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Virulence Genes and Antibiotic Resistance Profile of *Escherichia coli* Strains Isolated From Tuna Loins and Flakes Produced in Côte D'ivoire

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A B S T R A C T

This study was conducted to characterize virulence genes of *Escherichia coli* isolates from tuna loins and flakes produced in Côte d'Ivoire. In total, 460 *Escherichia coli* isolates were analyzed for the presence of diarrhea-associated genes (eae, stx, AggR, elt, and est) by multiplex PCR using specific primers and for their susceptibility to 10 different antimicrobials according to the disk diffusion susceptibility test. 44 isolates (21 from tuna loins and 23 from tuna flakes) were positive for virulence genes, including 22 positive for elt (ETEC), 8 positive for est (ETEC) and 14 positive for both elt and est. The resistance to amoxicillin was most prevalent (15.9%), followed by that to amoxicillin/clavulanic acid (9.1%), gentamicin (2.3%) and chloramphenicol (2.3%). 4.3% were resistant to amoxicillin and gentamicin; these were consequently defined as multidrug resistant. This study revealed the presence of diarrhea genic and multidrug resistant *E.coli* and potential public health risks if tuna products are not appropriately cooked.

Introduction

Escherichia coli are known a facultative anaerobic bacterium found in the normal flora of the intestinal tract of humans and most homeothermic or warm-blooded animals (Bettelheim, 1997). Currently, *E. coli* are widely used as a sanitation indicator of microbiological contamination in water and food (Lang *et al.*, 1999). While, *E. coli* is harmless in general, certain virulent strains are common causes of infectious diarrhea and other enteric diseases (Clements *et al.*, 2012). Each year, *E. coli* strains are responsible for 2 million deaths worldwide, whether through intestinal or extra intestinal infections (Russo and Johnson, 2003).

Extra-intestinal pathogenic *E. coli* (ExPEC) strains are usually able to cause infections in anatomical sites outside of the intestinal tract and are associated with urinary tract infections, neonatal meningitis and septicemia. ExPEC, like commensal *E. coli*, can colonize

the intestinal tract without causing gastroenteritis. In contrast, intestinal pathogenic *E. coli* (InPEC) strains can cause different types of gastroenteritis and can be divided into six pathogenic groups: enterohaemorrhagic (EHEC); enteropathogenic (EPEC); enteroaggregative (EAEC); enterotoxigenic (ETEC); enteroinvasive (EIEC) and diffusely adherent (DAEC) *E. coli* (Kaper *et al.*, 2004). Among the *E. coli* pathogenic strains, in most developing countries, EPEC, ETEC, and EAEC are the most common cause of infectious diarrhea in young children (Bii *et al.*, 2005).

Antibiotic resistance in pathogenic bacteria is recognized as a global problem in public health. It compromises the prevention and effective treatment of an increasing number of infections caused by bacteria. In addition, it increases the cost of health care by extending the length of hospital stays, requiring more intensive care and more expensive drugs. In Côte d'Ivoire, Enterobacteriaceae resistant to antibiotics including *E. coli* were different origin such as water (Coulibaly *et al.*, 2014), food (Dadié *et al.*, 2003), products from livestock (Koffi *et al.*, 2014; Bonny *et al.*, 2015) and human biological products (faeces, urine, blood) (Adele, 2015).

There are 2 types of tuna products exported in Côte d'Ivoire: Tuna finished products (canned) and 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 for fast food. A potential public health risk exists if these semi-finished products were contaminated by *E. coli* pathotypes on the one hand and on the other hand if these strains are resistant to antibiotics.

The aim of this study was to determine the profile of antimicrobial resistance and virulence factors in strains of *E. coli* isolated from tuna loins and flakes produced in Côte d'Ivoire.

Materials and Methods

Sample collection

Tuna loins and flakes were obtained from two industries located in Abidjan (economical capital of Côte d'Ivoire). Per sampling day, samples of about 500 g frozen tuna in a polyethylene bag were aseptically collected from each industry. Per month, the number of samples collected and analyzed depends on the importance of the tuna loins production. Quantities of 471 tuna loins samples and 222 tuna flakes were collected from both industries. Each sample was labeled, stored in an ice box and sent to the laboratory.

Isolation of *Escherichia coli* strains

A total of 460 of *E. coli* strains were isolated from tuna loins and flakes. On the 460 strains, 217 *E. coli* strains were isolated from 471 tuna loins and 246 *E. coli* strains were isolated from 222 tuna flakes. 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 were confirmed as *E. coli*. *E. coli* strain of American Type Culture Collection 25922 (ATCC 25922) was used as the control.

Detection of virulence genes by PCR

DNA of each isolate was extracted according to the boiling method. Approximately 5 to 10 colonies of an overnight bacterial culture were taken and suspended in 100μL of distilled water. The mixture was stored at −20°C for

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

Antibiotic susceptibility testing

The *E. coli* pathogens identified with PCR analysis were also screened for antibiotic susceptibility against 10 antibiotics (Table 2). They were chosen according to their importance in different treatments in humans (Amabile-Cuevas, 2010). Antibiotic susceptibility testing was performed on 44 *E. coli* isolates using the disk diffusion method on Mueller Hinton agar plates (Biorad, France). Antibiotic susceptibility test discs were purchased from LIOFILCHEM (Italy), and the results were classified as susceptible, intermediate, or resistant according to the zone diameter interpretive standards recommended by Antibiotic Committee of the French Society of Microbiology (CA-SFM, 2012). Intermediate resistance strains were considered resistant. The quality-control strains, *E. coli* ATCC 25922 was included in each run.

Results and Discussion

Virulence profile of *Escherichia coli* strains

Table 3 shows the distribution of virulent strains of *E. coli* in tuna loins and flakes. Forty-four strains (9.6%) of the 460 strains tested were positive for virulence genes. The prevalence of these strains are respectively 9.7% and 9.3% for tuna loins samples and tuna flakes samples analyzed. The presence of virulent strains of *E. coli* in tuna loins and flakes could result from human faecal contamination due to non-compliance with hygiene rules. According to Costa (2013), the presence of these strains could also be explained by microbial pollution of continental waters of fishing. The prevalence obtained in this study are described by Kambire *et al.*, (2017) and Canizalez-Roman *et al.*, (2013) respectively in crabs (2%) from the lagoon Aby in Côte d'Ivoire and in fish (1.98%) sold in Mexico.

The different virulent strains belong to the enterotoxigenic *E. coli* (ETEC). Twenty-one and twenty-three strains of this pathotype were identified respectively in the samples of tuna loins and flakes. No strain of enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (ECEP) and enteroaggregative *E. coli* (ECEA) were identified (Table 4). Previous studies conducted in Brazil, South Korea and Egypt reported the presence of ETEC in fish and seafood (Téophilo *et al.*, 2002; Koo *et al.*, 2012 ; Galal *et al.*, 2013). In Côte d'Ivoire, studies conducted by Kambire *et al.*, (2017), Toe *et al.*, (2018) respectively in the Aby

Lagoon and vegetable salads showed a predominance of ETEC pathotype. ETEC strains are associated with two major clinical syndromes: child diarrhea in developing countries and traveler's diarrhea (Turista) (Quadri *et al.*, 2005). According to Turner *et al.*, (2006), ETEC is regarded as a major cause of *E. coli* mediated diarrhea worldwide in human, affecting mainly children and travelers.

Table 5 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*", "*estt*" and "*elt* + est" genes. In tuna loins, 47.6% of the strains possess the "*elt*" gene, 23.8% "*est*" gene and 28.6% the two "*elt*" and "*east*" genes. Frequencies of 52.2%, 13%, and 34.8% were obtained respectively for the genes "*elt*", "*est*", and the strains possessing both "*elt*" and "*est*" genes, in tuna flakes. The amplicon sizes are shown in Figure 1.

According to Rigobelo *et al.*, (2006), ETEC causing traveler's diarrhea, particularly in developing countries are characterized by the presence of "*est*" gene encoding the synthesis of thermostable enterotoxin and the "*elt*" gene encoding the synthesis of thermolabile enterotoxin.

Table.1 Primers used for PCR in this study (Toma *et al.*, 2003)

Genes	Sequence (5' à 3')	Size (pb)	References
eae	F CCC GAA TTC GGC ACA AGC ATA AGC R CCC GGA TCC GTC TCG CCA GTA TTC G	881	Oswald *et al.*, (2000)
stx	F GAG CGA AAT AAT TTATAT GTG R TGA TGA TGG CAA TTC AGT AT	518	Yamasaki *et al.*, (1996)
aggR	F GTA TAC ACA AAA GAA GGA AGC R ACA GAA TCG TCA GCA TCA GC	254	Ratchtrachenc-hai *et al.*, (1997)
elt	F TCTCTATGTGCATACGGAGC R CCATACTGATTGCCGCAAT	322	Shacooriand Gougeon, (1994)
est	F TTAATAGCACCCGGTACAAGCAGG R CCTGACTCTTCAAAAGAGAAAATTAC	147	Hornes *et al.*, (1991)

Table.2 Antibiotic susceptibility test discs

Family	Group	Antibiotic	abbreviation	Disk load (µg)
ßlactam	Penam	Amoxicillin	AMX	25
		Amoxicillin + clavualnicacid	AMC	20/10
	Penem	Imipenem	IPM	10
	Cephalosporin	Cefotaxime	CTX	30
		Ceftazidime	CTZ	30
	Monobactam	Aztreonam	ATM	30
Aminoglycoside		Gentamicin	GEN	15
Phenicole		Chloramphenicol	C	30
Sulfanomide		Trimethoprim/ Sulfamethoxazole	SXT	1.25/23.75
Quinolone		ciprofloxacin	CIP	5

Table.3 Distribution of *Escherichia coli strains* in tuna loins and flakes

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

Table.4 Prevalence of *Escherichia coli* pathovars in tuna loins and flakes

Pathovars groups	Loins	Flakes	Total
ECET	21	23	44
ECEP	0	0	0
ECEH	0	0	0
ECEA	0	0	0
Total	21 (47.7%)	23 (52.3%)	44 (100%)

Table.5 Prevalence of virulence genes in tuna loins and flakes

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

N= number

Table.6 Antibiotic resistance profile of enterotoxigenic *Escherichia coli* strains isolated from tuna loins and flakes

Antibiotics class	Antibiotics	Resistant
Beta-lactam	Amoxicillin	7 (15.9%)
	Amoxicillin/clavulanicacid	4 (9.1%)
	Imipenem	0
	Cefotaxime	0
	ceftazidime	0
Monobactam	Aztreonam	0
Aminoglycoside	Gentamicin	1 (2.3%)
Phenicol	Chloramphenicol	1 (2.3%)
Sulfamide	Triméthoprim/sulfaméthoxazole	0
Quinolone	Ciprofloxacin	0
Total		13(29.5%)

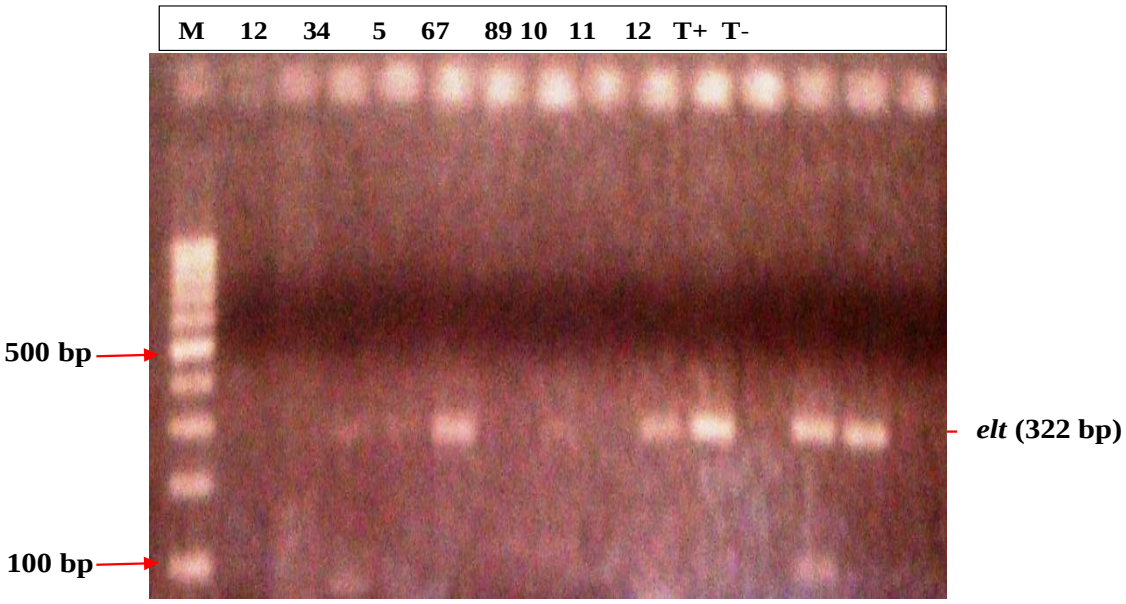
Table.7 Distribution of enterotoxigenic *E. coli* resistant strains to antibiotics in tuna loins and flakes

Antibiotics	Loins	Flakes
Amoxicillin	3 (14.3%)	4 (17.4%)
Amoxicillin/clavulanic acid	3 (14.3%)	1 (4.3%)
Chloramphenicol	1 (4.7%)	0
Gentamycin	0	1 (4.4%)
Total	7 (33.3%)	6 (26.1%)

Table.8 Distribution of enterotoxigenic *E. coli* multi-resistant strains to antibiotics in tuna loins and flakes

Antibiotics	Loins	flakes
Amoxicillin - gentamycin	0	1 (4.3%)
Total	0	1 (4.3%)

Fig.1 Gel electrophoresis profile of virulence genes of isolated *E. coli*



Lane M: weight marker, lanes 5, 9, 10, 12 (*elt*gene), lane T+ (positive control), lane T- (negative control).

ETEC possessing both the "*est*" and "*elt*" genes were detected. This result is similar to other reports (Toma *et al.*, 2003; Aranda *et al.*, 2007; Kalnauwakul *et al.*, 2007). According to Quadri *et al.*, (2005), ECET possessing the "*elt*" gene are less involved in disease cases than those with only the "*est*" gene or both "*est*" and "*elt*".

Antibiotic resistance profile of *Escherichia coli* strains

Antibiotic resistance of enterotoxigenic *E. coli* was evaluated and is shown in Table 6. Thirteen strains (29.5%) of the 44 virulent strains of enterotoxigenic *E. coli* showed resistance to one of the following four

antibiotics: amoxicillin, amoxicillin + clavulanic acid, chloramphenicol and gentamycin. The proportions of antibiotic resistance were 15.9%; 9.1%, 2.3% and 2.3% respectively for amoxicillin, amoxicillin + clavulanic acid, chloramphenicol and gentamycin. Resistance to amoxicillin was highest. No resistance of the strains was observed against imipenem, cefotaxime, ceftazidime, aztreonam, ciprofloxacin and trimethoprim/ sulfamethoxazole. Seven strains (33.3%) of the 21 virulent strains of *E. coli* enterotoxigenic isolated from tuna loins were resistant to antibiotics. The percentage of resistance was 14.3% to amoxicillin, 14.3% to amoxicillin + clavulanic acid and 4.8% to chloramphenicol (Table 7). Regarding tuna flakes, 6 strains (21.1%) of the 23 virulent strains were resistant to antibiotics. The percentage of resistance was 17.4% (amoxicillin), 4.3% (amoxicillin + clavulanic acid) and 4.3% (gentamycin). Similar results have been reported by Jeyasanta *et al.*, (2012) and Hleba *et al.*, (2013) for bacterial strains isolated from fish and seafood respectively, in India and Slovakia.

The proportion of antibiotic resistance in *E. coli* has increased in recent years (Guessennd *et al.*, 2008; Trystam, 2012). According to Okéké (2009), this is a problem that is gaining momentum especially in enterotoxigenic *E. coli.* Two factors are responsible for the emergence and dissemination of bacterial resistance to antibiotics: the pressure of selection exerted by antibiotics and once acquired resistance, the diffusion of these bacteria by cross-transmission (Arsalane *et al.*, 2010). The virulent strains *E. coli* of isolated in this study showed high resistance to amoxicillin (15.9%) compared to other antibiotics tested. This resistance could be related to the massive use of this molecule due to self-medication. It could also be related to acquired resistance. High resistance of *E. coli* strains to amoxicillin in seafood in India has already been reported by Jeyasanta *et al.*, (2012). Resistance was also observed against amoxicillin / clavulanic acid (9.3%), gentamycin (2.3%) and chloramphenicol (2.3%). In Côte d'Ivoire, the studies conducted by Toé *et al.*, (2017) on vegetable salads have also shown resistance of *E. coli* strains to these antibiotics.

Indeed, these authors obtained proportions of 2.3%, 6.9%, 2.3% respectively for amoxicillin/ clavulanic acid, chloramphenicol and gentamycin. No strain of the 21 virulent strains isolated from tuna loins showed multi-resistance to antibiotics. Regarding the tuna flakes, 1 strain of the 23 virulent strains presented a multi-resistance to antibiotics. The percentage of resistance was 4.3% to amoxicillin / gentamicin (Table 8). A low multi-resistance and sensitivity of strains to antibiotics (ceftazidime, cefotaxime, trimethropime/ sulfametazole, ciprofloxacime, aztreonam and imipenem) were obtained.

This result could be related to the origin of the strains. Indeed, according to Duredoh *et al.*, (2012), high resistance to these antibiotics is generally observed in strains resulting from hospital discharges.

The objective of this study was to search for virulence genes and to establish the resistance profile of *E. coli* strains isolated from tuna loins and Flakes. At the end of this study, only one pathotype was identified namely enterotoxigenic *E. coli*. This pathotype has been identified in tuna loins and flakes. The resistance profile of virulent strains to antibiotics indicates resistance (29.5%) to four antibiotics: amoxicillin, amoxicillin / clavulanic acid, chloramphenicol and gentamycin. For companies producing these tuna loins and flakes, hygiene measures are necessary during production to preserve the health of the consumer.

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