with sterile forceps and placed on the surface of the Mueller-Hinton agar plate. The disks made contact with the medium. The plates were incubated at 37^0C for 18 hours. The zone of inhibition was measured with a ruler in millimeters. The *E. cloacae* were interpreted as resistant or sensitive to the antibiotics based on zone of inhibitions measurement using the CLSI guide (CLSI, 2020).

ACCESSION NUMBERS

The sequence accession numbers: OP595768.1 (*Enterobacter Cloacae* LGNG1) and OP595746.1 (*Enterobacter Cloacae* LGNG2)

Statistical analysis

The incidence of *Enterobacter cloacae* isolated from diseased *Clarias gariepinus* was presented in percentage. The level of statistical significance was assessed at $p < 0.05$ using the analysis of variance (ANOVA).

Table 2: Incidence of Enterobacter cloacae isolated from diseased Clarias gariepinus in Lagos

Local Government Area	No. of isolates	No. of Samples	Incidence (%)
Surulere	12	20	60
Ikorodu	46	60	76.7
Alimosho	26	40	65
	84	120	

RESULTS

The incidence of E. cloacae isolates in diseased African catfish from Lagos State was 60% in Surulere, 65% in Alimosho, and 76.7% in Ikorodu (Table 2).

Table 3: Enterobacter cloacae isolates Biochemical Tests

S/N	Biochemical Tests	*Enterobacter cloacae*
1	Citrate	+ve
2	Motility	+ve
3	Oxidase	-ve
4	Catalase	+ve
5	Gram staining	gram-negative short rods
6	Indole	-ve
7	MacConkey	pinkish and mucoid colonies
8	Voges proskauer	+ve
9	Urea hydrolysis	-ve
10	Hydrogen sulphide production	-ve
11	OF (Oxidative-Fermentative)	facultative anaerobes
12	TSI agar	alkaline slant, acidic butt, and presence of gas
	Sugar Fermentation Test	
1	Arabinose	+ve
2	Sucrose	+ve
3	Xylose	+ve
4	Mannitol	+ve
5	Lactose	-ve
6	Maltose	+ve
7	Glucose	+ve

Positive = +ve, Negative = -ve

All *E. cloacae* isolates were lactose-fermenter, gram-negative, and short-rod under the microscope. The isolates showed negative reactions to indole, hydrogen sulfide, methyl-red, and oxidase. Meanwhile, positive reactions to catalase, xylose, arabinose, mannitol, glucose, citric acid, and Voges-Proskauer. On MacConkey agar, E. cloacae isolates showed pinkish colonies (Table 3).

The phylogenetic tree showed the evolutionary relationship of the 16S rRNA gene of *E. cloacae* observed in this study (OP595768.1 *Enterobacter Cloacae* LGNG1, and OP595746.1 *Enterobacter Cloacae* LGNG2) with strains from Indonesia, Vietnam, Malaysia, India, China, Columbia and Botswana (Figure 1).

According to the antimicrobial susceptibility test results, E. cloacae isolates were completely resistant to tetracycline, ampicillin-cloxacillin, metronidazole, streptomycin, and chloramphenicol. The isolates of E. cloacae showed resistance to gentamycin, doxycycline, ampicillin, and colistin sulfate. Meanwhile, the isolates of E. cloacae were susceptible to ciprofloxacin, ceftriaxone, and enrofloxacin (Figure 2).

DISCUSSION

The results of this study indicated circulation of multi-drug resistant Enterobacter cloacae within farmed diseased Clarias

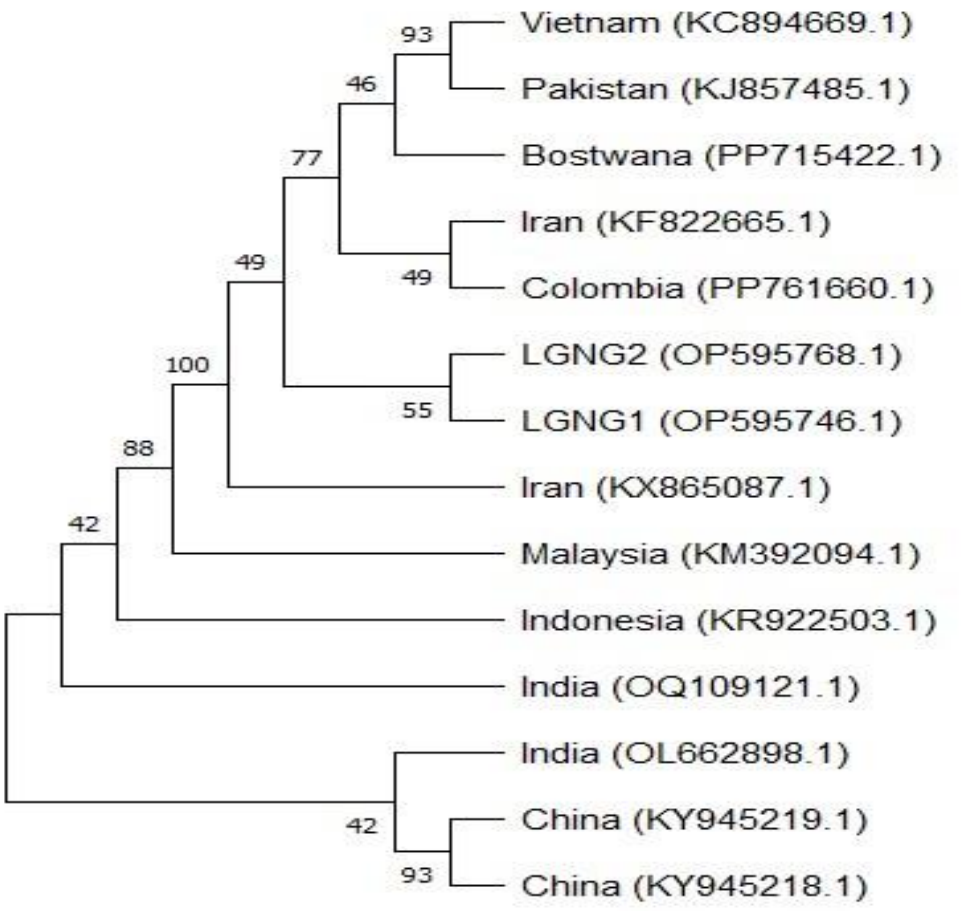

Figure 1: *Phylogenetic tree of 16S rRNA gene of* Enterobacter cloacae *isolated from diseased Clarias gariepinus in Lagos*

gariepinus in Lagos. The strain of Enterobacter cloacae found is keenly associated with reported infectious strains from different nations. The industry's progress depends on a continuous evaluation of the pathogens that are circulating in aquaculture farms. This is because it may aid early disease identification and give current data on the changing capacity of the pathogen community. Currently, African catfish is a popular fish farmed throughout Nigeria, making a significant contribution to the volume of animal protein. The pathogen isolation frequency in this investigation was comparable, albeit higher, to the 11.8% and 49.5% findings in the Tilapia by Matya (Matya, 2016), and Abdel-Latif and Sedeek, (2017) in the Eastern Mediterranean fish. The lower report by previous studies on isolation and

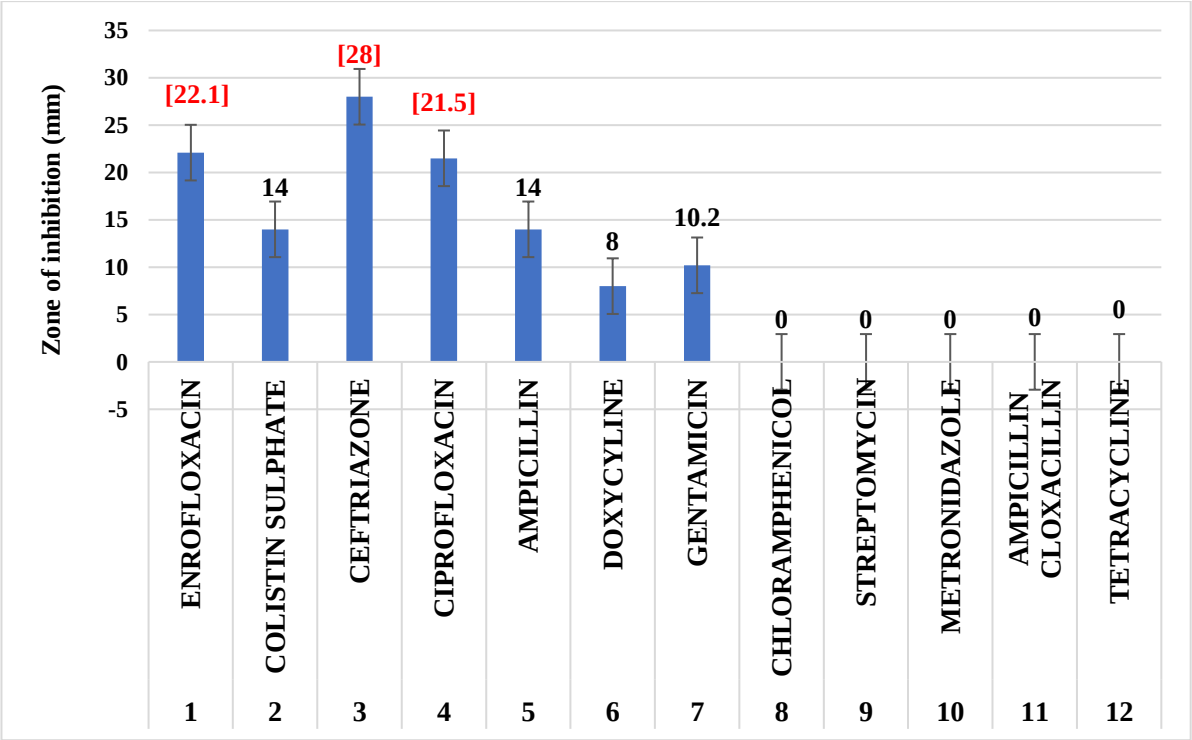

Figure 2: *Antimicrobial susceptibility test of Enterobacter cloacae isolates with the mean zone of inhibitions. MAR Index = 0.72*

characterisation may be due to the result of environmental conditions, host resistance variations, and fish culture process variations.

The isolates of Enterobacter cloacae observed in this study were tested negative for methyl red and indole, but positive for motility and the simmon citrate test. The urea hydrolysis test yielded negative results for the isolates. Furthermore, as Table 3 shows, the Enterobacter cloacae isolates were not able to digest lactose. Similar biochemical characteristics findings have been reported by Abdel-Latif and Sedeek, (2017) in Tilapia fish, moribund fish, Etroplus maculatus (Nair et al., 2021), Mugil cephalus (Thillai-Sekar et al., 2008), and freshwater fish (Matter et al., 2018).

As a standardized method, the 16S rRNA gene was employed in this investigation to identify and characterize Enterobacter cloacae from diseased Clarias gariepinus in Lagos State, Nigeria. Characterisation of E. cloacae isolated from diseased fish such as Oreochromis niloticus, Claris gariepinus, and Mugil cephalus by Matter et al., (2018) and in rainbow trout by Duman et al., (2019) using 16S rRNA gene was earlier reported. Gene marker employed in this study was due to its useful consistency and the existence of preserved and varying sequence areas that are developing at distinct rates (Clarridge, 2004). The investigation of the phylogenetic tree using the highest probability method revealed that the strain closest to E. cloacae found in this study was earlier reported in Malaysia. Additionally, the phylogenetic tree

of the E. cloacae isolates highlighted an evolutionary association with reported global strains from different nations; Vietnam, Colombia, Botswana, Indonesia, China, and India. Traveling along the commercial route may be responsible for the spread of the bacteria isolates between nations.

Bacteria sourced from different habitats and conditions may be important antibiotic-resistance reservoirs (Matya, 2016). The global report of rampant disorder caused by antibiotic resistance was earlier reported. Previous report of E. cloacae resistance to major antibiotics such as beta-lactamases, aminoglycosides, erythromycin, and chloramphenicol was earlier reported (Preena et al., 2021). The Nigerian aquaculture industry has witnessed widespread usage of antibiotics as disease-preventing and growth-promoter agents without proper application and notification of withdrawal period (Anifowose et al., 2024).

This may cause resistance of E. cloacae to multiple antibiotics observed in this study. The high Multiple Antibiotic Resistance (MAR) Index of 0.72 observed in this study is an indication of the bacterial isolates to many antibiotics on the fish farms. This is the first study regarding the genetic characterisation and antibiotic resistance of Enterobacter cloacae isolated from moribund African catfish in Lagos State, Nigeria.

CONCLUSION

This study highlights the importance of isolation, phenotypic, and genotypic characterization of *Enterobacter cloacae* strain LGNG1 from diseased *Clarias gariepinus* in Lagos State. *Enterobacter cloacae* isolates observed in this study were multi-drug resistant and showed evolutionary association with reported global strains from different nations; Vietnam, Colombia, Botswana, Indonesia, China, and India. There is a need for isolation and characterization of antibiotic resistance and virulence.

antibiotic resistance and virulence.

DECLARATIONS

Funding Support

There was no financial support from any organization or individual concerning this research work

Competing interest

There are no competing interests to declare concerning this research work

Availability of Material and Data

The data for this research work is available on request

Consent for Publication

This does not apply to this research work

Contribution of Authors

Anifowose and Ajiboye contributed to the study concepts. All authors contributed to the

data generation and fieldwork. Anifowose made the data analysis and a draft manuscript. Ajiboye and Olakojo edited the manuscript. the authors approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was received from the University of Ibadan, Animal Care and Use Research Ethics Committee (UI-ACUREC) with the assigned number UI-ACUREC/056-0622/10

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