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ANTIMICROBIAL RESISTANCE OF ENTEROTOXIGENIC ESCHERICHIA COLI STRAINS ISOLATED FROM ABY LAGOON IN IVORY COAST

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ABSTRACT

The aim of the present research was to assess the resistance to antibiotitcs of enterotoxigenic *Escherchia coli* (ETEC) from surface water, sediment, crab and fish. The genes (*elt* and *est*) of ETEC were assessed by duplex PCR using specif primers. The antimicrobial resistance of the isolates was conducted according to the method of disc diffusion on Mueller Hinton agar. Fifty six (56) isolates were identified as ETEC strains. The resistant were 9.7% for amoxicillin, 3.3 % for amoxicillin / clavulanic acid, 19.4% for chloramphenicol, 1.7% for nalidixic acid and 13% for tetracycline. No resistance of the strains was observed with the different antibiotics: imipenem, cefotaxime, ceftriaxone, aztreonam, gentamicin and colistin. The presence of antibiotic-resistant strains of *E. coli* indicates that other antibiotic-resistant fecal bacteria may be present.

Keywords: Enterotoxigenic *Escherichia coli*, antibiotics, resistance, Aby lagoon

INTRODUCTION

Diarrhea is a major cause of morbidity and mortality worldwide, especially in infants and young children (**Canizalez-Roman *et al.*, 2016**). Diarrhea is one of the major causes of death in children under 5 years of age in developing countries. *Escherichia coli* is mainly the agent responsible of these diarrhea. *Escherichia coli* strains are normally present in the intestinal tract of humans and warm-blooded animals. This bacteria is used as an indicator in the determination of microbiological quality of water for human consumption and other human needs. Generally harmless, some strains of *E. coli* have been able to acquire virulence genes giving them the capacity to cause illness for humans and animals (**Kambire *et al.*, 2017**). According to **Seyedeh *et* al. (2017)**, diarrheagenic *E. coli* (DEC) is one of the most important bacterial agent which causes diarrhea. Based on epidemiological, clinical, and pathogenic characteristics, diarrheagenic *E. coli* strains have been classified into six groups of pathotypes : enteropathogenic *E. coli* (EPEC), shiga-toxin producing *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffuse adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). The present of *E. coli* in the aquatic environment may derive from the contact with human and animal excreta.

In from of the emergence of resistant strains to most antimicrobial agents in the last decades, the treatment for Enterobacteriaceae family including *E. coli* has been increasingly complicated **(Canizalez-Roman *et al.*, 2016)**. Each year in the United States, at least 2 million people acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections and at least 23,000 people die each year as a direct result of these antibiotic-resistant infections **(CDC, 2013)**. Many antibiotic failures are correlated to the origin (animal and human) of *E. coli*. In fact, antibiotics are used abusively in human and animal medicine thus creating resistant strains. Antimicrobial resistance in *E. coli* has been reported worlwide **(Alizade, 2018 ; Mokaba *et al.*, 2018; Kerlly *et al.*, 2017).** However, information concerning the extent of the problem of antimicrobial resistance in African Region is limited because surveillance of drug resistance is carried out in only a few countries **(WHO, 2014)**. In Côte d'Ivoire antibiotic resistance has been observed in strains of *Escherichia coli* isolated from Vegetables Salads **(Toé *et al.*, 2017)**, Porcine **(Kouadio *et al.*, 2018)**, urine **(Moroh *et al.*, 2014)**. The objective of the present research was to assess the resistance of DEC from surface water, sediment, crab and fish to antibiotics.

MATERIAL AND METHODS

Sampling Sites

The Aby Lagoon is located between 2°51 and 3°21 eastern longitudes and 5°05 and 5°22 northern latitudes southeast (**Fig 1**). Six sampling stations spread throughout the Aby Lagoon were selected because these stations were subject to various discharges (wastewater, excreta). In total six sampling campaigns were carried out between June 2010 to March 2011.

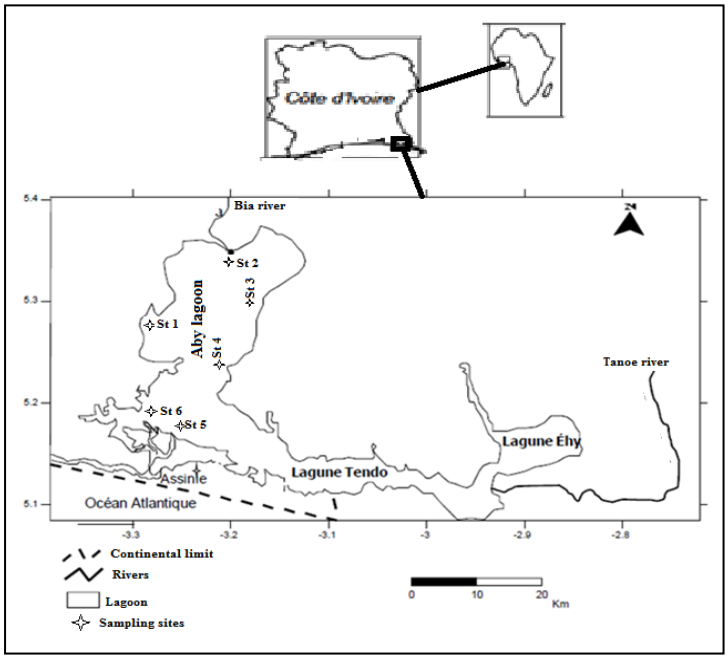

Figure 1 Study area and sampling stations

Sampling

Six campaigns were carried out for the collection of water, sediment, fish, and crab samples. At each sampling point, water were collected in sterile glass bottles and the sediments in sterile stomacher bags. Fish and crabs samples obtained from fishermen in Aby Lagoon were stored in stomacher bags. A total of 72 water samples, 36 sediment samples, 36 fish samples and 36 crab samples were analyzed. For transport to the laboratory, all samples were preserved in cold boxes.

Isolation of *Escherichia coli* strains

A total of 113 strains of *E. coli* were isolated from samples of water, sediment, fish and crab. *E. coli* isolates from water and sediment were obtained on Eosin Methyl Blue agar through the membrane filtration method. Briefly, 1 mL, 5 mL and 10 mL of water samples were filtered (**CEAEQ, 2014**) through 0.45 μm cellulose membrane filters (Millipore, Sartorius Stedim Biotech, Germany) and placed on Eosin Methyl Blue agar. For sediment analysis, dilutions were first performed with sterile buffer peptone water and then volume of 5 mL and 10 mL of each diluted sample were filtered as previously described and the filters were subsequently placed on Eosin Methyl Blue agar. For fish and crab analysis, 25 g of gut, flesh and gills of fish and 25 g of gut and shell of crab from each sample was mixed to 225 mL of sterile buffer peptone water contained in plastic stomacher bag and mixed. Decimal dilutions, were plated on desoxycholate agar. The incubation of all the Petri dishes was 44.5°C for 24 hours. In addition, isolates were purified on Eosin Methyl Blue agar, a selective medium for enterobacteria and incubated as before. Metallic sheen colonies with a dark central spot **(Nguyen, 2000)** were considered as presumptive *E. coli*. 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 boiling method. Approximately 5 colonies of an overnight bacterial culture were taken and suspended in 100 µL of distilled water. The mixture of bacterial cell and distilled water was stored at -20°C for ten min and then boiled at 100°C for 10 min. After centrifugation in a Mikro 220R Herttich centrifuge at 14000 RPM for 10 min, supernatants were used for PCR amplification. The amplification reactions were performed in reaction mixture of 25 µL containing 10 µL of Master Mix 1X (5PRIME Hot Master Mix 2.5 X DOMINIQUE Dutscher) (France); 1.4 µM concentration (each) of primers (**table 1**) and 5 µL 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 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 visualized and photographed under UV illumination (UV UVITEC, U.K.). The buffer in the electrophoresis chamber (PCR SCIE-PLAS, China) and in the agarose gel was 1×Tris-borate-EDTA (89 mM Tris-borate, 2.5 mM EDTA).

Table 1 Primers used for PCR in this study

Genes	Sequence (5' à 3')	size (bp)	References
Elt	F TCTCTATGTGCATACGGAGC R CCATACTGATTGCCGCAAT	322	**Tamanai-Shacoori and Joivet-Gougeon, 1994**
Est	F TTAATAGCACCCGGTACAAGCAGG R CCTGACTCTTCAAAAGAGAAAATTAC	147	**Hornes *et al.*, 1991**

Antimicrobial susceptibility testing

The antibiotic susceptibility of *E. coli* isolates was conducted using the diffusion method on Mueller Hinton agar as described by **Bauer et al. (1966)**. After incubation at 37 ° C for 24 hours, diameters were measured and strains were classified as sensitive, intermediate or resistant according to the Antibiogram Committee of French Society of Microbiology (CA-SFM) guidelines. All *E. coli* isolates were tested for resistance to 11 different antibiotics shown in **table 2**.

Table 2 Antibiotic disc

Antibiotic class	Antibiotics	Abbreviation	Concentration (µg)
Beta-lactam	Amoxicillin	**AMX**	25
	Amoxicillin/clavulanic acid	**AMC**	20/10
	Imipenem	**IPM**	10
	Céfotaxime	**CTX**	10
	Ceftriaxone	**CRO**	30
Monobactam	Aztreonam	**ATM**	30
Aminoglycoside	Gentamicin	**GM**	15
Cyclin	Tétracycline	**TE**	30
Phenicol	Chloramphénicol	**C**	30
Quinolone	Nalidixic acid	**AN**	30
Polypeptide	Colistin	**CS**	50

Statistical Analysis

Statistica 7.1 software was used for statistical analysis. The different percentages were calculated using the descriptive statistics

RESULTS

A total of 113 *E. coli* isolated from all the samples were examined. Among them, 56 isolates (49.50%) were positive for virulence genes of enterotoxigenic *Escherchia coli* (ETEC). Eighteen strains (66.6%) were isolated in the sediment, followed by 37 strains (59.6%) from water and 1 strains (9%) from crab. No ETEC was detected in fish samples (**table 3**). ETEC strains harboring "*est* " gene for synthesis of a heat-stable toxin was found predominantly with a frequency of 80.3 %. Thirty-three strains, or 58.9% of the ETEC strains possessed "*elt*" gene coding for synthesis of a heat-labile toxin. The frequency of strains possessing both genes at once was 39.2% (**table 4**).

Table 3 Distribution of *E. coli* strains

	Source				
	Water	**Sédiment**	**Fish**	**Crab**	**Total**
Number of strains	62	27	13	11	113
Virulence strains N(%)	37(59.6)	18(66.6)	0	1(9)	56 (49.5)

Table 4 Virulence genes

	Virulence genes		
Sources	***elt***	***est***	***elt+est***
Water	19(33.9)	30(53.5)	12(21.4)
Sediment	13(23.2)	14(25)	9(16)
Crab	1(1.7)	1(1.7)	1(1,7)
Fish	0	0	0
Total N(%)	**33(58.9)**	**45(80.3)**	**22(39.2)**

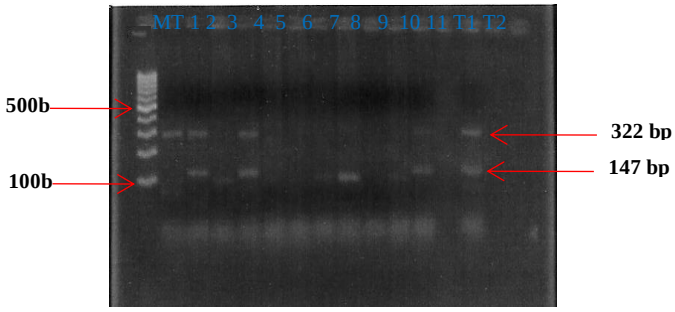

Figure 2: Presentation of the different virulence genes on electrophoresis gel
Lane 1 shows a strain possessing the "*elt"* gene, lane 8 and 11 both strains with the gene "*est"*. Lanes 2 and 4 indicate two strains simultaneously possessing the genes "*elt*" and "*est*". Lanes T1 and T2 respectively represent negative and positive controls.

Figure 2 shows the electrophoretic profile of the virulence genes. This profile illustrates the diversity of virulence genes present in *E. coli* strains of the different samples.

The overall percentage of antibiotic resistance is shown in table 5. An average of 46.8% of ETEC strains displayed acquired resistance towards to one of five antibiotics: amoxicillin, amoxicillin/clavulanic acid, chloramphenicol, nalidixic acid and tetracycline. Resistance to chloramphenicol was the most common in 12 (19.4%) isolates, followed by tetracycline (13%), amoxicillin (9.7%), amoxicillin/clavulanic acid (3.3%), and nalidixic acid (1.7%). No resistance of the strains was observed with the following antibiotics: imipenem, cefotaxime, ceftriaxone, aztreonam, gentamicin and colistin.

Table 5 Resistance profile of virulent strains isolated from different samples

		Frequencies N(%)		
Antibiotic class	**Antibiotics**	**I**	**R**	**I + R**
	Amoxicillin	0	6(9,7)	6 (9,7)
	Amoxicillin/clavulanic acid	2 (3,3)	0	2 (3,3)
	Imipenem	0	0	0
Beta-lactam	Cefotaxime	0	0	0
	Ceftriaxone	0	0	0
Monobactam	Aztreonam	0	0	0
Aminoglycoside	Gentamicin	0	0	0
Cyclin	Tétracycline	0	8(13)	8(13)
Phenicol	Chloramphénicol	0	12(19,4)	12(19,4)
Quinolone	Nalidixic acid	0	1(1,7)	1(1,7)
Polypeptide	Colistin	0	0	0
	Total N(%)	**2 (3,2)**	**27(43,6)**	**29(46,8)**

I : intermediate, R : resistant

DISCUSSION

ETEC was identified as the common cause of infections among tourists visiting Asia, South America and Africa. This pathovar is also involved in childhood diarrheal diseases in many developing countries on these three continents **(Tornieporth *et al.*, 1995, Quadri *et al*., 2005)**.

ETEC strains displayed acquired resistance towards to five antibiotics: chloramphenicol, amoxicillin, amoxicillin/clavulanic acid, tetracycline and nalidixic acid. Antibiotic resistance in this study is clearly low compared to that obtained by **Boukef et al. (2007)** in Tunisia. These authors reported that enteropathogenic *E. coli* strains isolated from the Bizerte lagoon (Tunisia) were resistant to more than 10 antibiotics. These bacterial resistance to antibiotics could be due to the rejection of resistant bacteria via faeces, antibiotic residues in the Aby lagoon. According to **Arsalane et al. (2010),** two factors are at the origin of the emergence and spread of bacterial resistance: the selection pressure exerted by antibiotics and once resistance is acquired, the spread of these bacteria by cross transmission.

The strains tested showed greater resistance to chloramphenicol (19.4%) than the other four antibiotics. This resistance could be linked to the massive use of this molecule due to self-medication in humans. In Côte d'Ivoire, resistance to this molecule has been observed in *Salmonella* isolated from chicken livers **(Coulibaly *et al.*, 2010)**.

About 13% of *E. coli* strains tested were resistant to tetracycline. The very low resistance recorded against tetracycline here is in reasonable agreement with results of 10.3% from the study of Paveen et al. (1997) in Apalachicola Bay, Florida**. Adingra et al. (2010)** also investigated the antimicrobial resistance of *E. coli* isolates from fish samples. The percentage of resistance to tetracycline obtained by these authors was 100%. According to **Ndiaye (2005)**, tetracycline has been widely used in the past, allowing bacteria to develop resistance against this antibiotic. A frequency of 9.7% of the strains tested were resistant to amoxicillin. This resistance could be related to the natural resistance with the production of a weak cephalosporinase which confers to the bacterium a weak resistance to amoxicillin. This resistance rate is lower than that obtained by **Ben Abdallah et al. (2008)** in Monastir region (Tunisia) which is 61%.

Clavulanic acid integrated into amoxicillin allowed the restoration of its activity partly, hence reduced the resistance frequency of this association to 2.1%. This result was also obtained by **Seck (2005)** in the study on the resistance of strains of *Escherichia coli* and *Klebsiella pneumoniae* implicated in urinary tract infections. Amoxicillin / clavulanic acid has increased activity on some β-lactamase producing strains.

Strains were less resistant to nalidixic acid with a rate of 1.7%. This result is identical to that obtained by **Watkinson et al. (2007**) on wastewater and surface water. According to **Trystram et al. (2002)**, this resistance may be related to the selection pressure exerted by fluoroquinolones. No isolates were resistant to ceftriaxone, cefotaxime, gentamicin, colistin, aztreonam and imipenem. This result could be related to the origin of the strains. Indeed, the strong resistances are generally observed in the strains coming from the hospital discharges.

CONCLUSION

Majority of the enterotoxigenic *Escherchia coli* isolates from Aby lagoon were resistant to five: nalidixic acid, amoxicillin/clavulanic acid, amoxicillin, tetracycline and chloramphenicol. The existence of antibiotic-resistant strains of *E. coli* in the lagoon indicates that other antibiotic-resistant fecal bacteria may be present. It is important that the antibiotics use by humans and in animal production be monitored to prevent the development of resistant *Escherichia coli*.

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