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Phenotypic characterization of bacteria isolated from dried crayfish (*Procambarus clarkii*) and fresh catfish *Clarias gariepinus*) in Nsukka, Nigeria

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Abstract
Microorganisms in any ecological niche exhibit some characteristics that contribute to their survival in the ecosystem. A good example of such survival strategies is the production of antibiotic hydrolyzing enzymes such as ESBL and MBL enzymes in Gram negative bacteria. The study was conducted to isolate, and characterize by phenotypic methods, bacterial isolates from dried crayfish and fresh catfish sold in Nsukka, Nigeria. Isolation, characterization and antibiotic sensitivity tests were done by using standard methods. Out of the 30 randomly sampled dried crayfish in Brain-Heart infusion enrichment medium, a total of 32 bacterial isolates cultured comprised of 10 (31.3%) *Klebsiella pneumonia*, 5 (15.6%) *Pseudomonas aeruginosa*, 5 (15.6%) *Serratia marcescens*, 2 (6.7%) *Enterobacter* species, 7 (21.8%) *Staphylococcus aureus* and 3 (9.4%) *Staphylococcus epidermidis.* Similarly. 30 swab samples collected from catfish yielded a total of 21 bacteria comprising 5 (23.8%) *Klebsiella pneumonia*, 5 (23.8%) *Enterobacter* species, 3 (14.3%) *Staphylococcus aureus*, 3 (14.3%) *Staphylococcus epidermidis*, and 5 (23.8%) *Escherichia coli.* All the Gram negative bacteria studied were resistant to amoxicillin (R=100%), while resistance patterns varied across other antibiotics. Generally, most of the test organisms were multi-drug resistant. Of all the Gram negative bacteria isolates tested for the production of ESBL and MBL, only one strain of *Klebsiella pneumoniae* from crayfish was able to produce ESBL enzyme. One strain of *Klebsiella pneumoniae* isolated from catfish produced both ESBL and MBL whereas one strain of *Escherichia coli* from catfish produced ESBL only. Remedial strides such as antibiotic resistance public health education should therefore be considered and enforced so as to mitigate this globally emerging threat called antibiotic resistance.

Keywords: Bacteria, crayfish, catfish, Nsukka, ESBL, MBL

Introduction
Microorganisms, especially bacteria have occupied a top position in the ecological niche, in terms of their distribution. This ubiquitous spread of microorganisms means that each microbiota will contain a large population of microbial species that are imported by random dispersal (Finlay *et al.*, 2001) [15]. Bacteria are key components of the microbial loop in aquatic ecosystems (Silva *et al.*, 2016) [20] and thus are expectedly seen in crayfish and catfish, some of which are Gram positive, and some Gram negative.

Procambarus clarkii (Crayfish) live in a vast array of freshwater habitats including lakes, rivers, streams, and ponds. In these habitats, crayfish tend to live in the water's sediment in self-made burrows. Crayfish feed on multiple sources, thus increasing their likelihood of ingesting various bacteria (Finch, 2022) [14]. They are thus potentially exposed to not only antibiotics and antibiotic-resistant bacteria in water, but also to the sediment (Finch, 2022) [14]. Dried crayfish are normally prepared from fresh crayfish caught. When dried they are packaged and transported to different places so that they can get to the final consumers. In many places, the dried crayfish are consumed raw. In Nsukka, Nigeria, many buyers would like to test it by chewing at least a handful of the dry one before choosing the one to purchase.

Clarias gariepinus or African sharp tooth catfish is a species of catfish of the family Clariidae, the airbreathing catfishes. Fish is a major source of food and income globally (Tacon and Metian 2013) [22]. It feeds on living, as well as dead, animal matter, small mammals, other fishes, and eggs and plant matter such as fruit and seeds. The majority of disease related deaths in the Catfish industry originate from bacterial diseases (Wise *et al.*, 2021) [25].

Catfish producers often experience high-level mortality events due to bacterial pathogens, thus, antibiotics are usually used in catfish farming as therapeutic and prophylactic agents (Chua *et al.* 2016) [7]. In an experiment carried out in Kano, Nigeria, a number of isolates were obtained, namely; *Escherichia coli, Salmonella species, Klebsiella species, Proteus* and *Enterobacter species.* Their study established the presence of some *Enterobacteriaceae* and the development of multi-drug resistance by these microorganisms (Usman *et al.*, 2021) [23]. In Nsukka, there is a paucity of information regarding the reports on microbial contamination of dried crayfish and presence/absence of bacteria with/without the propensity of producing antibiotics hydrolysing enzymes such as extended-spectrum beta-lactamase (ESBL) and metallo beta-lactamase (MBL). Further, considering the nutritional relevance of these proteinous foods, Crayfish and African catfish, this research aimed at evaluating the presence of bacterial isolates from the samples (Crayfish and catfish) and their antibiotic susceptibility patterns as well as the contribution of antibiotic degrading enzymes in mediating drug resistance in them.

Materials and Methods

Materials

Study Area: This study was conducted in Nsukka, Nigeria

Test Samples: Dried crayfish (Figure 1) and fresh catfish (Figure 2)

Test organisms: The organisms studied include: *Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Enterobacter species, Serratia marcescens* and *Enterococcus faecalis.*

Culture media Apart from Eosin methylene blue agar (Lab M, UK), other culture media were purchased from Titan biotech Ltd, (India), and they include Nutrient agar, MacConkey agar, Cetrimide Mannitol salt agar

Standard antibiotic disk

The selected antibiotic discs used for the study were procured from Polytes laboratories, (Nigeria) represent the antibiotics commonly used in the study area for the treatment of infections and they include ampicillin (30 ug), erythromycin (10 ug), gentamicin (10 ug), ciprofloxacin (10 ug), chloramphenicol (10 ug), ofloxacin (30 ug), meropenem (10 ug), ceftriaxone (10 ug) and septrin (30 ug). However, all the antibiotic discs used for the phenotypic detection of the presence of drug degrading enzymes (ESBL and MBL) were gotten from Oxoid Company (Oxoid UK) and they include Meropenem (10u g), Imipenem 10 (ug), Amoxiclav (30 ug) and ceftriaxone (30 ug).

Fig 1: Dried crayfish (*Procambarus clarkii*)

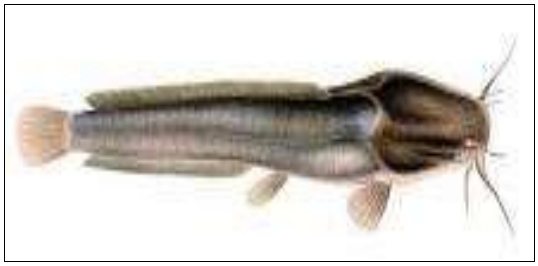

Fig 2: Fresh Catfish (*Clarias gariepinus*)

Methods

Preparation and sterilization of media

The media used were prepared according to the manufacturers' instructions.

Sample Collection: By random sampling method, five pieces of crayfish were collected from each of the twenty (20) different markets/villages in Nsukka cultural zone. Similarly, thirty sterile swab-sticks were used to collect samples from the skin and intestinal contents of fifteen different catfish from eight (8) different fish ponds in the study area. All these samples were kept at a refrigerator temperature pending the time for culturing.

Cultivation, isolation and characterization

Each of the samples collected was inoculated into 5 ml of brain heart infusion in a tube for enrichment and incubated for 16 h at 37°C. Following the enrichment, sub-culturing of the broth culture was done unto different selective media for presumptive isolation of different bacteria. Due incubation was done at optimum temperature of 37°C for 24 h. Then, the organisms isolated were characterized based on morphology, biochemical, cultural and metabolic characteristics. Cetrimide agar, Mannitol salt agar and Eosin methylene blue (EMB) agar were used for presumptive isolation of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* respectively. The isolates were further subjected to biochemical characterization such as indole test, methyl red, urease, citrate utilization, catalase, coagulase etc.

Antibiotic sensitivity test

Sensitivity test was carried out by disk agar diffusion method following the guidelines of the technique described by Clinical and laboratory standards institute (CLSI, 2023) [8]. The IZD (inhibition zone diameter) was measured using a graduated meter rule and result recorded and interpreted following the guidelines of CLSI (2023) [8].

Phenotypic detection of Extended Spectrum Beta-lactamase (ESBL)

In order to detect the presence of ESBL, we followed the protocols of the double disk synergy test (DDST) technique as described elsewhere (Iroha *et al*., 2009) [9]. All the Gram negative isolates which showed a diameter of less than 25 mm for both ceftazidime and ceftriaxone were selected for screening for the production of the enzyme- ESBL. A lawn culture of the test bacteria on a Mueller-Hinton agar plate was prepared and used for the tests. A drug disc which contained amoxicillin-clavulanate (20/10 µg) was placed in the center of the plate. The discs of third generation cephalosporins (ceftazidime (30 µg) and ceftriaxone (30 µg) were placed 15mm apart respectively, center to center, to

that of the amoxicillin-clavulanate disc (Iroha *et al*., 2009) [9]. Any increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production.

Screening for Metallo betalactamase (MBL)

Metallo-beta-lactamase (MBL) enzyme production in Gram negative isolates was confirmed by the inhibitor-based assay as was previously described (Renata *et al*., 2009) [19]. Two meropenem disks (10 µg) and two imipenem disks (10 µg) were placed 25 mm apart on Mueller-Hinton (MH) media plate (s) inoculated with the test bacteria (maintaining 0.5 McFarland turbidity standards). Sterilized EDTA solution (5 µl) was added to one of the imipenem disk and meropenem disk respectively using a micropipette, and the plates were incubated at 37 °C for 18-24 h. After incubation, the zones of inhibition around the imipenem and imipenem+ EDTA disks, and meropenem and Meropenem +EDTA disks were measured using a meter rule, recorded and compared. MBL production in the screened bacteria was inferred if the zone of inhibition of Imipenem +EDTA disk and Meropenem +EDTA disk compared to imipenem and meropenem disks alone respectively is greater than 7mm (Renata *et al*., 2009) [19].

Result

Ethical approval: The samples were collected with the consent of the vendors of the Crayfish and Catfish.

Isolation of bacteria from the samples

Out of the 30 randomly sampled pieces of crayfish and 30 swabs from Catfish, a total of 53 organisms cultured comprised 32 and 21 bacteria respectively (Table 1). The Crayfish organisms were comprised of 10 (31.3%) *Klebsiella pneumonia*, 5 (15.6%) *Pseudomonas aeruginosa*, 5 (15.6%) *Serratia marcescens*, 2 (6.7%) *Enterobacter* species, 7 (21.8%) *Staphylococcus aureus* and 3 (9.4%) *Staphylococcus epidermidis*. Similarly, organisms isolated from Catfish swab samples yielded a total of 21 bacteria comprising 5 (23.8%) *Klebsiella pneumonia*, 5 (23.8%) *Enterobacter* species, 3 (14.3%) *Staphylococcus aureus*, 3 (14.3%) *Staphylococcus epidermidis*, *a*nd 5 (23.8%) *Escherichia coli*. The antibiotic susceptibility patterns (%) of bacteria isolated from both Crayfish and Catfish are shown in Tables 2 and 3 respectively.

All the Gram negative bacteria (100%) isolated from Crayfish showed resistance to ampicillin and at least 50% of these isolates exhibited resistance to nitrofurantoin and chloramphenicol. Conversely, at least 50% of these Gram negative bacteria were sensitive to ofloxacin, meropenem and pefloxacin. All the Gram positive isolates showed resistance to ampicillin and nitrofurantoin and an appreciable number of these organisms also exhibited non susceptibility to other antibiotics tested.

Tables 4 and 5 show the respective results of screening tests for the production of ESBL and MBL enzymes by Gram negative isolates. A total of 2 ESBL consisting of two enzymes from catfish and none from crayfish was investigated. The results of MBL production showed that one strain of *Klebsiella pneumoniae* could produce the enzyme whereas all other Gram negative bacteria tested could not express the protein.

Table 1: Isolation rate of bacteria from Crayfish and Catfish

Isolate	No of isolates from crayfish (%)	No of isolates from Catfish (%)
Klebsiella pneumoniae	10 (31%)	5 (29%)
Pseudomonas aeruginosa	5 (16%)	0 (0%)
Serratia marcescens	5 (16%)	0 (0%)
Enterobacter sp	2 (6%)	5 (29%)
Staphylococcus aureus	7 (33%)	3 (14%)
Staphylococcus epidermidis	3 (14%)	3 (14%)
E. coli	0 (0%)	5 (29%)
Total	32	21

Table 2: Antibiotic susceptibility pattern (%) of bacteria isolated from crayfish.

Test Bacteria	SUST	AM	ERY	GN	CPX	CH	OFX	MP	CEF	SPT
Klebsiella spp	S	0	10	80	70	30	90	100	90	80
	I	0	40	10	30	10	0	0	0	20
	R	100	100	1 0	0	60	10	0	10	0
Pseudomonas aeruginosa	S	0	0	20	100	40	100	100	80	100
	I	0	0	0	0	0	0	0	0	0
	R	100	100	80	0	60	0	0	20	0
Enterobacter Spp	S	0	0	100	50	60	50	90	50	0
	I	0	0	0	0	0	0	0	50	0
	R	100	100	0	50	100	50	10	0	100
Serratia marcescens	S	0	20	80	40	20	80	80	40	60
	I	0	0	20	20	0	0	0	0	0
	R	100	80	0	40	80	20	20	60	40
S. aureus	S	0	0	60	25	30	80	50	50	60
	I	0	0	0	0	0	0	0	0	0
	R	100	100	40	75	40	20	50	50	40
S. epidermidis	S	0	0	50	50	30	80	50	50	40
	I	0	0	30	10	0	0	0	0	0
	R	100	100	20	40	40	20	50	50	60

Key: SUST= Susceptibility**,** R= Resistance, I=intermediate, S =sensitive susceptible, AM=amoxicillin Ery=Erythromycin, GN=gentamcin CPX=ciprofloxacin CH=chloramphenicol, OFX= ofloxacin., MP= meropenem, CET= ceftriaaxone, SPT =Septrin

Table 3: Antibiotic susceptibility pattern (%) of bacteria isolated from Catfish.

Test Bacteria	SUST	AM	ERY	GN	CPX	CH	OFX	MP	CEF	SPT
Klebsiella spp	S	0	0	60	20	0	20	50	9	80
	I	0	0	0	0	80	20	0	0	0
	R	100	100	40	80	20	60	50	100	20
E.coli	S	0	0	0	0	0	0	0	0	10
	I	0	0	20	20	0	20	0	0	0
	R	100	100	80	80	100	80	100	100	90
Enterobacter Spp	S	0	0	80	50	0	0	0	0	0
	I	0	0	0	0	0	0	0	0	0
	R	100	100	20	50	100	100	100	100	100
S. aureus	S	0	0	0	25	30	100	100	50	100
	I	0	0	0	0	0	0	0	0	0
	R	100	100	100	75	40	0	0	50	40
S. epidermidis	S	10	0	0	50	30	80	100	0	40
	I	0	0	0	0	0	0	0	0	0
	R	90	100	100	0	40	20	0	100	60

Key: SUST= Susceptibility**,** R= Resistance, I=intermediate, S =sensitive susceptible, AM=amoxicillin Ery=Erythromycin, GN=gentamcin CPX=ciprofloxacin CH=chloramphenicol, OFX= ofloxacin., MP= meropenem, CET= ceftriaaxone, SPT =Septrin

Table 4: Extended spectrum beta-lactamase (ESBL) enzyme in Gram negative isolates from crayfish and catfish

Isolate	Number of Crayfish isolate	Number of ESBL+ ve isolate in Crayfish (%)	Number of Catfish isolate	Number of ESBL+ ve isolate in Catfish (%)
Klebsiella	10	1 (10%)	5	1(20%)
Pseudomonas	5	0(0%)	Nil	Nil
Serratia	5	0(0%)	Nil	Nil
Enterobacter	2	0(0%)	5	0(0%)
E. coli	Nil	Nil	5	1(20%)
Total	22	1	15	2

Key: nil = the bacterium was not isolated. +ve means ESBL enzyme present

Table 5: Metallo Beta-lactamase (MBL) enzyme in Gram negative isolates from crayfish and catfish

Isolate	Number of Crayfish isolate	Number of MBL+ve isolate in Crayfish (%)	Number of Catfish isolate	Number of MBL+ve isolate in Catfish (%)
Klebsiella	10	0 (0%)	5	1(20%)
Pseudomonas	5	0	Nil	Nil
Serratia	5	0	Nil	Nil
Enterobacter	2	0	5	0(0%)
E. coli	Nil	Nil	5	0(0%)
Total	22	0	15	1

Key: nil = the bacterium was not isolated. +ve means ESBL enzyme present

Discussion

Bacteria are cosmopolitan in distribution. They colonize both animate and inanimate materials including different foodstuff, meat, different fishes, wine and inert surfaces. Due to high prevalence of diseases caused by bacteria, it is necessary to know the causative agent and treatment options for proper management and control of infectious disease in a population like Nsukka. In the present study, we isolated different bacteria that contaminate dried crayfish as well as bacteria infecting *C. gariepinus* and determined the antibiotic susceptibility pattern of catfish sold in local communities of Nsukka cultural zones, Nigeria. This study reports a total of 6 and 5 bacterial species from crayfish and catfish samples respectively. The samples from crayfish yielded 32 bacteria comprising 10 (31.3%) *Klebsiella pneumonia,* 5 (15.6%) *Pseudomonas aeruginosa,* 5(15.6%) *Serratia marcescens,* 2(6.7%) *Enterobacter* species, 7(21.8%) *Staphylococcus aureus* and 3(9.4%) *Staphylococcus epidermidis.* The kinds of bacteria cultured from these samples are not in agreement with the work done elsewhere (Osuntokun *et al.,* 2020; Usman *et al.,* 2021) [18, 23]. The reason for the disparity may be due to effects of geographical location and level of human contamination as crayfish is being transported from one place to another before it gets to the final consumers. Osuntokun and his colleagues (2020) [18] isolated *Micrococcus luteus* (4.3%), *Alcaligenes latus* (4.3%), *Citrobacter* diverticus (4.3%), Listeria grayi (4.3%), *Bacillus cereus* (4.3%), *Citrobacter freundii* (4.3%), *Proteus vulgaris* (4.3%), *Salinicoccus roseus* (4.3%) etc. from fresh crayfish in a riparian area of Osun State, Nigeria. Many of the bacteria isolated in Osun State are not common human pathogens. The reasons may be that these bacteria are common environmental bacteria and might have contaminated the fresh crayfish. Conversely, Nsukka has no riparian ecosystem for the growth of crayfish. Crayfish sold in Nsukka must have passed through many human hands and materials that might have contributed to the kind of bacteria isolated in this study. We cultured human and animal pathogens which belong to the group of bacteria called the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter spp.* These ESKAPEs are mainly hospital-acquired pathogens that affect the *gastrointestinal* tract, middle ear, oral infections, wound infections, and urinary tract infections (Adonu *et al*., 2013)

[1]. In the same vein, catfish samples yielded a total of 21 bacteria comprising 5(23.8%) *Klebsiella pneumonia*, 5(23.8%) *Enterobacter* species, 3(14.3%) *Staphylococcus aureus*, 3(14.3%) *Staphylococcus epidermidis a*nd 5(23.8%) *Escherichia coli*, similar to the results of work done elsewhere. (Akaniro *et al*. 2020; Wamala *et al*., 2018) [2, 24].

The organisms isolated showed different sensitivity and resistance patterns to different antibiotics. There was 100% sensitivity of *Pseudomonas aeruginosa and Klebsiella sp* to meropenem and more than 50% susceptibility to ciprofloxacin, septrin and ofloxacin Such high susceptibility levels of fish bacteria have been reported elsewhere (Aravena-Román *et al*. 2012) [4]. Conversely, all the Gram negative bacteria isolated from catfish were resistant (R=100%) to amoxicillin, erythromycin and ceftriaxone. Apart from *Serratia marcesencs* that showed 80% resistance, all other Gram negative bacteria isolated from crayfish showed 100% resistance to amoxicillin and erythromycin. Such high levels of drug resistance have also been reported by other researchers (El-salam *et al*.,2016; Akaniro *et al.,* 2020; Breijyen *et al.,* 2020) [10, 2, 5]. Generally, some of the test organisms were reported to be multi-drug resistant. The excessive use (or misuse) of antibiotics in animal production has severe consequences for public health and the environment (Caudell *et al.,* 2020; Amit *et al.,* 2017) [6, 7]. Following the discovery of growth promoting and disease fighting capabilities of antibiotics, fish farmers and livestock producers began using such drugs in animal feeds (FAO 2005) [12]. Thus antibiotics have been reported to be widely used in the treatment of diseases in the aquaculture industry (Ning *et al.,* 2022) [17]. Markedly, the antibiotic consumption patterns in agriculture vary across regions and countries in the developing world, and even antibiotics that have been banned in other countries, including the developed countries, are still being used in most developing countries (Manyi-Loh, 2018) [16].

An important aspect of medical microbiology is the mechanism of drug resistance, one of which is the production of antibiotic degrading enzymes such as extended-spectrum beta-lactamase (ESBL) and metallo beta-lactamase (MBL) found in Gram negative bacteria. In the present study, one stain of *Klebsiella pneumoniae* out of all the test Gram negative isolates from crayfish was able to produce ESBL only. This low proportion of ESBL producers in bacterial isolates from fish was also reported by other researchers (Sola *et al.,* 2022) [21]. Similarly, one strain each of *Klebsiella pneumoniae* and *E. coli* isolated from catfish were able to produce ESBL. Our findings support the findings of other researchers, who stated that the worldwide spread of *Klebsiella pneumonia* producing extended spectrum beta-lactamase (ESBL) is a significant threat (Fils *et al.,* 2021) [13]. Moreover, only one strain of *Klebsiella pneumoniae* from catfish was able to express MBL. Our finding on the presence of MBL in bacteria from fish is in agreement with the report from a similar work done in Ibadan, Nigeria (Falodun *et al*., 2022) [11]. Our results might suggest that bacteria from humans, fish packaging materials or other materials, animate or inanimate can contaminate dried crayfish and/or fresh catfish during the process of packaging, transportation, hawking/sales, preparation for human consumption etc. Further, bacteria from farm or industrial effluents can reach the open sea or body of water and contaminate fish/aquatic organisms that are living or reared nearby. Consequently, care should be taken to limit contamination of these food items by human activities as some of these bacteria are reservoirs of antibiotic resistant genes that can be transferred to human normal flora and thus spread drug resistance.

Conclusion and Recommendation

This study detected different bacteria contaminating dried crayfish in Nsukka. It also identified diverse pathogens that infect African catfish in the area. From the several phenotypic studies conducted, we found out that some of these bacteria are multi-drug resistant super-bugs, with some being able to produce ESBL and/ or MBL enzymes. These findings pose a serious threat to public health.

In view of the foregoing, we, therefore, recommend that efforts should be put in place to limit the emergence and dissemination of antibiotic resistance in dried crayfish and fresh catfish in Nsukka through:

1. Public health education on the strategies to prevent bacterial contamination.
2. Dangers of using antibiotics and other growth promoters to rear fishes.

Implementation of rigorous quality control measures by fish farmers and marketers as well as the consumers in the production – distribution chain.

Conflict of interest

Not available

Financial support

Not available

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