ANTIBIOGRAM OF BACTERIA ISOLATED FROM *TYMPANOTONUS FUSCATUS VAR*. RADULA SOLD IN MARKETS IN NASARAWA STATE, NIGERIA

ABSTRACT

An investigation was conducted to determine the antibiogram of bacterial isolates from *Tympanotonus fuscatus* var. radula sold in markets in Nasarawa State. Nigeria. Samples of *Tympanotonus fuscatus var*.radula periwinkles were bought from soup ingredient sellers at different sale locations in Keffi, Masaka and Orange markets and were analyzed using standard bacteriological methods. The bacterial isolates were identified using morphological, cultural and biochemical techniques. The total bacteria count varied from 1.18–3.20 x 10^8 cfu/g for the raw samples while the total bacterial count for the boiled samples varied from 0–1.57 x 10^8. Periwinkle samples with shells from Masaka market had the highest bacterial load with a mean total bacterial count of 2.94 x 10^8cfu/g and mean total coliform count of 2.80 x 10^6cfu/g. Raw periwinkle samples with shells had a higher bacterial load than samples without shells. There was also a drastic reduction in the bacterial load in the periwinkle samples after boiling under laboratory conditions. The bacteria isolated were *Bacillus sp and Staphylococcus aureus were the gram positive bacteria isolated. Enterobacter* sp., *Escherichia coli, Salmonella* sp., *Pseudomonas* sp., *Serratia* sp. *and Proteus* sp. The most frequently occurring gram positive bacteria was *Escherichia coli* with an isolation frequency of 6(24%), the least frequently occurring was *Bacillus* sp., 8(32)%. Antibiotic susceptibility test showed that all the gram negative organisms exhibited sensitivity to ciprofloxacin: *Escherichia coli* (32mm), *Enterobacter* sp. (41.5mm), *Proteus* sp. (40.0mm), *Salmonella* sp. (37.0mm), *Serratia* sp. (26.0mm), *Pseudomonas* sp. (23.0mm). All the gram negative organisms showed marked resistance to vancomycin: *Escherichia coli* (12.0 mm), *Enterobacter* sp. (10.0mm), *Proteus* sp. (11.0mm), *Salmonella* sp. (5.0mm), *Serratia* sp. (10.0mm) and *Pseudomonas* sp. (4.5mm).

Keywords: Bacteria, antibiotics, coliform, *Tympanotonus fuscatus* var. radula, Nigeria.

INTRODUCTION

Globally, foodborne diseases and infections have become a growing health challenge. Each year, as many as 600 million or almost 1 in 10 people in the world fall ill after consuming contaminated foods, 420,000 people die including 125,000 children under the age of 5 years (WHO, 2015). Food borne diarrheal diseases kills 1.9 million children globally every year (Ameme *et al*., 2016). In the developing world, food borne infection leads to the death of many children and the resulting diarrheal disease can have long term effects on children's growth as well as their physical and cognitive development (Okonko *et al.,* 2008). In the industrialized world, food borne infections cause considerable illness, heavily affecting health care systems (Adak *et al.,* 2009). In major part of the world, about 10 - 19% of food-borne illness involved shellfishes as a vehicle and between 1993 and 1997, 6.8% of the food borne illnesses involved consumption of fish and shellfishes (Huss *et al.,* 2003). Some of these food borne infections are resistant to known antibiotics culminating in high morbidity and mortality, there by aggravating the escalating healthcare costs worldwide. Despite the availability of newer antibiotics, emerging antimicrobial resistance has become an increasing problem in many pathogens throughout the world (Keith and John, 2005). The past two decades have witnessed a tremendous increase in emergence and spread of multidrug – resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and some cephalosporins (Levy and Marshall, 2004).

Survey on the microbiological quality of shellfishes shows that they harbor pathogenic organisms (Ukpong and Utuk., 1992; Adebayo-Tayo *et al,* 2006; Nrior *et al.,* 2016). This is because the water bodies from which the shellfishes are harvested are heavily polluted. *Tympanotonus fuscatus* var. radula are invertebrates belonging to the kingdom animalia, phylum Mollusca, class Gastropoda, subclass Prosobranchia, family Potamididae, genus *Tympanotonus* (Egonwan., 2008; Jamabo *et al.,* 2009 ; Ehigiator and Oterai., 2012). They are common in many brackish water creeks, estuaries and mangrove swamps (Egonwan., 2008; Moruf and Lawal-Are., 2015). Periwinkles are a delicacy used to prepare delicious Nigerian dishes such as ekpang nkukwo in Akwa Ibom and Cross River States, Isemi fulo (periwinkle soup) and Foi isemi in Nembe, Bayelsa State and Keke-fiyai in Ijaw, Bayelsa State. Bob-Manuel (2012) revealed that they are highly medicinal for cases like endemic goiter. Grolie

(1980) reported that grounded periwinkle shell is used as powder for pimples, fertilizers and calcium for animal feeds. The shells compete favorably in construction, cosmetics and ornamental industries (Jamabo and Chinda, 2010).

Tympanotonus fuscatus var. radula is a relatively cheap source of high quality animal protein and minerals. The aim of this work is therefore to determine the antibiogram of bacteria isolated from *Tympanotonus fuscatus* var. radula sold in markets in Nasarawa State, Nigeria.

MATERIALS AND METHODS

Description of Study Area

Nasarawa State is located in the central part of Nigeria otherwise known as the middle belt with a land area of 27, 137.81 km^2. It is bounded in the north by Kaduna State, in the west by Kogi State and Federal Capital Territory, Benue States and Plateau States in the East (NIMET, 2005). It has a total population of about 1, 863,275 people according to 2006 population census.. It lies on latitude 7◦ 45^1 and 9◦25^1 N of the equator and between longitude 7◦ and 9◦37^1 E of the Greenwich meridian (Agidi *et al.*, 2018). Lafia is the capital of Nasarawa State. Three markets in Nasarawa State were randomly selected. Keffi market in Keffi Local Government Area; Masaka and Orange markets both in Karu Local Government Area of Nasarawa State.

Sample Collection and Processing

The samples were divided into four groups:

Group 1: Periwinkle Samples with Shells:- At the laboratory, the periwinkle samples with shells were extensively scrubbed, washed and rinsed using normal saline solution to remove dirt, debris and surface contaminants (Edun *et al*, 2016). The pointed ends were cut off using a sterile knife (Omenwa *et al.*, 2011). All aseptic techniques were carried out under the Purifier Biosafety Cabinet (Model Delta series, LABCONCO, USA).

Group 2: Periwinkle Samples without Shells:- The periwinkle samples without shells were extensively scrubbed, washed and rinsed using normal saline solution to remove dirt, debris and surface contaminants (Edun *et al*, 2016). The pointed ends were cut off using a sterile knife (Omenwa *et al.*, 2011). The fleshy part was extracted aseptically using a specially fabricated sterile needle. All aseptic techniques were carried out under the Purifier Biosafety Cabinet (Model Delta series, LABCONCO, USA).

Group 3: Boiled periwinkle samples with shells:- Periwinkle samples in Group 1 were boiled at laboratory conditions of 100^{o}C for 5 minutes using a hot plate (Jenway, United Kingdom).

Group 4: Boiled Periwinkle samples without shells.

Periwinkle samples in Group 2 were boiled at laboratory conditions of 100^{o}C for 5 minutes using a hot plate (Model Jenway, United Kingdom).

Bacteriological Analyses

Bacteriological analyses were carried out in triplicates on 50g each of periwinkles from Groups 1, 2, 3 and 4. They were homogenized with 450ml of 0.1% sterile peptone water (CONDA, Spain) using a sterile blender/grinder (Model QASA, QLink, China) (Adebayo-Tayo *et al*., 2006). Thereafter, 1ml of fresh sterile dilutors (10^{-8}) from 10-fold dilutions of the samples were used to prepare pour plates in autrient agar (Merck, Germany) for total bacteria count, MacConkey agar (FLUKA, India) for total coliform count, *Salmonella/Shigella* agar (L-S Biotech) for *Salmonella/Shigella* count and Mannitol Salt Agar (FLUKA., India) for *Staphyloccus aureus* count. After incubation at 37^{o}C for 24 hours, colonies were enumerated and selected randomly. Bacteria cultures were characterized and identified using various morphological and biochemical tests such as gram stain, motility, catalase, coagulase, indole, MR-VP, urease, citrate, oxidase, blood agar haemolysis, hydrogen sulphide test and sugar fermentation tests (Adebayo-Tayo *et al.*, 2006; Omenwa *et al.*, 2011). The isolates were characterized and identified with reference to Cowan and Steel's Manual for the identification of Medical Bacteria (Cowan, 1985) and Fawole and Oso's laboratory manual (Fawole and Oso, 1988). Mean colony counts were calculated and expressed as colony forming units per gram (cfu/g) of the sample analyzed (ICMSF., 1982; Harrigan and McCance, 1990).

Coliform Forming Unit/Gram was calculated as:

Average number of colonies × Total dilution factor divided by volume plated (aliquot)

Purification and Preservation of Bacterial Isolates

Bacterial isolates were aseptically picked with a sterile wire loop based on their morphological appearance and were sub-cultured onto freshly prepared nutrient agar plates to obtain pure cultures. They were incubated for 24 hours at 37°C after which pure cultures were stored in McCartney bottles and stored in a laboratory refrigerator at 4°C (Nrior *et al.*, 2016).

Antimicrobial Susceptibility Testing of the bacterial Isolates

The antimicrobial susceptibility testing was carried out as described by Clinical and Laboratory Standards Institute (CLSI, 2017). Pure colonies of the bacterial isolates were inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) and the turbidity was adjusted to the turbidity equivalent to 1.5 McFarland standard. The McFaland's standard was prepared by adding 0.5ml of 1.172% (w/v) $BaCl_2 2H_2O$ was added into 99.5 ml of 1% (w/v) H_2SO_4. A sterile swab stick was soaked in standardized bacteria suspension and streaked on Mueller Hinton agar (Titan Biotech., India) plates and the antibiotic discs were placed aseptically at the center of the plates and allowed to stand for one hour for pre-diffusion. The plates were incubated at 37▫C for 24 hours. The antibiotics discs used were Augmentin (30µg), Gentamycin (10µg), Erythromycin (10µg), Chloramphenicol (30µg), Septrin (30µg), Tetracycline (25µg), Ciprofloxacin (10µg), Vancomycin (10µg), Ampicillin (10µg) and Streptomycin (30µg). After the incubation period, the diameter of zone of inhibition (clearance) was measured using a millimeter rule from the center of the disc to the edge of the circumference of the clearance zone and recorded to the nearest millimeter. The result was interpreted in accordance with the susceptibility breakpoint as described by Clinical and Laboratory Standard Institute (CLSI, 2017).

Statistical Analysis

Data were presented as means standard deviation of triplicate determinations. All statistical analyses were carried out using SPSS for windows version 21.0 statistical package (SPSS Incorporated. USA). One way analysis of variance was done to determine significant difference as $P< 0.05$.

RESULTS

Table 1: Mean Bacteria load of Raw *Tympanotonus fuscatus var. radula* sold in Markets in Nasarawa State, Nigeria (cfu/g)

Source	Total Bacterial Count (10^8)	Total Coliform Count (10^6)	Total Salmonella/ Shigella Count (10^6)	Total Faecal Coliform Count (10^6)
Keffi shells	1.90	2.70	1.70	1.90
Keffi meat	1.20	1.20	1.00	1.10
Masaka shells	2.94	2.80	1.40	2.00
Masaka meat	1.57	1.30	1.00	1.30
Orange shells	2.92	2.20	1.50	2.20
Orange meat	1.23	1.60	1.00	1.10
Standard Deviation	0.79	0.70	0.31	0.49
Range	1.18-3.20	1.20 – 2.80	1.00 – 1.85	1.10 – 2.30

Table 2: Mean Bacteria load of Boiled *Tympanotonus fuscatus var. radula* sold in Markets in Nasarawa State. Nigeria (cfu/g)

Source	Total Bacterial Count (10^8)	Total Coliform Count (10^6)	Total Salmonella/ Shigella Count (10^6)	Total Faecal Coliform Count (10^6)
Keffi shells	1.13	1.50	1.30	1.10
Keffi meat	0.00	0.00	0.00	0.00
Masaka shells	1.56	1.70	1.60	1.20
Masaka meat	0.00	0.00	0.00	0.00
Orange shells	1.51	1.10	1.10	1.20
Orange meat	0.00	0.00	0.00	0.00
Standard Deviation	0.78	0.81	0.75	0.64
Range	0.00 – 1.57	0.00 – 1.56	0.00 – 0.77	0.00 -1.24

Table 3: Cultural, Morphological and Biochemical Characteristics of Bacterial Isolates from *Tympanotonus fuscatus* var. radula sold in markets in Nasarawa State Nigeria

Cultural Characteristics				**Biochemical Characteristics**							**Sugar Fermentation Tests**					
Colony Edge	**Appearance after Culture**	**Shape**	**Grain Stain**	**Indole**	**Catalase**	**Coaglulase**	**Oxidase**	**Methyl Red**	**Voges Proskauer**	**Citrate**	**Glucose**	**Lactose**	**Fructose**	**Maltose**	**Sucrose**	**Probable organism**
Circular and entire	Greenish-metallic sheen on EMB	Rods	-	+	+	-	-	+	-	-	+	+	+	+	+	*Escherichia* sp.
Irregular	Smooth pale on Mac	Rods	-	-	+	-	-	+	-	+	+	+	-	+	+	*Salmonella* sp.
Entire	Bluish green on NA	Rods	-	-	+	-	+	-	-	+	-	-	+	-	-	*Pseudomonas* sp.
Entire	Golden-yellow on MSA	Cocci in clusters	+	-	+	+	-	+	-	-	-	+	+	+	+	*Staphylococcus* sp.
Entire	Colourless on EMB	Rods	-	-	+	-	-	+	-	+	+	-	-	-	-	*Proteus* sp.
Irregular & Rhizoid	Creamy white on NA	Rods	+	-	+	-	-	-	-	+	+	+	-	-	-	*Bacillius* sp.
Circular	Red glistening colony on NA	Rods	-	-	-	-	-	-	+	+	+	-	-	+	+	*Serratia* sp.
irregular	Red colony on NA	Rods	-	-	+	+	+	-	-	+	+	-	+	+	-	*Enterobacter* sp.

Key: + = Positive, – = Negative, EMB= Eosine Methylene Blue, NA= Nutrient Agar, Mac= MacConkey Agar, MSA= Manitol Salt Agar

Table 4: Frequency of Occurrence of Bacterial Isolates

Isolate	Frequency	Percentage (%)
Escherichia coli	6	24.0
Bacillus sp.	8	32.0
Pseudomonas sp.	4	16.0
Serratia sp.	2	8.0
Staphylococcus aureus	1	4.0
Proteus sp.	1	4.0
Enterobacter sp.	2	8.0
Salmonella sp.	1	4.0

Table 5: Antibiotic Susceptibility Profile of Gram Negative Bacterial Isolates

Antibiotics (µg)	Zones of Inhibition (mm)						Overall (%)		
	Pseudomonas sp.	*Salmonella* sp.	*E. coli*	*Enterobacter* sp.	*Proteus* sp.	*Serratia* sp.	**S**	**I**	**R**
Ciprofloxacin (10)	23.0	37.0	32.0	41.5	40.0	26.0	6(100)	0(0)	0(0)
Gentamicin (10)	43.0	2.0	19.0	27.0	3.5	26.4	4(66.6)	0(0)	0(0)
Ampiclox (30)	43.5	16.0	29.0	38.0	19.4	35.0	5(83.3)	0(0)	2(33.3)
Septrin (30)	2.0	38.0	34.0	28.0	18.0	32.0	5(83.3)	0(0)	1(16.5)
Tetracycline (25)	16.0	6.0	27.0	41.5	4.0	32.0	3(50.0)	2(33.3)	1(16.5)
Chloramphenicol (30)	28.2	5.0	29.0	18.0	6.0	14.0	3(50.0)	2(33.3)	1(16.5)
Erythromycin (30)	38.0	17.0	2.0	6.0	34.0	17.0	2(33.3)	2(33.3)	2(33.3)
Augmentin (30)	26.6	20.0	45.7	38.0	37.0	18.6	6(100)	0(0)	0(0)
Vancomycin (10)	4.5	5.0	12.0	10.0	11.0	10.0	0(0)	0(0)	6(100)
Ampicillin (10)	22.0	10.0	34.0	10.0	4.0	18.0	3(50.0)	3(50.0)	0(0)

Key: Zone of inhibition ≥ 18mm = Susceptible (S)

Zone of inhibition 13 – 17mm = Intermediate (I)

Zone of inhibition <17mm = Resistant (R)

Table 6: Antibiotic Susceptibility Profile of Gram Positive Bacterial Isolates

Antibiotics	Concentration (µg)	Zones of Inhibition (mm)		Overall (%)		
		Bacillus spp.	*Staphylococcus aureus*	**Susceptible**	**Intermediate**	**Resistance**
Ciprofloxacin	10	28.0	31.0	2(100)	0(0)	0(0)
Gentamicin	10	24.0	36.0	2(100)	0(0)	0(0)
Ampiclox	30	12.0	24.0	1(50)	0(0)	1(50)
Septrin	30	4.0	21.0	1(50)	0(0)	1(50)
Tetracycline	25	2.0	28.5	1(50)	0(0)	1(50)
Chloramphenicol	30	10.0	15.0	0(0)	1(50)	1(50)
Erythromycin	30	8.0	28.5	1(50)	0(0)	1(50)
Augmentin	30	18.5	19.3	2(100)	0(0)	0(0)
Vancomycin	10	6.0	22.0	1(50)	0(0)	1(50)
Ampicillin	10	17.0	37.7	0(0)	0(0)	0(0)
% Susceptibility		3(30)	9(90)			
% Intermediate		1(10)	1(10)			
% Resistance		6(60)	0(0)			

Key: Zone of inhibition ≥ 18mm = Sensitive (S)

Zone of inhibition 13 – 17mm = Intermediate (I)

Zone of inhibition <17mm = Resistant (R)

RESULTS AND DISCUSSION

Table 1 depicts the mean bacterial load of Raw *Tympanotonus fuscatus var*. radula sold in markets in Nasarawa State. The total bacterial count (TBC) varied from 1.18 – 3.20 x 10^8 cfu//g, total coliform count (TCC) varied from 1.20 – 2.80 x 10^6cfu/g, total Salmonella/Shigella (TSS) varied from 1.00 - 1.85 x 10^6cfu/g and total faecal coliform (TFC) varied from 1.10 x 2.30 x 10^6cfu/g in the raw periwinkles. Raw periwinkles with shells from Masaka market had the highest bacterial load with a TBC of 2.94 x 10^8cfu/g and a TCC of 2.80 x 10^6cfu/g. Raw periwinkle samples without shells from Keffi market had the least bacterial load with a TBC of 1.20 x 10^8cfu/g and TCC of 1.20 x 10^6cfu/g. Table 2 depicts the mean bacterial load of boiled *Tympanotonus fuscatus var.* radula sold in markets in Nasarawa State. The TBC varied from 0 - 1.57 x 10^8 cfu/g, TCC varied from 0 – 1.56 x 10^6cfu/g. Boiled periwinkle with shells from Masaka market had the highest TBC of 1.56 x 10^8cfu/g and a TCC of 1.70 x 10^6cfu/g. The raw periwinkles with shells had a higher bioload due to the spiral shaped nature of the shells which makes it easy for the bacteria to harbor the periwinkles. The high bioload recorded in the periwinkle samples could be attributed to the fact that water bodies from which *Tymapanotonus fuscatus var* .radula are harvested are contaminated and since the periwinkles are filter feeders there is a tendency that they will accumulate high levels of pathogens as a result of cross contamination. Ekanem and Adegoke (1995) stated that the level of pollution of the cultivation waters determines the level of contamination of shellfish. The presence of enteric organisms in the presence study is an indication of pollution of their underlying waters with untreated faecal waste and sewage. This result is in consonance with previously reported works (Adebayo-Tayo *et al* 2006; Nwiyi and Okonkwo, 2013).

After boiling under laboratory condition of 100^{o}C for 5 minutes, the bacterial load in the shelled samples reduced drastically. On boiling the bacteria in the periwinkles without shells were significantly lower than those in the boiled periwinkles with shells ($P<0.05$). This result is in consonance with the work of Omenwa *et al*. (2013). The bacterial load in the periwinkle samples exceeded the acceptable limit as suggested by the International Commission on Microbiological Specifications for food (ICMSF., 1982) and the US Food and Drug Administration (FDA., 1991) that a maximum microbial count of not greater than 1 x 10^5 cfu/g and coliform levels not more than 1 x 10^2 cfu/g for shellfish.

The bacteria isolated from the periwinkle includes: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* species, *Proteus* species, *Enterobacter* species, *Serratia* species, *Salmonella* species and *Bacillus* species. They are all significant to human health. Enteric organisms such as *Enterobacter* specie caused septicemia and neonatal meningitis. *Staphylococcus aureus* is a major cause of cerebrospinal fluid shunts in children. The presence of *Salmonella sp* in the periwinkle samples is significant as this organism is one of the most important foodborne pathogens and are indicators of sewage contamination and is found to be associated with a number of non-human hosts such as reptiles (Adebayo-Tayo and Okpo, 2010). *Salmonella* survives and persist in the aquatic environment. It has been detected in periwinkles from different creeks (Adebayo-Tayo *et al.*, 2006). The presence of *Escherichia coli* in the samples is an indication of secondary contamination. As *E. coli* are known to be associated with gastro-intestinal tracts of warm blooded animals and are known to be present in the environment as natural flora. This secondary contamination may be as a result of sewage contamination of the harvesting areas. *E. coli* causes infantile diarrhea and newborn meningitis, pneumonia and kidney infections (Kumar *et al.,* 2015). *Pseudomonas* specie commonly thrives in burns,

wounds and some blood infections. They are likely to have been introduced into the environment through swimmers and infected individuals who use the original habitats of these periwinkles for recreation. Therefore, pseudomonas may have occurred due to bathing of the locals with open wounds or other infections. *Bacillus cereus* causes a toxin mediated disease rather than infection (Bergdoll, 1981).

Table 3 depicts the morphological, cultural and biochemical characteristics of bacterial isolates from *Tympanotonus fuscatus var radula.* Table 4 depicts the frequency of occurrence of bacterial isolates. The most frequently occurring bacteria were *Bacillus* species, 8(32%); *Pseudomonas* species, 4(16%); *Escherichia coli*, 6(24%). The least frequently occurring were *Proteus* species, 1(4%), *Staphylococcus aureus*, 1(4%*)* and *Salmonella* species, 1(4%). Table 5 depicts the antibiotic susceptibility pattern of gram negative bacterial isolates from *Tympanotonus fuscatus var*. radula sold in markets in Nasarawa State. The result shows that all the gram negative organisms were susceptible to Ciprofloxacin and Augmentin; however, they displayed a 100% resistance to Vancomycin. The high performance of these antibiotics can also be due to their molecular sizes a factor which enhances their solubility in diluents thus promoting their penetration power through cell wall into the cytoplasm of the target microorganism as elucidated by Lin *et al.* (2015). Table 6 depicts the antibiotic susceptibility pattern of gram positive bacterial isolates from *Tympanotonus fuscatus var*. radula sold in markets in Nasarawa State. The gram positive organisms *Bacillus* species and *Staphylococcus* species were both susceptible to Ciprofloxacin, Gentamycin and Augmentin. The susceptibility pattern observed for the isolates in this study are comparable to those reported by Urassa *et al.* (2010), Isibor and Ekundayo (2012), Makut *et al.* (2014) and Ishaleku *et al.* (2015).

Conclusion

Tympanotonus fuscatus var radula periwinkles sold in markets in Nasarawa State are a good source of high quality animal protein, consumption should therefore be encouraged. Nonetheless, the presence of antibiotic resistant pathogens in the periwinkles is an indication that not cooking periwinkles properly could result in a health risk which could culminate in chemotherapeutic failure of commonly used antibiotics.

Recommendations

From the findings of this study, government should sponsor public enlightenment programmes on the inherent dangers of consuming raw or improperly cooked periwinkles with or without shells. The public should be made to understand how past outbreaks of food borne diseases occurred. More so, it should be emphasized that the storage and handling procedures should be done properly as most pathogenic organisms are transmitted by hands. Emphasis must be laid on adequate sanitary measures, good personal and environmental sanitary practices in the market and health education**.** Indiscriminate use of antibiotics should also be discouraged.

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