Detection of Extended Spectrum Beta-Lactamase *Escherichia coli* and Histamine Contents in Raw Mackerel (*Scomber japonicus*) Sold in Open Markets in Sagamu, Nigeria

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ABSTRACTS

Background: A food borne sickness called histamine fish poisoning is frequently brought on by eating some fish species that have high levels of histamine and other biogenic amines in their tissues. When fish is improperly stored and preserved, its natural makeup makes it polluted.

Objectives: This study was carried out to examine the histamine contents, determine the extended spectrum beta-lactamase producing isolates of *Escherichia coli* from the gut of raw mackerel fish obtained in Sagamu markets and relate the plasmid isolated, if present, with Extended Spectrum Beta Lactamase (ESBL).

Materials and Methods: Fifty (50) mackerel fish were dissected and their guts were removed for the isolation of *E. coli* on eosin methylene blue agar medium. The isolates obtained were Gram stained, biochemically characterized, and thereafter plated on Mueller Hinton agar impregnated with ESBL discs by double disc synergy methods. Ten gram (10 g) of each part of fish weighing 100 g was blended for histamine determination by spectrophotometry and plasmid analysis of some selected ESBL resistance amongst the isolates were determined.

Results: *Escherichia coli* were found in all fifty mackerel fish and 31 (62%) of those were ESBL producers. All of these were plasmid-free. In 14 (28%) of the 50 fish analysed, histamine concentrations of more than 100 mg/100 g exceeded the tolerance threshold of 10 mg/100 g,

Conclusion: The results from the study showed that some of the fish sold in the markets of Sagamu contain ESBL producing *Escherichia coli* which may be considered as reservoirs for resistant bacteria. Significant level of histamine recorded surpassed histamine tolerance level in fish for human consumption. There is a need to provide storage facilities and raise hygiene awareness in markets where fish is sold.

Keywords: Fish, Histamine, ESBL, *Escherichia coli,* Sagamu.

INTRODUCTION

Fish are aquatic, limbless cold blooded craniate, gill-bearing vertebrates that lack limbs with digits living wholly in water. Fish, a source of vitamin A, are edibles and are needed for healthy skin and good eye sight. Fish also contains vitamin D which enables the body to absorb calcium for strong bones and healthy teeth. Fish are rapidly perishable food; it deteriorates rapidly due to the enzymatic and metabolic processes mediated by bacteria within the gut and every other anatomical loci of the fish (1).

Fish, as important marine animal, possess an ideal intestinal breeding ground for bacteria to thrive and multiply, and may ingest antibiotic resistant bacteria from aquatic surroundings. Fish comes in contact with bacteria from the aquatic environment through natural activities and/or human imputes. The ingested bacteria could find fish gut as a suitable environment for horizontal transfer of antibiotic resistant gene and thereby help the spread of resistant factors (2).

Fish and fish products are of enormous relevance for human growth globally and can serve as source of transmission of food-borne pathogens with potential to spread marine related infection. According to a report by the Food and Agriculture Organization of the United Nations dubbed "GLOBEFISH-Information and Analysis on World Fish Trade", Nigeria is the world`s fourth largest importer of fish in terms of volume after China, Japan and the United States (3).

Approximately 80% of antimicrobials used in aquaculture to enhance fish production enter the environment with their potency intact and bacteria within the gut develop resistance to them by mutations or other means, and transfer these mobile genetic elements containing multiple resistance determinants to other bacteria (4).

Escherichia coli, a member of the bacterial family of *Enterobacteriaceae,* is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, as well as one of the most important pathogens (5). As a commensal, it lives in a mutually beneficial association with hosts and rarely causes disease. It is, however, also one of the most common human and animal pathogens as it is responsible for a broad spectrum of diseases. *E. coli* remains one of the most frequent causes of several common bacterial infections in humans and animals. The therapeutic treatment of *E. coli* infections could be threatened by the emergence of antimicrobial resistance (6).

Extended-spectrum β-lactamase (ESBL) production is one of the resistance mechanisms in *Enterobacteriaceae.* It can reduce the efficacy of modern expanded spectrum cephalosporins and monobactams drugs, with the exceptions of cephamycins and carbapenems. The emergence and spread of Extended-Spectrum β-Lactamases (ESBLs) among members of *Enterobacteriaceae* family originating from food-producing animals is a major public health issue worldwide (7). The persistent exposure of bacterial strains to a multitude of β-lactams has induced continuous production and mutation of newly developed β-lactamases, expanding their activity even against the newly developed ESBLs. ESBLs confer resistance to β-lactam antibiotics and are also associated with resistance to other classes of penicillin antibiotics, including fluoroquinolones, aminoglycosides trimethoprim, sulfamethoxazole, and ß-lactam/ß-lactamase inhibitor combinations (8).

Histamine (2-[4-emidazolyl] ethylamine) is an organic nitrogenous compound involved in local immune responses, as well as regulating physiological function in the gut and acting as a neurotransmitter for the brain, spinal cord, and uterus. Histamine belongs to the biogenic amine class and is synthesized by the pyridoxal phosphate (vitamin B6)-containing L-histidine decarboxylase (HDC) from the amino acid histidine. Histamine is a potent mediator of numerous physiologic reactions. Histamine exerts its effects on target cells in various tissues by binding to its receptor (9). Histamine fish poisoning is a food borne illness commonly caused by certain species of marine fish that have high level of histamine and other biogenic amines in their tissues. Harmful level of histamine can build up in fish before any spoilage develop, such as a bad smell or taste. A wide range of bacteria that are naturally found in fish environment are capable of producing histamine. Certain kinds of fish are more prone to cause histamine toxicity. These include tuna, mackerel, anchovy, herring, bluefish and amberjack (10).

The production of histamine is directly related to the mishandling of food as a result of poor storage system. The enzymes that produce histamine can be inactivated by freezing or cooking. But once histamine has been produced, it cannot be destroyed or eliminated by normal cooking or freezing temperatures, and its toxicity remains intact. Histamine fish poisoning or Scombroid fish, is a syndrome resembling an allergic reaction that occurs after eating fish and includes vomiting, diarrhoea, abdominal cramp, a peppery taste sensation, itching, hypotension, burning sensation of the mouth and lips, skin rash, head ache and skin rash. All these symptoms could threaten consumer's health and subsequent economic loss as a result of treatment costs (11). Fresh high-quality fish usually contains less than 10 mg/kg histamine, while the concentration can rise to over 1,000 mg/kg in older fish. Poisoning symptoms, known as histamine fish poisoning, may already appear when fish with a histamine level of 400 mg/kg is consumed.

This study was carried out to isolate ESBL producing *Escherichia coli* from locally marketed mackerel fish (*Scomber japonicus*) in Sagamu, Nigeria to determine the histamine contents by spectro-photometry and plasmid profiles, and relate the molecular weight obtained, if present, to the ESBL obtained from isolates of *E. coli* from the samples of the fish gut.

MATERIALS AND METHODS

Collection of Samples

Every day at 10am, ten samples of raw mackerel fish were bought, 5 each at the Sabo and Falawo markets in Sagamu, Nigeria for a period of 10 days out of which 50 samples were selected for this study due to financial constraint. The number of fish for each sampling trips were selected randomly from carton pack from various sellers and weighed to be 100 g each. The selected fish were transported immediately with a sterile nylon pack to the Department of Pharmaceutical Microbiology Laboratory, Olabisi Onabanjo University, Ogun State, Nigeria for microbiological analysis.

Gut Preparation for the Isolation of *Escherichia coli*

The gut material was aseptically removed from the raw fish by dissecting the fish using a sterile scalpel and forceps, and thereafter vortex mixed for 15 min. The subnatant was removed, serially diluted using peptone water to a dilution factor of 10^{-10} and was subcultured on eosin methylene blue (EMB) agar for isolation of *Escherichia coli* (12).

Biochemical Tests for *Escherichia coli*

The isolates obtained from EMB culture plates were observed morphologically and Gram stained. Conventional biochemical characterization test which include methyl red, indole, Voges Proskauer, oxidase, coagulase, catalase tests and other metabolic sugar test were carried out on the isolates.

Preparation of Fish for Histamine Determination

Separation of Histamine

Representative samples were prepared by blending 10 g of fish in an electric mixer with 90 mL of 10% trichloroacetic acid (TCA) for 1 to 2 min. The homogenate was filtered through a filter paper #3. Ten millilitres of TCA was transferred

into a test tube and the pH was adjusted to 4.62 with 5.6 mL of acetate buffer and 5–7 mL of KOH. The mixture was then transferred onto a 150 × 10 mm chromatographic column previously condi-tioned at pH 4.62 with 20 mL of acetate buffer that contained 1 g of the cationic exchange resin (Amberlite CG50, type 1, 100-200 mesh). The column was washed with 100 mL of acetate buffer and after all the drip had stopped, and the washing discarded. The retained histamine was eluted with 20 mL of 0.4 N H_2S0_4 (13).

Determination of Histamine

A volume of 2 mL of the above eluate was transferred into a test tube that was placed in an ice bath. Before that, 10 mL of a paranitroaniline solution at 0.1 g/100 mL of 0.1N HCl was placed in the ice bath for 5 min before adding 1 mL of an aqueous sodium nitrite solution at 4 g% (w/w), agitating and waiting for an additional 5 min at least. A volume of 0.5 mL of the diazonium reagent thus formed was added to the eluate, followed by the addition, 5 min later, of 0.5 mL of the coupling buffer that was previously prepared by mixing 7.15 g of sodium metaborate and 5.7 g of sodium bicarbonate in 100 mL of distilled water. Five minutes later, 0.25 g of disodium tetraborate ($Na_2B_40_7$, 10 H_20) was added and the mixture was agitated for 30 sec (final pH 8.6). After 15 min in ice, 5 ml of methyl isobutyl ketone was added and the mixture was again well agitated. Immediately after, both layers were transferred to a 16 × 150 mm test tube and left at room temperature for 10 min. The upper layer was then transferred to another 16 × 150 mm test tube containing 5.0 mL of Barbital buffer prepared by dissolving 1 g of sodium barbital in 100 mL of distilled water and adjusting the pH at 7.7 with diluted (1/12) acetic acid solution. The mixture was well agitated for 30 sec. The upper layer was again trans-ferred to a spectrophotometric cell using a fine-tip dropper and optical density (O.D.) was read at 475 nm on a spectrophotometer Varian DMS 70 against methyl isobutyl ketone. The external histamine standard used 2 mL of a solution containing 10 g histamine/mL 0.4 N H_2S0_4 and the blank consisted of 2.0 mL of eluate (14).

Histamine content was calculated as follows:

$$\text{mg\%} = \frac{\text{Sample O.D.} - \text{Blank O.D.}}{\text{Standard O.D.} - \text{Blank O.D.}^{x}}$$

Data Analysis

Descriptive statistics were utilized to determine the standard deviation and to analyse the data obtained.

Detection of ESBL

Double disk synergy method was used as the phenotypic method for the detection of ESBL. Ceftriaxone (30 µg) and Ceftazidime (30 µg) were placed 25 mm apart from Augmentin (Amoxicillin 20 µg/Clavulanate 10 µg) positioned at the centre of Müeller-Hinton agar medium seeded with the test organism. The plates were incubated for 24 h at 37°C. A clear extension of the edge of the zone of growth inhibition towards the disk containing Clavulanate was interpreted as synergy, indicating the presence of ESBL (15).

Plasmid DNA Extraction

The selected ESBL positive isolates were suspended in nutrient broth and incubated at 37ºC for 18-24 h. Thereafter, the bacterial suspension was vortex mixed for 15 min. About 1.5 mL of the broth was measured into a sterile Eppendorf tube and centrifuged at 13,000 rpm for 2 min. The supernatant was gently decanted and what was left of the broth was vortex mixed at high speed until a pellet was completely suspended in the broth. Thereafter, 300 µg of Tris 25 mM, EDTA 10 mM, NaOH 0.1 N and SDS 0.5% were added to the broth and mixed until the solution became slimy. A volume of 150 µL of 3.0 M sodium acetate (pH 5.2) was added to the mixture and vortex mixed for about 10 sec, followed by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred into another 1.5 mL Eppendendorf tube and 900 µL of ice-cold absolute ethanol was added. Following this, the supernatant obtained was vortex mixed and centrifuged at 13,000 rpm for 5 min, after which the supernatant was discarded leaving a white pellet at the bottom of the tube. One millilitre of ice-cold 70% ethanol was added to the pellet followed by centrifugation at 13,000 rpm. The process was repeated before air-drying the pellets (16).

Agarose Gel Electrophoresis

The dissolved pellets were mixed with 1 µL of a loading buffer. Thereafter, the mixture was loaded on 0.8% agarose in TBE buffer, stained with 0.01% ethidium bromide alongside *Hind* III marker. This was run at 100 V for 1.5 h after which the gel was viewed using the photo gel documentation system.

RESULTS

Histamine Contents Distribution and Statistical Summary of the Contents

Various histamine contents were elicited from a total of 50 mackerel samples selected. According to the information in Table 1, histamine levels ranged from 0.4 mg% in samples 1 and 37 from Sabo and Falawo markets to 102 mg% in sample 13 from Sabo market. Thirty-six (72%) had a tolerable histamine content of 0-10 mg%, while 14 (28%) provoked histamine toxicity with an average of 10.88 mg% and a standard deviation of 19.05 mg% as shown in Table 2.

Percentage Distribution of ESBL

A total of 50 *Escherichia coli* isolates selected from 100 gut samples of fish dissected were subjected to ESBL examination. The percentage of ESBL producer and ESBL negative of the isolates of *Escherichia coli* were in ratio of 62 and 38 percent as expressed in Figure 1.

Plasmid Analysis

The isolates did not elicit any plasmid since there were no visible bands from plasmid gel electrophoresis as shown in Figure 2.

DISCUSSION

One of the most frequent food poisonings brought on by consuming fish and fishery products is histamine poisoning.

Table 1: Histamine Contents in each Fish Sample

S/N	Market	Fish Type	Histamine Content (mg%)
1	Sabo	Mackerel	0.4
2	Sabo	Mackerel	1.34
3	Sabo	Mackerel	0.67
4	Sabo	Mackerel	10.4
5	Sabo	Mackerel	0.5
6	Sabo	Mackerel	1.2
7	Sabo	Mackerel	0.46
8	Sabo	Mackerel	1.68
9	Sabo	Mackerel	0.41
10	Sabo	Mackerel	0.76
11	Sabo	Mackerel	11.5
12	Sabo	Mackerel	12.6
13	Sabo	Mackerel	102
14	Sabo	Mackerel	2.6
15	Sabo	Mackerel	0.81
16	Sabo	Mackerel	0.96
17	Sabo	Mackerel	13.7
18	Sabo	Mackerel	1.3
19	Sabo	Mackerel	0.42
20	Sabo	Mackerel	1.39
21	Sabo	Mackerel	14.2
22	Sabo	Mackerel	11.8
23	Sabo	Mackerel	0.9
24	Sabo	Mackerel	7.08
25	Sabo	Mackerel	50.4
26	Falawo	Mackerel	6.01
27	Falawo	Mackerel	0.93
28	Falawo	Mackerel	15.9
29	Falawo	Mackerel	8.07
30	Falawo	Mackerel	0.85
31	Falawo	Mackerel	3.4
32	Falawo	Mackerel	25.54
33	Falawo	Mackerel	1.2
34	Falawo	Mackerel	45.65
35	Falawo	Mackerel	0.69
36	Falawo	Mackerel	0.67
37	Falawo	Mackerel	0.4
38	Falawo	Mackerel	35.9
39	Falawo	Mackerel	60.13
40	Falawo	Mackerel	0.71
41	Falawo	Mackerel	1.34
42	Falawo	Mackerel	4.64
43	Falawo	Mackerel	2.71
44	Falawo	Mackerel	9.1
45	Falawo	Mackerel	8.04
46	Falawo	Mackerel	2.16
47	Falawo	Mackerel	3.6
48	Falawo	Mackerel	0.64
49	Falawo	Mackerel	23.69
50	Falawo	Mackerel	32.76

Table 2: Statistical Summary of Histamine Contents in Mackerel

Range of Histamine Levels (mg%)	Number of Fish Sample	% of Sample within the Range
0 – 10	36	72
11 – 20	6	12
21 – 30	2	4
31 – 40	2	4
41 – 50	1	2
51 and above	3	6
Total Average		10.88 mg%
Standard deviation		19.05 mg%
Range		0.4–102 mg%

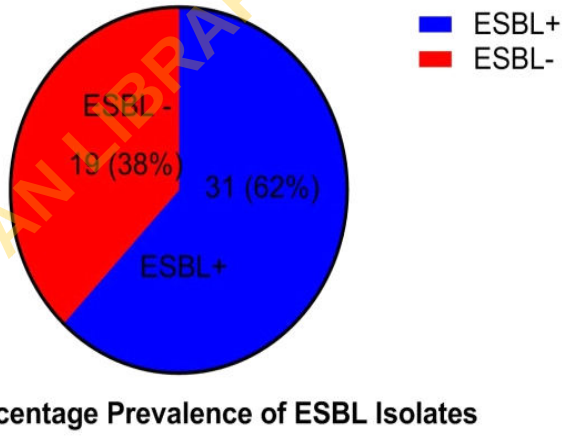

Fig. 1: Percentage Distribution of ESBL Isolates of *Escherichia coli*.

(L–R: 100 BP marker, lanes 28-42)

M 28 29 30 31 32 34 35 36 37 38 39 40 41 42

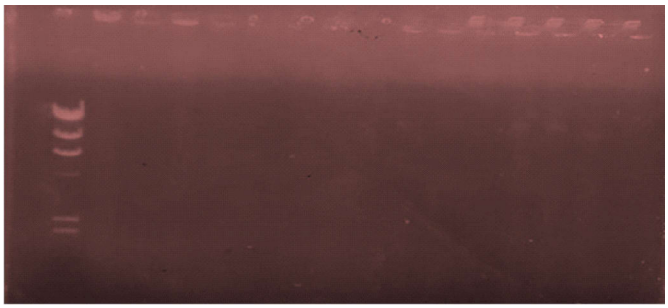

Fig. 2: Plasmid Analysis of the selected ESBL Positive showing no Profiles after Trans-illumination.

The key determinants of the toxicological reaction in consumers that may result in atopic dermatitis, allergic rhinitis, and allergic asthma are the amount of histamine in food, individual sensitivity, and detoxifying activity; these spectrums of discomfort has the potential to impair human health (17).

A total of 50 raw mackerel fish selected from a pool of 100 were processed for commercial purpose (each weighing 100 g); these were examined for histamine contents and ESBL producing *Escherichia coli*. The fish were randomly selected from the carton packs from different sellers in Sabo and Falawo markets within the morning hours, at exactly 10am purposely to reduce the possibility of deterioration as time advances due to the lack of modern storage facility (4). A total of 14 (28%) of the 50 fish sampled had high histamine contents, while the 36 (72%) within the range of 10 and 50 mg% were considered tolerable. Sample number 13 had histamine value above 100 mg%/100 g which is considered to be extremely toxic in agreement with a review of the literature on 250 cases of histamine poisoning which stated that intoxication of 5-10 mg of histamine /100 g of fish is safe, 10-100 mg of histamine /100 g is toxic, and above 100 mg of histamine /100 g of fish is too toxic in the study of histamine poisoning from ingestion of fish or scombroid syndrome (18). However, susceptible individuals could develop allergic reaction and other histamine intolerant symptoms at 5 mg%. The variation in histamine contents obtained in this study could be attributed to inherent property of the microbes in the fish gut brought by process of decarboxylation induced mainly by metabolic enzymes of *E. coli* and other enteric bacterial in the intestine and skin of the fish, which corroborates the findings of the study of Thangam *et al.* (18) on histamine poisoning from ingestion of fish, an allergy-like intoxication. The reports on histamine intoxication generally involve only a small number of individuals, so it is difficult to estimate the dose/exposure level in order to construct quantitative assessment of dose versus adverse response. Histamine is not found in live fishes, but it is produced after been killed, harvested and stored in an inadequate cooling and preservation. There was no remarkable difference in histamine contents obtained from the two markets which could be due to similar problems of lack of storage facility both markets were experiencing. Histamine food poisoning accounts for about 5% of all reported food poisonings in the United States and about 40% of poisoning resulting from ingestion of fish (19). The variability of symptoms can be linked to both the amount of histamine ingested and individual sensitivity. The ingestion of food containing small amounts of histamine has little effect in healthy individuals, but it can result in histamine intolerance in persons characterized by impairment of diamine oxidase activity, either due to genetic predisposition, gastrointestinal diseases, or medication with monoamine oxidase inhibitors (20). However, due to poor data storage and preservation equipment in Nigeria, it is believed that the incidence may be higher than the above stated data, which may not have been reported to the existing authorities that are virtually non-existing in Nigeria due to the lack of manpower, funding and investigative tools (21).

Bacterial spoilage and production of histamine can occur at any stage of the food chain (fishing, fish landing, processing, distribution system, or handling during catering at home). Histamine is produced as a product of microbial enzyme metabolic decarboxylation of histidine in the gut of processed fish. *Escherichia coli* was found in all the samples examined, which was expected as a result of its digestive roles in fish gut. In this study, 62% of ESBL positive were observed as shown in Figure 1 with zones of overlapping synergy. The percentage obtained in this study is remarkable, an indication of prevalence of resistant factor rapid spreads within the aquaculture; this corroborates the findings of Carbello *et.al.* (22) in the study on antimicrobial use in aquaculture and its relevance to antimicrobial resistance. Histamine forming bacteria are able to grow more rapidly at high abuse than at moderate abuse temperatures.

Gel electrophoresis of some selected ESBL positive isolates showed no bands of plasmid as shown in Figure 2. However, the absence of plasmids does not imply that the isolates obtained were not resistant to antimicrobials. The resistant factor could be chromosomally mediated or through other mobile genetic element. This study proves that the raw mackerel fish sold in Sagamu markets serve as a reservoir for resistant bacteria. This correlates with the findings of Elhadi (23) in the study on the prevalence of extended spectrum β-lactamase producing *E. coli* in fish in Eastern province of Saudi Arabia. Oral administration of pure histamine does not cause systemic effects; it is inactivated in the intestine prior to entering the portal circulation and thereafter converted by the enteric flora and when consumed with fishes, the presence of diamine enhancers interferes with the protective action of intestinal mucin which binds histamine and prevents enteric absorption (24, 25).

CONCLUSION

Twenty eight percent (28%) of the samples examined exceeded histamine tolerant threshold in fish for human consumption and the alarming 62% ESBL producer obtained though were not plasmid borne, could be caused by chromosome and other genetic factors which could mediate antimicrobial resistance as a precursor for therapeutic failure. The data obtained in this study suggest the possibility of mishandling the fish during processing. There is therefore need to provide storage facilities to prevent rapid deterioration to reduce the episode of histamine and ESBL production, thereby minimize the associated health hazard implication and economic lost.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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