PREVALENCE AND ANTIBIOGRAM OF *PSEUDOMONAS* SPECIES ISOLATED FROM WEST AFRICAN MUD CREEPER (*TYMPANOTONUS FUSCATUS*)

ABSTRACT

The menace of opportunistic infections arising from food contamination can be tackled with knowledge of agents effective against the etiologic agents. A bacteriological examination was carried out on *Tympanotonos fuscatus* (West African Mud Creeper) sold at different markets in Port Harcourt, Nigeria with interest on *Pseudomonas* species and their antibiogram using standard microbiological methods. Results showed that parboiled samples had the mean total heterotrophic bacterial counts between 26.33±11.02x10^6 and 85.67±3.79x10^6 cfu/g, total coliform counts, 6.0±1.73x10^6 and 13.0±7.21x10^6 cfu/g and total Pseudomonad counts, 0.00 and 0.67±1.16x10^2 cfu/g. Roasted samples had total heterotrophic bacterial counts between 40.0±1.73x10^6 and 45.75±3.86x10^6 cfu/g, total coliform counts, 10.0±7.211x10^6 and 13.0±9.539x10^6cfu/g and total Pseudomonad counts, 6.67±4.619x10^2 and 20.75±22.824 x10^2cfu/g. There was difference (p<0.05) in the total heterotrophic bacteria counts of parboiled samples from the different markets but none in coliform and Pseudomonads counts. No difference (p>0.05) was recorded in counts from the roasted samples. Thirty-two (32) *Pseudomonas* isolates were obtained from the markets occurring least in the parboiled 6 (18.75%) samples compared to roasted 26 (81.25%) samples. Mile3 market had the highest occurrence 3 isolates (50%) for parboiled samples whereas Mile1 market with 1 isolate (16.67%) was lowest. Results of Antibiogram revealed that *Pseudomonas* species had varying sensitivity to all the antibiotics tested and was most susceptible to Ofloxacin (90.6%) and most resistant to Nalidixic acid (84.4%). Although the counts obtained are within the upper limits (5x10^7cfu/gm) recommended by the International Commission on Microbiological Specifications for Foods (ICMSF), there is need to properly cook *Tympanotonus fuscatus* before consumption to reduce risks of opportunistic pathogens. Infections from *Pseudomonas* species may be managed with appropriate doses of ofloxacin.

Keywords: Antibiotic Susceptibility, Pseudomonas species, Tympanotonus fuscatus, Market

1. INTRODUCTION

The microbes belonging to the genus *Pseudomonas* are ubiquitous in nature; of particular mention is the species *Pseudomonas aeruginosa* being one of the most relevant pathogens causing human opportunistic infections [1]. *Pseudomonas aeruginosa* is the most common driver of chronic respiratory infections in patients suffering from cystic fibrosis or other long lasting underlying diseases [2]. It has been shown to cause food-borne diseases as well as food spoilage. The innate or inherent resistance of this organism makes it difficult to treat these infections coupled with the organism's remarkable ability to acquire further mechanisms of resistance to a multiple group of antimicrobial agents [3]. It is amongst the microorganisms that are genetically equipped with excellent inherent antibiotic resistance capabilities [4,5,6]. The production of an inducible AmpC cephalosporinase, the constitutive or inducible expression of efflux pumps, and the reduced permeability of its outer membrane are believed to be largely responsible for the basal lower susceptibility of *Pseudomonas aeruginosa* to antibiotics when compared with that of other Gram-negative pathogens [7].

An antibiogram is an overall profile of antimicrobial susceptibility testing results of a specific microorganism to a group of antimicrobial agents/drugs [8,9]. The subject of antimicrobial sensitivity is of enormous importance to the field of public health microbiology as microorganisms have been shown to device strategies through which they resist the effects of many antimicrobial agents [10]. Successful treatment of diseases caused by microorganisms rely heavily on the ability of public health

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experts to ascertain antimicrobial agents effective on such microbes hence, the need to continuously study the sensitivity patterns of microbes to antimicrobial agents.

The overall importance of the human alimentary canal as it pertains to health cannot be overemphasized. There is therefore a need for greater care towards ensuring that opportunistic pathogens do not find their way into the human gut and cause diseases thereof. Food is a well known vehicle of infection for many microorganisms; in the environment, food-borne pathogens are widely distributed and may significantly bring about health issues and death in human population [11].

Tympanotonos is one of the most readily and relatively easily harvested shell fishes are they fishes or snail within the coastal areas particularly in the southern part of Nigeria and some riverine areas in West Africa where it is a relatively cheap source of animal nutrients [12] and used in the preparation of several delicacies. Perewinkles as they are popularly called in Nigeria are well represented in the mangrove swamps and lagoons of the Niger Delta in Nigeria [13]. Communities and settlements around these areas harvest and use this organism for food and commercial purposes. Sea foods like *Tympanotonos fuscatus* (West African Mud Creeper) can get contaminated in riverine areas where the water bodies are used as dumping grounds for excreta and refuse. They can also become contaminated through contact with the market environment as well as unhygienic practices by vendors and processors [14,15]. Nigerians, especially inhabitants of the southern (Riverine)=should be removed parts as earlier mentioned consume the West African Mud Creeper on a very large scale and are hence prone to infection when this organism replaced with food item becomes contaminated by microorganisms in high populations.

This study was carried out to ascertain the prevalence of *Pseudomonas* species in edible parts of the West African Mud Creeper and also their antimicrobial susceptibility pattern to conventional antibiotics.

2. MATERIALS AND METHODS

2. 1 Sample Description and Sample Collection

A total of 42 samples comprising () mention the numbers parboiled and () roasted samples of *Tympanotonos fuscatus* (West African Mud Creeper) were collected from three different locations in Rivers State Nigeria; Mile 1 market in Port Harcourt City Local Government Area (4.7918^{o} N, 6.9986^{o} E), Rumueme Market in Obio/Akor Local Government Area (4.8273^{o} N, 6.9820^{o} E) and Mile 3 Market in Port Harcourt City Local Government Area (4.8042^{o} N, 6.9924^{o} E) for a period of three months. These were properly labelled, put into an ice-chest and transported to the Microbiology laboratory for bacteriological examination.

2.2 Enumeration and Isolation of Bacteria

Stock analytical unit was prepared by weighing 10 grams of edible (internal parts) of the parboiled and roasted *Tympanotonos fuscatus* samples respectively and homogenizing in 90ml of sterile normal saline. Tenfold serial dilution method was continued by pipetting 1ml of the sample into 9ml of sterile normal saline up to 6 dilutions (dilution factor from 10^{-1} to 10^{-6}). This was done for all samples collected [16].
After serial dilution, two dilution factors (10^{-2} and 10^{-3}) were inoculated in duplicates into already prepared sterile plates of Cetrimide Agar for total Pseudomonads counts and 10^{-5} and 10^{-6} dilution factors on Nutrient Agar for total heterotrophic bacterial count (THB) and MacConkey Agar for total coliform this should be bacteria encountered, not in the title counts using the spread plate technique. The inoculated plates were incubated at 37°C for 16 to 24 hours after which discrete colonies that grew on the plates were counted and recorded [17]. Representative colonies were described and subcultured onto nutrient agar plates and incubated for 24 hours at 37°C to obtain pure cultures. Pure cultures were stored in sterile 10% v/v glycerol for preservation and subsequently used for identification.

2.3 Identification of Bacteria

This was done as described by [18]. The following tests were performed on each of the isolates to confirm their identity: Gram staining, Sugar fermentation tests, oxidase test, catalase test, indole test, methyl red test, vogues proskaeur test, citrate utilization test, haemolysis test, motility test, lecithinase test and starch hydrolysis test. Molecular identification using the 16s rRNA subunit of the DNA was

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also carried out to verify the identity of the isolates molecularly should be removed, procedure not in the methodology [19].

2.4 Antibiotic Sensitivity Testing

Standardization of isolates was carried out by adjusting to preparing 0.5 Macfarland solutions and adjusting the turbidity of isolates in test tubes to that of a 0.5 Macfarland standard. The antimicrobial susceptibility profiles of the isolates to conventional antibiotics were determined using the Kirby Bauer disk diffusion method [9] on sterile Mueller-Hinton agar. The surface solid media plate was inoculated with bacterial suspension by streaking the swab over the agar plate surface; being sure that no zone of the surface is left free of inoculum. This procedure was repeated several times, rotating the agar plate 60° each time to ensure even distribution of the inoculum to the edge of the agar. The plates were left to dry for 3–5 min to allow absorption of any moisture prior to applying the antibiotic disks. Antibiotic disks of ten conventional antibiotics (Cephalexin (CEP) – 10µg, Ofloxacin (OFX) -10µg, Nalidixic acid (NA) – 30µg, Pefloxacin (PEF) - 10µg, Gentamycin (CN) - 10µg, Amoxicillin/Clavulanic acid (AU) - 30µg, Ciprofloxacin (CPX) - 10µg, Trimethoprim (SXT) - 30µg, Streptomycin (S) - 30µg and Ampicillin (PN) - 30µg) were aseptically placed on the surface of the inoculated agar plate with sterile forceps. Each disk was pressed down to ensure full contact with the surface of the agar. At least 24 mm was left between the centres of the disks, and not less than 15 mm from the border of the plate too. The plates were then inverted and placed in an incubator within 15 min of applying the disks. Finally, the plates were incubated for 24 hours at 33 to 35°C [9]. After incubation, the plates (control and test plates) were examined to ensure growth was confluent or near confluent. On the underside of the plate, the diameter of each zone of inhibition for those that had zones of inhibition were measured in millimetre (mm) using a meter rule. The measurement included the diameter of the disc. For interpretation MIC Analysis and Susceptibility Testing, the criteria provided by CLSI were followed.

2.5 Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 22 was used to analyse the data obtained from the plate counts as well as those from the measurement of the zones of inhibition. Descriptive statistics was used to summarize all data obtained. Analyses (Analysis of variance was carried out to test for significant difference in the bacterial counts in the various markets and sites. Where differences occurred, Duncan multiple range test was used to separate the means [20].

3. RESULTS AND DISCUSSION

The current study revealed as seen in Table 1. that out of 42 samples of *Tympanotonos fuscatus*, the total heterotrophic bacteria counts for parboiled samples was highest in the Mile 1 market samples (85.67±3.79 x 10^6 cfu/g) followed by the samples from Rumueme and Mile 3 markets with mean counts of 29.33±11.50 x 10^6cfu/g and 26.33 ±11.02 x 10^6cfu/g respectively. The difference ($p>0.05$) observed in counts for total heterotrophic bacteria can be attributed to several factors such as resident normal microbial flora and microorganisms introduced from its natural habitat not eliminated during parboiling, unsanitary handling by vendors in the market and the water source with which the *T. fuscatus* are stored in the markets [21]. The hands of the food handlers, sea foods inclusive are the most important vehicle for the transfer of organisms from faeces, nose and skin to the food [21]. Results of total coliform counts from Table 1 showed that Mile 3 market samples had the highest counts with 13.00±7.21 x 10^6 cfu/g followed by Rumueme market and Mile 1 market with 10.67±9.29 x 10^6 cfu/g and 6.00±1.73 x 10^6 cfu/g respectively. However, the difference in total coliform counts was not statistically significant ($p<0.05$). Poor environmental conditions such as broken sewage pipes, overcrowded market and waste disposal sites were characteristic of the three markets hence a probable reason for the similar coliform counts obtained. [22] confirms that periwinkles carry with them a lot of coliform bacteria from their natural environment and may even harbour more in the market place if exposed to coliform sources such as water and soil contaminated with sewage. Total Pseudomonads counts were highest in Rumueme market (0.67±1.16 x 10^2cfu/g) followed by Mile 3 market with 0.33±0.58 x 10^2 cfu/g as also seen in Table 1. No counts were recorded for Mile 1 market (0.00±0.00 x 10^2 cfu/g). There was also no difference ($p>0.05$) in the mean total Pseudomonads counts.

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Table 1: Bacterial population of parboiled samples from the various markets sampled.

Location	THB (10^6 Cfu/g)	TCC (10^6 Cfu/g)	TPC (10^2 Cfu/g)
Mile 1	85.67±3.79^b	6.00±1.73^a	0.00±0.00^a
Mile 3	26.33±11.02^a	13.00±7.21^a	0.33±0.58^a
Rumueme	29.33±11.50^a	10.67±9.29^a	0.67±1.16^a

Key: THB (Total Heterotrophic Bacteria), TCC (Total Coliform Count), TPC (Total *Pseudomonas* Counts). The table above did not present the number of samples collected from various locations

Results of Total Heterotrophic Bacteria and Pseudomonads count among vendors as presented in Table 2 revealed that for the total heterotrophic bacterial counts, Vendor B with 45.75±3.86 x 10^6 cfu/g slightly had the highest counts followed closely by Vendor C and Vendor A with 45.67±10.79 x 10^6 cfu/g and 40.00±1.73 x 10^6 cfu/g respectively. There was no difference ($p>0.05$) in the total heterotrophic bacteria counts from the roasted samples. Vendor C with 13.00±9.54 x 10^6 cfu/g had the highest total coliform counts followed by Vendor B with 10.50±5.75 x 10^6 cfu/g and Vendor A (10.00±7.21 x 10^6 cfu/g). Total Pseudomonads counts were highest in the samples from Vendor B (20.75±22.82 x 10^2 cfu/g) followed by Vendor C and Vendor A with 8.33±5.86 x 10^2 cfu/g and 6.67±4.62 x 10^2 cfu/g respectively. There was no difference ($p>0.05$) in the counts recorded for roasted samples however, Vendor A had the least counts for all three categories of microbes examined for.

Table 2: Bacterial population of roasted samples collected randomly from mile 1 market.

Location (Mile 1)	THB (10^6 Cfu/g)	TCC (10^6 Cfu/g)	TPC (10^2 Cfu/g)
Vendor A	40.00±1.73^a	10.00±7.21^a	6.67±4.62^a
Vendor B	45.75±3.86^a	10.50±5.75^a	20.75±22.82^a
Vendor C	45.67±10.79^a	13.00±9.54^a	8.33±5.86^a

Key: THB (Total Heterotrophic Bacteria), TCC (Total Coliform Count), TPC (Total *Pseudomonas* Counts).

T. fuscatus after collection is usually sold in different forms either as a whole with its shell intact or it undergoes processing which may be through parboiling and subsequent separation of the edible internal parts from the shell or roasting in moderate heat. The study further revealed that *Pseudomonas* species was more prevalent in the roasted samples than the parboiled *T. fuscatus* samples. *Pseudomonas* species have been shown to be most prevalent in fresh seafood however; they are more prevalent in roasted or smoked foods than parboiled seafood [23,24]. Fig. 1 shows that out of 32 *Pseudomonas* isolates, 26 (81.25%) were isolated from the roasted samples while 6 (18.75%) were from parboiled samples.

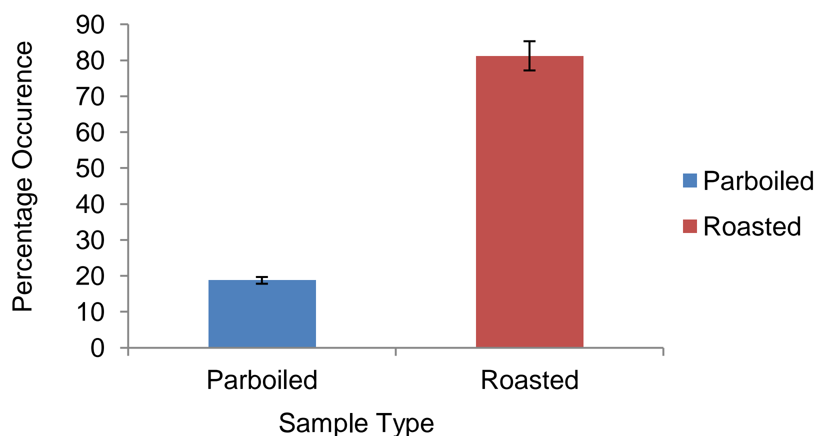

Figure 1: Percentage Occurrence of *Pseudomonas* species isolates only single specie was indicated in this work; hence, *Pseudomonas aeruginosa* should be adopted

Fig. 2 shows that Mile 3 market had the highest occurrence of *Pseudomonas* species isolates with 50% occurrence. It is followed closely by Rumueme market with 33.33% and then by Mile 1 market with 16.67% (Fig. 2).

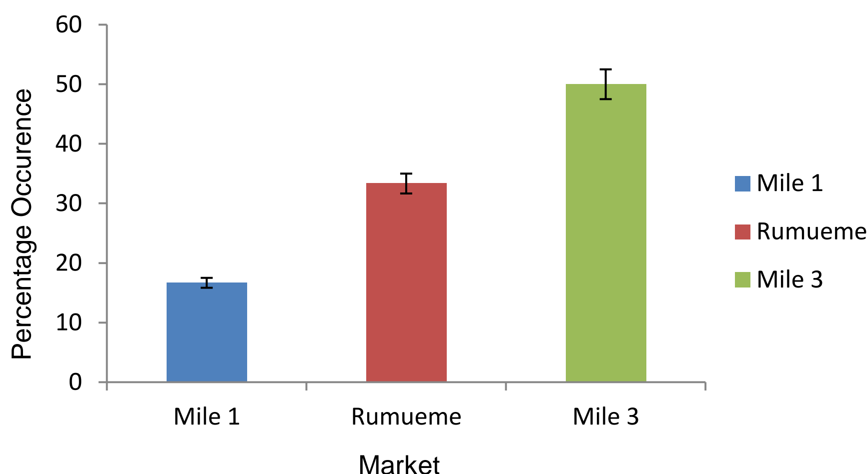

Figure 2: Percentage Occurrence of *Pseudomonas* species isolates in Parboiled Samples from various Market.

From Fig. 3 on the other hand the counts obtained from samples from the various vendors at Mile 1 market were highest from Vendor B with 46.15%. Vendor A and Vendor C both had 26.92%. Recall that vendor B also has the highest occurrence for total heterotrophic bacteria count and second highest counts of coliform. These high bacteria counts may be associated with the method of handling employed by the vendor for the products as well as the sanitary condition of his selling spot. The water source used for storing the *T. fuscatus* before they are sold may also be a source of contamination as these water sources are not screened and are mostly public boreholes around the market, some of which are not potable for drinking and hence not fit to be used for food products [25]. [21] explained that poor personal hygiene and orderliness on the part of food vendors could expose to food consumers to biological hazards posing very serious public health concern.
The pathogenesis after introduction of *Pseudomonas aeruginosa* into the body through such foods as periwinkles may partly be due to the proteins secreted by the organism as the bacterium is known to possess a wide range of secretion systems, which export numerous proteins relevant to the pathogenesis of clinical strains [26]. Diseases arising from *Pseudomonas* such as soft tissue infections, bacteraemia, gastrointestinal infections and systemic infections are more common in immunocompromised individuals, the aged and young children [27].

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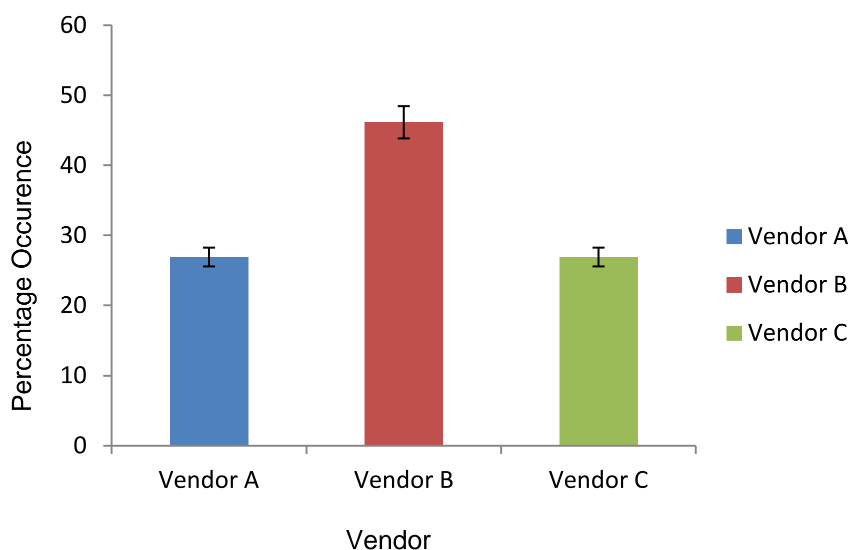

Figure 3: Percentage Occurrence of *Pseudomonas* species isolates in Roasted Samples from different Vendors in Mile 1 Markets.

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Results of antibiotic sensitivity test as interpreted using [8] revealed that all the *Pseudomonas* species are uniquely resistant to most antibiotics as all the isolates had varying susceptibility to the antibiotics tested. Table 3 shows that Ofloxacin-10µg was effective on 29 (90.6%) *Pseudomonas* species isolates tested making it the antibiotic with the highest efficacy on the isolates. Gentamycin-10µg and Pefloxacin-10µg followed closely having 81.3% and 75.0% effectiveness respectively on *Pseudomonas* isolates. On the other hand, the *Pseudomonas* isolates tested were more resistant to Nalidixic acid-30µg with 27 (84.4%) out of the 32 isolates tested. Isolates were also very resistant to Amoxicilin/clavulanic acid-30µg 26 (81.3%) and Cephalexin-10µg 24 (75.0%). Similar studies by [28] also showed that *Pseudomonas* is highly susceptible to gentamycin. A study from [29] also confirms high susceptibility of *Pseudomonas* to ofloxacin and gentamycin as well as its high resistance to Amoxicilin/Clavulanic acid (Augmentin).

Table 3: Percentage Susceptibility/Resistance of *Pseudomonas* species Isolates

	Antibiotics (Concentration)	**Susceptible**	**Intermediate**	**Resistant**
1	CEPHALEXIN (10µg)	4 (12.5%)	4 (12.5%)	24 (75.0%)
2	OFLOXACIN (10µg)	29 (90.6%)	1 (3.1%)	2 (6.3%)
3	NALIDIXIC ACID (30µg)	3 (9.4%)	2 (6.3%)	27 (84.4%)
4	PEFLOXACIN (10µg)	24 (75.0%)	4 (12.5%)	4 (12.5%)
5	GENTAMYCIN (10µg)	26 (81.3%)	2 (6.3%)	4 (12.5%)
6	AMOXICILIN/CLAVULANIC ACID (30µg)	3 (9.4%)	3 (9.4%)	26 (81.3%)
7	CIPROFLOXACIN (10µg)	10 (31.3%)	8 (25.0%)	14 (43.8%
8	TRIMETHOPRIM (30µg)	18 (56.3%)	10 (31.3%)	4 (12.5%)
9	STREPTOMYCIN (30µg)	17 (53.1%)	11 (34.4%)	4 (12.5%)
10	AMPICILIN (30µg)	11 (34.4%)	6 (18.8%)	15 (46.9%)

4. CONCLUSION

The West African Mud Creeper (*Tympanotonos fuscatus*) has been shown to be very highly populated with microorganisms after purchase from vendors in the market. There is therefore need to ensure that its processing before and after purchase is carried out in the most hygienic manner possible to avert contamination by opportunistic pathogens such as *Pseudomonas* species. The market places need to be regularly sanitized to ensure that the air and surrounding environment is completely clean and free from contaminants. Market authorities and Government needs to ensure that there is access to clean and safe water in the market places. Awareness on the dangers of indiscriminate use of antibiotics is of utmost importance as this is one means by which microbes acquire resistance to these antibiotics.

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