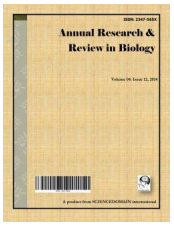

Annual Research & Review in Biology

29(1): 1-7, 2018; Article no.ARRB.44236
ISSN: 2347-565X, NLM ID: 101632869

Identification and Biotyping Enterotoxigenic *Escherichia coli* Strains from Tuna Loins and Flakes Produced in Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/44236
Editor(s):
(1) Dr. Viduranga Y. Waisundara, Faculty of Applied Sciences, Rajarata University of Sri Lanka, Mihintale, Sri Lanka.
(2) Dr. George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) R. Jasmine, Bishop Heber College, India.
(2) P. A. Tsaku, Nasarawa State University, Nigeria.
(3) Ene Paschal Chuka, School of Health Technology, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/26642

Original Research Article

Received 30 August 2018
Accepted 29 September 2018
Published 15 October 2018

ABSTRACT

Aims: The study aims to explore the enterotoxigenic *Escherichia coli* (ETEC) strains isolated from tuna loins and flakes produced in Côte d'Ivoire and identify their biotypes.
Study Design: Tuna loins and flakes were obtained from two industries located in Abidjan. Samples

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of about 500 g frozen tuna were collected in a polyethylene bag and labelled. Samples were stored in an ice box and sent to the laboratory for determination of virulence genes..
Place and Duration of Study: The study was conducted at Central Laboratory for Food Hygiene and Agribusiness, LANADA, Abidjan and Laboratory of Biotechnology and Food Microbiology, Department of Food Science and Technology, University of Nangui Abrogoua Abidjan, Côte d'Ivoire during June 2013 to September 2013.
Methodology: 460 *Escherichia coli* strains isolates were analysed for the presence of diarrhoea-associated genes (*elt* and *est*) by multiplex PCR using specific primers and for the biotyping of ETEC strains based on the characters highlighted with the API 20E gallery.
Results: Forty-four isolates (21 from tuna loins and 23 from tuna flakes) were identified for ETEC, including 22 positive for *elt*, 8 positive for *est* and 14 positive for both *elt* and *est*. Four biotypes (biotype 1, 2, 3 and 4) were observed in this study. Biotype 2 [LDC (-), ODC (-)] was the most prevalent in the strains with frequencies of 56.8% followed by biotype 1 (31.8%), biotype 3 (6.8%) and biotype 4 (4.5%).
Conclusion: This study revealed the presence of different biotypes diarrhoeagenic *E. coli* (ETEC) and potential public health risks if tuna products are not properly cooked.

Keywords: Tuna loins; tuna flakes; Escherichia coli; virulence; biotype; Côte d'Ivoire.

1. INTRODUCTION

Escherichia coli are known as a facultative anaerobic bacterium found in the normal flora of the intestinal tract of humans and most homeothermic or warm-blooded animals [1]. Currently, *E. coli* are widely used as a sanitation indicator of microbiological contamination in water and food [2]. While *E. coli* is harmless in general, certain virulent strains are common causes of infectious diarrhoea and other enteric diseases [3]. Each year, *E. coli* strains are responsible for 2 million deaths worldwide, through intestinal or extraintestinal infections [4]. Enterotoxigenic *Escherichia coli* (ETEC) is a bacteria that colonise the small intestine and cause severe diarrhoea, dysentery, abdominal cramps, and fever. ETEC can be life-threatening due to the significant fluid loss and severe dehydration. Beyond its burden in endemic countries, ETEC is the leading cause of diarrhoea in travellers from developed regions returning from vacation in low resource countries. Enterotoxigenic *E. coli* were discovered in the course of a clinical investigation of patients with *Vibrio cholerae* culture- negative stools presenting with clinical cholera characterised by acute onset of watery diarrhoea and severe dehydration [5]. ETEC remains among the most common bacterial causes of diarrhoea-associated morbidity and mortality [6,7]. ETEC is often the first bacterial illness that children experience in endemic areas, with infants and young children experiencing two to five diarrhoea episodes due to ETEC during their first three years of life [8]. Recent studies in sub-Saharan Africa and south Asia conducted under the Global Enteric Multicenter Study (GEMS) re-affirmed the continuing importance of ETEC as one of the top four causes of moderate-to-severe diarrhoea (MSD) among children less than five years of age seeking care for their illness at health centres in both the regions [6].

Cultural characterisation of *E. coli* by using different media and biochemical characterisation by observing the variable reaction to different sugars and chemicals are the basic rules for their identification [9]. *E. coli* can ferment a variety of carbohydrate substrates, generally by converting them to glucose or a substrate on the fermentative chain of the breakdown of glucose. The ability to ferment a given sugar of the types described above by a strain of *E. coli* is dependent on the strain having the requisite enzymes to convert it into glucose or a substance on the degradative chain from glucose [10].

There are 2 types of tuna products exported from Côte d'Ivoire: (1) Tuna finished products (canned) and, (2) tuna semi-finished products (tuna loins, tuna flakes, tuna skin and tuna pulp). The tuna loins are portions of the tuna flesh usually skinless and boneless and ready to use, and tuna flakes are pieces of tuna got back during trimming of tuna loins. They are intended for canning factories and fast food. A potential public health risk exists if these semi-finished products were contaminated by enterotoxigenic *E. coli*. In addition, the detection and identification of *E. coli* biotypes in tuna product have not been reported in the study area.

The present work was designed to identify different biotypes of enterotoxigenic *E. coli* (ETEC) isolated from tuna loins and flakes produced in Côte d'Ivoire isolates using standard biochemical and genotypic tests.

2. MATERIALS AND METHODS

2.1 Sample Collection

Tuna loins and flakes were obtained from two industries located in Abidjan (economic capital of Côte d'Ivoire) during September 2011 to September 2013. Per sampling day, samples of about 500 g frozen tuna was collected aseptically in a polyethylene bag from each industry. Per month, the number of samples collected and analysed depends on the importance of the tuna loins production. A total of 471 tuna loins samples and 222 tuna flakes were collected from both industries. Each sample was labelled and stored in an ice box and sent to the laboratory for further analysis.

2.2 Isolation of *Escherichia coli* Strains

The *E. coli* isolation was carried out on RAPID' *E. coli* 2 selective chromogenic medium (Bio-rad, France) according to ISO 16140. Presumptive *E. coli* strains with positive indol, negative citrate, and negative urea was confirmed as *E. coli*. *E. coli* strain of American Type Culture Collection 25922 (ATCC 25922) was used as control.

2.3 Determination of Enterotoxigenic *Escherichia coli* (ETEC) by PCR

DNA of each isolate was extracted according to the boiling method. Approximately 5 to 10 colonies of overnight incubated bacterial culture were taken and suspended in 100 μL of distilled water. The mixture was stored at −20°C for 10 min and then boiled at 100°C for 10 min. After centrifugation in a Mikro 220R Hettich centrifuge at 14000 RPM for 10 min, the supernatants were separated and used for PCR amplification. The amplification reactions were carried out in a reaction mixture of 25 μL containing 10 μL of Master Mix 1x (5PRIME Hot Master Mix 2.5x Dominique DUTSCHER, France), 1.4 μM concentration (each) of primers (Table 1), and 5 μL of the DNA template. The PCR amplification was performed by using a thermocycler system (Applied Biosystems, 2720 Thermal Cycler, USA). The amplification program included an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation (94°C for 1 min), primer annealing (52°C for 1 min), and extension (65°C for 1 min), with a final extension at 65°C for 10 min. PCR products (10 μL) were resolved by electrophoresis on a 2.5% agarose gel (Promega, USA) at 120 mV for 80 min. Agarose gel was then stained with ethidium bromide (Sigma Aldrich, USA), and the DNA bands were visualised and photographed under UV illumination (UV UVItec, UK). The buffer used in the electrophoresis chamber (PCRSCIE-PLAS, China) and agarose gel was 1x Tris-borate-EDTA (89 mM Tris-borate, 2.5 mM EDTA).

2.4 Biotyping of Enterotoxigenic *Escherichia coli* (ETEC) Strains

The biotyping of ETEC strains consists in establishing a biochemical profile of the strains, based on the characters highlighted with the API 20E gallery. The analysis of the results of the test, taking into account the biochemical variants, served as a discriminating factor from one strain to another for determination of biotypes. These different biochemical tests are the search tool for: lysine decarboxylase (LDC); ornithine decarboxylase (ODC). These tests also highlight the study of acidification of carbohydrates and derivatives: inositol (INO), sorbose (SOR), rhamnose (RHA), sucrose (SAC), and amygdalin (AMY).

3. RESULTS AND DISCUSSION

3.1 Identification of Enterotoxigenic *Escherichia coli* (ETEC) Strains

A total of 44 ETEC were detected from 460 *E. coli* strains (Table 2). Twenty-one and twenty-three strains of this pathotype were identified in

Table 1. Primers used for PCR in this study [11]

Genes	Sequence (5 to 3')	Size (pb)	References
elt	F TCTCTATGTGCATACGGAGC R CCATACTGATTGCCGCAAT	322	[12]
est	F TTAATAGCACCCGGTACAAGCAGG R CCTGACTCTTCAAAAGAGAAAATTAC	147	[13]

the samples of tuna loins and flakes, respectively.

The prevalence of ETEC was 9,6% with 9.7% and 9.3%, respectively, for tuna loins and flakes. Previous studies conducted in Brazil, South Korea and Egypt reported the presence of ETEC in fish and seafood [14,15,16]. In Côte d'Ivoire, Kambire et al. [17] andToe et al. [18] reported a predominance of ETEC pathotype in the Aby Lagoon and vegetable salads respectively. ETEC strains are associated with two major clinical syndromes: child diarrhea in developing countries and traveller's diarrhoea (Turista) [19]. ETEC is the most frequent bacterial cause of diarrhoea among travellers to Africa, Asia and Latin America, including military personnel deployed to these areas [8]. According to Turner et al. [20], ETEC is regarded as the major cause of *E. coli* mediated diarrhoea in human worldwide, affecting mainly children and travellers. Recent data strongly suggest that ETEC infections in travellers can increase the risk of subsequent functional bowel disorders. In fact, 10-14% of travellers recovering from ETEC-associated travellers' diarrhoea may go on to develop irritable bowel syndrome [21].

Table 3 shows the distribution of virulence genes in tuna loins and flakes. Genes belonging to the enterotoxigenic *E. coli* (ETEC) strains were detected with frequencies of 50%, 18.2% and 31.8% respectively for the "*elt*", "*est*" and "*elt* + *est*" genes. In tuna loins, 47.6% of the strains possess the "*elt*" gene, 23.8% "*est*" gene and 28.6% of both "*elt*" and "*est*" genes. Frequencies of 52.2%, 13%, and 34.8% were obtained for the genes "*elt*", "*est*", and the strains possessing both "*elt*" and "*est*" genes respectively, in tuna flakes. According to Rigobelo et al. [22], ETEC causing traveller's diarrhoea, particularly in developing countries are characterised by the presence of "*est*" gene encoding the synthesis of thermostable enterotoxin and the "*elt*" gene encoding the synthesis of thermolabile enterotoxin. ETEC possessing both the "*est*" and "*elt*" genes were detected. This result is similar to other reports [11,23,24]. According to Quadri et al. [19], ETEC possessing the "*elt*" gene are less involved in disease in compared to those with only the "*est*" gene or both "*est*" and "*elt*".

3.2 Biotype of Enterotoxigenic *Escherichia coli* (ETEC) Strains

Biotypes of enterotoxigenic *E. coli* (ETEC) strains were presented in Table 4, where four biotypes were observed. The biochemical differences were in the decarboxylation reactions of lysine (LDC) and ornithine (ODC) as well as in the use of sugars such as sucrose and amygdalin. Biotypes 1, 3 and 4 were LDC (+) while biotype 2 was LDC (-). Biotypes 1, 2 and 4 were ODC (-) and sucrose (-) while biotype 3 was ODC (+) and sucrose (+). Biotypes 1, 2 and 3 were amygdalin (-) while biotype 4 was amygdalin (+). Biotype 2 was the most prevalent in the strains with frequencies of 56.8% followed by biotype 1 with frequencies of 31.8%. Biotypes less encountered were biotypes 3 and 4 with respective frequencies of 6.8% and 4.5%. Table 5 shows the distribution of different biotypes in tuna loins and flakes. In tuna loins, 42.9% of strains belongs to biotype 1; 52.4% to biotype 2 and 4.8% to biotype 3. Biotype 4 was not identified in tuna loins. In tuna flakes, 21.7% of strains belongs to biotype 1; 60.9% to biotype 2; 8.7% to biotype 3 and 8.7% to biotype 4. The distribution of isolates in different biotypes indicates a wide variety of enzymes that ferments a given sugar which further implies the diverse nature of the bacteria. Biochemical reactions have conventionally been used for identification of bacteria to the species level. Extensive studies of sugar fermentation reactions of bacteria have been performed to introduce biochemical typing systems in epidemiological studies of bacteria [25]. The ability to ferment a given sugar described above by a strain of *E. coli* is dependent on the strain having the requisite enzymes to convert it into glucose or a substance on the degradative chain from glucose. It has been recorded that different strains of *E. coli* differ in their ability to perform these conversions [10]. The low diversity of biotypes in this study could be explained by the fact that these strains could have the same (human) origins. In contrast, several studies

Table 2. Distribution of enterotoxigenic *Escherichia coli* (ETEC) strains in tuna loins and flakes

	Loins	Flakes	Total
Number of strains	217	246	460
ETEC strains	21 (9.7%)	23 (9.3%)	44 (9.6%)
Non-pathogenic strains	196 (90.3%)	223 (90.7%)	416 (90.4%)

Table 3. Prevalence of virulence genes in tuna loins and flakes

Source	Virulence genes N (%)		
	elt	***est***	***elt + est***
Tuna Loins	10 (47.6%)	5 (23.8%)	6 (28.6%)
Tuna Flakes	12 (52.2%)	3 (13%)	8 (34.8%)
Total	22 (50%)	8 (18.2%)	14 (31.8%)

Table 4. Differential characteristics and frequencies of biotypes of enterotoxigenic *E. coli* (ECET)

	Biotype 1	Biotype 2	Biotype 3	Biotype 4
Lysine Decarboxylase	+	-	+	+
Ornithine Decarboxylase	-	-	+	-
Inositol	-	-	-	-
Sorbose	+	+	+	+
Rhamnose	+	+	+	+
Sucrose	-	-	+	-
Amygdalin	-	-	-	+
Digital profile	50445525	10445525	51445725	50445535
Fr Biotype N (%)	14 (31.8)	25 (56.8)	3 (6.8)	2 (4.6)

(-) = negative reaction; (+) = positive reaction

Table 5. Distribution of biotypes of enterotoxigenic *E. coli* (ETEC) strains in tuna loins and flakes

	Biotype 1	Biotype 2	Biotype 3	Biotype 4
Tuna loins	9 (42.9)	11 (52.3)	1 (4.8)	0 (0)
Tuna flakes	5 (21.7)	14 (60.9)	2 (8.7)	2 (8.7)
Total N (%)	14(31.8)	25 (56.8)	3 (6.8)	2 (4.6)

reported the presence of14 biotypes in the Fresco lagoon and fish in Côte d'Ivoire [26]; 7 biotypes in cattle in Coimbatore [27] and 15 biotypes in cases of diarrhoea in Ethiopia [28]. Biotypes 1, 2 and 3 recorded in this study were similar to those reported on fish and the Fresco lagoon in Côte d'Ivoire [26].

4. CONCLUSION

Present study aimed to identify different biotypes of enterotoxigenic *E. coli* (ETEC) strains isolated from tuna loins and Flakes. It can be concluded that, identification of "*est*" and "*elt*" genes revealed the presence of enterotoxigenic *E. coli* (ETEC). Four biotypes were recorded in this study and biotype 2 [LDC (-), ODC (-)] was the most prevalent. This biotyping indicated the diverse nature of the studied organism in this area. For companies producing these tuna loins and flakes, hygiene measures are necessary during production to preserve the health of the consumer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26642