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Molecular Characterization Of Virulence Genes In Extended-Spectrum Beta-Lactamase-Producing Escherichia Coli Strains Isolated From Bovine Faeces In The Abidjan District, Ivory Coast

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Abstract:
The increase in antibiotic resistance, due to the rise of multi-resistant bacteria, is a global public health problem with serious consequences. This situation compromises the effectiveness of treatments for bacterial infections and jeopardizes the progress of modern medicine.
The aim of this study is to characterize virulence genes in ESBL-producing E. coli strains isolated from bovine faeces in the district of Abidjan (Côte d'Ivoire).
E. coli strains were isolated from various samples of fresh bovine faeces on Rapid E. coli 2 medium containing 2 mg/L of ceftazidime, and the identity of the strains was then confirmed by MALDI-TOF mass spectrometry. An antibiotic sensitivity test and a synergy test on Mueller Hinton medium were performed to identify the ESBL phenotype. The various virulence genes were identified using conventional PCR techniques with specific primers (stx1, stx2, eae, sta and lt).
Thus, out of a total of 420 faecal samples analyzed, 45 E. coli strains were confirmed as producing extended spectrum beta-lactamase (ESBL), representing a prevalence of 52.9%. PCR testing for virulence genes showed that the ESBL-producing E. coli strains tested possessed at least one virulence gene. The stx1, stx2, eae, sta and lt genes were detected at rates of 40%, 60%, 86.7%, 2% and 13.3%, respectively.
Keywords: *E. coli, ESBL, virulence, cattle, Abidjan, Ivory Coast*

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I. Introduction

Antibiotic resistance is a growing global public health concern, threatening the effectiveness of treatments for bacterial infections (Salam et al., 2023). This crisis is exacerbated by the excessive use of antibiotics in agriculture, food production and healthcare, which promotes the emergence and spread of antimicrobial resistance and risks increasing global mortality (Lior et al., 2014; Sirwan et al., 2024). Among resistant bacteria, extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBLES), and more specifically *E. coli*, a species common in humans and animals, are of particular concern because of their ability to inactivate a wide range of beta-lactam antibiotics, which are often used as first-line treatments. Resistant *E. coli* strains are the leading cause of antibiotic resistance-related deaths worldwide (Murray et al., 2022). In fact, the World Health Organization (WHO) has classified them as critical pathogenic bacteria requiring the development of new antibiotics (Milenkov and Armand-Lefevre, 2025).

In Ivory Coast, antibiotic resistance rates are on the rise. A study conducted by the National Antibiotic Reference Centre indicates that the percentage of antimicrobial resistance (AMR) rose from 9% in 2002 to 46% in 2018 (WHO, 2021). In this context, cattle farming, an important economic activity in the Abidjan district, could contribute to the spread of resistant bacteria. Indeed, cattle- can harbor ESBL-producing *E. coli* strains in their intestinal flora and spread them into the environment via their faeces, which could expose populations to an increased risk of infection. (Madec, and Haenni, 2018; Ewers et al., 2012; Kola et al., 2018).

Although antibiotic resistance is a major problem, the virulence of *E. coli* strains remains a key factor in their ability to cause disease. Virulence genes, which are primarily responsible for bacterial invasion, control the production of key factors for host colonization, such as cell adhesion, the production of harmful toxins and

mechanisms for evading the immune system (Kaper et al., 2004; Sansonetti, 2004). Therefore, a detailed molecular analysis of the virulence genes present in extended-spectrum beta-lactamase (ESBL)-producing *E. coli* strains is essential. It allows us not only to gain an in-depth understanding of their pathogenic potential, but also to accurately assess the risks to human and animal health. Furthermore, it is crucial to note that these virulence and resistance genes can be simultaneously transmitted to other strains, creating virulent strains that are potentially more difficult to treat (Li et al., 2023).

In Ivory Coast, although research has been conducted on the prevalence of ESBL-producing *E. coli* strains in bovine faeces (Yao et al., 2018), data on the characterization of virulence genes in ESBL-producing *E. coli* strains isolated from bovine faeces remain limited.

The overall objective of this study is to characterize virulence genes in ESBL-producing E. coli strains isolated from bovine faeces in the district of Abidjan (Ivory Coast).

II. Material And Methods

Samples Collection of and isolation of ESBL producing *Escherichia coli* strains from bovine faeces

Samples were collected in five municipalities in the Abidjan district (Port-Bouët, Abobo, Adjamé, Yopougon and Bingerville) between April and September 2016. A total of 420 samples of fresh faeces from apparently healthy cattle were collected directly after defecation at the various sites on a random basis. The samples, collected in sterile coproculture pots, were stored cold (+4°C) and transported to the microbiology laboratory of the Pasteur Institute for testing for extended-spectrum beta-lactamase-producing *E. coli* strains. Initial detection of resistant *E. coli* strains was performed according to the methodology described by Yao *et al.*, 2018. To this end, twenty-five (25) grams of faecal matter were diluted in 225 mL of buffered peptone water and seeded on Rapid' *E. coli* 2 agar containing 2 mg/L ceftazidime, then incubated at 37°C for 24 hours. Three to five characteristic *E. coli* colonies, recognisable by their purple colour, were selected and identified using the Leminor reduced rack. The identified *E. coli* strains were then confirmed by MALDI-TOF mass spectrometry (BioMérieux, France). This study isolated and identified a total of 85 resistant *E. coli* strains, representing a prevalence of 20.2% during this period.

Antibiotic sensitivity testing

Antibiotic susceptibility was assessed for all *E. coli* strains using the agar disc diffusion method, and the results were interpreted according to the standards of the Antibiogram Committee of the French Society of Microbiology (EUCAST/CA-SFM, 2016). The reference strain of *E. coli* ATCC 25922 was used for internal quality control. The following antibiotic discs (Bio-Rad France) were used: ampicillin (10 µg), amoxicillin + clavulanic acid (30 µg), cephalothin (30 µg), cefepime (30 µg), aztreonam (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), imipenem (10 µg), tetracycline (30 µg), minocycline (30 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), nalidixic acid (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), colistin (50 µg) and trimethoprim/ sulfamethoxazole (25 µg).

Detection of the ESBL phenotype

The search for extended-spectrum beta-lactamase (ESBL)-producing *E. coli* strains was carried out using synergy tests according to the methodology described by Jalier *et al*., 1988, on all isolated *E. coli* strains. These tests were performed by placing amoxicillin/clavulanic acid in the center of a Petri dish containing Mueller Hinton agar, surrounded by third-generation cephalosporins (ceftazidime, ceftriaxone, cefotaxime), as well as aztreonam and cefepime (Yao *et a*l., 2018).

Detection of virulence genes by PCR

Virulence genes were detected by PCR on all strains of extended-spectrum beta-lactamase-producing *E. coli*. Bacterial DNA was extracted from 500 µL of suspension of *ESBL-producing E. coli* isolates using nuclease-free water (pure water), and total DNA extraction was performed by heat shock (Lee et al., 2005). The various DNA extracts obtained were then used as a template for PCR reactions using specific primers listed in **Table 1**. Genomic amplification was performed in a final reaction volume of 50 µl. This mixture contained a colored 5X buffer and an uncolored 5X buffer (Promega, USA), 25 mM MgCl2 (Promega, USA), 10 mM of each dNTPS (Biorad, France), 10 µM specific sense and antisense primers, 5U Taq polymerase Go taq ® G2 Flexi DNA Polymerase (Promega, USA) and 5 µL of DNA. Reference strains from the National Food Institute (DTU Food) collection were used as positive controls for PCR (Table 2), while a mixture without DNA was used as a negative control. The amplification parameters are detailed in Table 3.

The PCR products were analysed by agarose gel electrophoresis. The gels, prepared with 10X TAE (Tri-Acetate EDTA) buffer and 5 µL of EZ-vision® solution (Inqaba biotec, West Africa), had a concentration of 2%. Electrophoresis was conducted at 120 V/cm for 1 hour.

A 100 bp molecular weight marker was used to determine the approximate size of the fragments to be analyzed. After migration, the DNA was observed under UV light (λ = 312 nm) using the Gel Doc EZ Imager automated system (BioRad, USA).

Table no 1: Specific primer for the detection of resistance genes

Gene	Primer	Sequence (5'-3')	Hybridisation temperature	Amplicon size (bp)	Reference
stx	stx1-f stx1-r	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT	50	555	Franck *et al*., 1998
	stx2-f stx2-r	GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC	50	118	Franck *et al*., 1998
sta	sta-f sta-r	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	50	190	Franck *et al*., 1998
eae	eae-f eae-r	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA	50	425	Franck *et al*., 1998
ehA	ehxA-f ehxA-r	GGTGCAGCAGAAAAAGTTGTAG' TCTCGCCTGATAGTGTTTGGTA	57	1551	Schmidt *et al*., 1995
lt	lt-f lt-r	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	57	450	López-Saucedo *et al*., 2023

Table no 2: Reference strain

Species	Use	Reference/Origin
E. coli ATCC25922	controls for culture media and antibiograms	DTU Food (Denmark)
Shigella sonei	Positive control for detection *of stx1*, *eae*, *lt* genes	DTU Food (Denmark)
Shigella flexneri	Positive control for the detection of *stx1/stx2*, *ehxA*, *sta* genes	DTU Food (Denmark)

Table no 3: PCR program for the detection of pathogenicity genes

Amplification step	Temperature condition/duration		
	stx1, stx2	*eae, sta*	*lt, ehxA*
Initial denaturation	94°C/2 min	94°C/2 min	94°C/2 min
Cyclic denaturation	94°C/30s	94°C/30s	94°C/60s
Hybridisation	50°C/45s	50°C/45s	57°C/60s
Cyclic elongation	70°C/90s	70°C/90s	72°C/60s
Final elongation	72°C/10 min	72°C/10 min	72°C/10 min
Number of cycles	45	45	45

III. Result

Frequency of isolation of resistant E. coli strains

The identification of *E. coli* strains present in cattle faeces has been significantly improved by the use of MALDI-TOF mass spectrometry (Vitek-MS). This system has not only enabled rapid and reliable confirmation of the identity of these strains, but has also revealed a prevalence of antibiotic-resistant strains. Thus, out of a total of 420 stool samples collected and analysed, a total of 85 resistant E. coli strains were isolated, corresponding to a prevalence of 20.2%. These resistant strains were isolated specifically on Rapid'*E. coli* culture medium supplemented with ceftazidime, suggesting a possible involvement of this antibiotic in the selection and proliferation of these resistant microorganisms.

Resistance profile of ceftazidime-resistant *Escherichia coli* strains

Analysis of the antibiotic resistance of the 85 E. coli strains showed a very high level of resistance to betalactams, with the following percentages: 92.9% for ampicillin, 96.5% for cefalotin, 95.3% for aztreonam, 85.9% for cefuroxime, 89.4% for ceftriaxone, 84.7% for ceftazidime, 87.1% for cefepime and 61.2% for amoxicillin + clavulanic acid. In contrast, resistance to cefoxitin was relatively low (31.8%). Similarly, a low level of resistance was observed to quinolones, with rates ranging from 16.5% to 17.6%.

With regard to cyclines, polypeptides, sulphonamides and phenicols, very high resistance was observed to tetracycline (97.6%), minocycline (69.4%), colistin (58.8%) and trimethoprim/sulfamethoxazole (69.4%). However, lower resistance rates were observed for aminoglycosides and phenicols, particularly gentamicin (34.1%), tobramycin (22.4%), amikacin (10.6%) and chloramphenicol (7.1%). No resistance was observed with imipenem (Table 4).

Table 4: Resistance of *E. coli* strains to antibiotics

	Number/Resistance rate (%)	
Antibiotic family	**Number (n=85)**	**Rate (R%)**
Betalactams AMP	**79**	92.9
AMC	52	61.2%
CEF	82	96.5%
CXM	73	85.9%
FOX	27	31.8%
CRO	76	89.4%
CAZ	72	84.7%
FEP	74	87.1%
ATM	81	95.3%
IMP	0	0
Quinolones NAL	15	17.6
CIP	15	17.6
NOR	14	16.5 ( )
Cyclines TET	83	97.6
MNO	59	69.4
Aminoglycosides AKN	9	10.6
TMN	19	22.4
GEN		34.1
Polymyxins CST	50	58.8
Phenicols CHL	6	7.1
Sulphonamides SXT	59	69.4

AMP = Ampicillin**;** AMC = Amoxicillin + clavulanic acid**; CEF =** Cefalotin**;** CXM = Cefuroxine**; FOX =** Cefoxitin**; CRO =** Ceftriaxone**; CAZ =** Ceftazidime**; FEP =** Cefepime**; ATM =** Aztreonam**; IMP =** Imipenem; NAL = Nalidixic acid**;** CIP = ciprofloxacin**;** NOR = norfloxacin**;** TET = tetracycline; MNO = minocycline; AKN = amikacin; TMN = tobramycin; GEN = gentamicin; CST = colistin; CHL = chloramphenicol; SXT = trimethoprim/sulfamethoxazole. R = resistant

Detection of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* strains

Of the 85 resistant *E. coli* strains isolated from bovine faeces, 45 were identified as producing extended spectrum beta-lactamase. This classification was confirmed by double synergy tests with the presence of a "champagne cork" between the disc containing amoxicillin + clavulanic acid (AMC) and at least one third generation cephalosporin (C3G) (Figure 1).

The prevalence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* strains in this study was 52.9%.

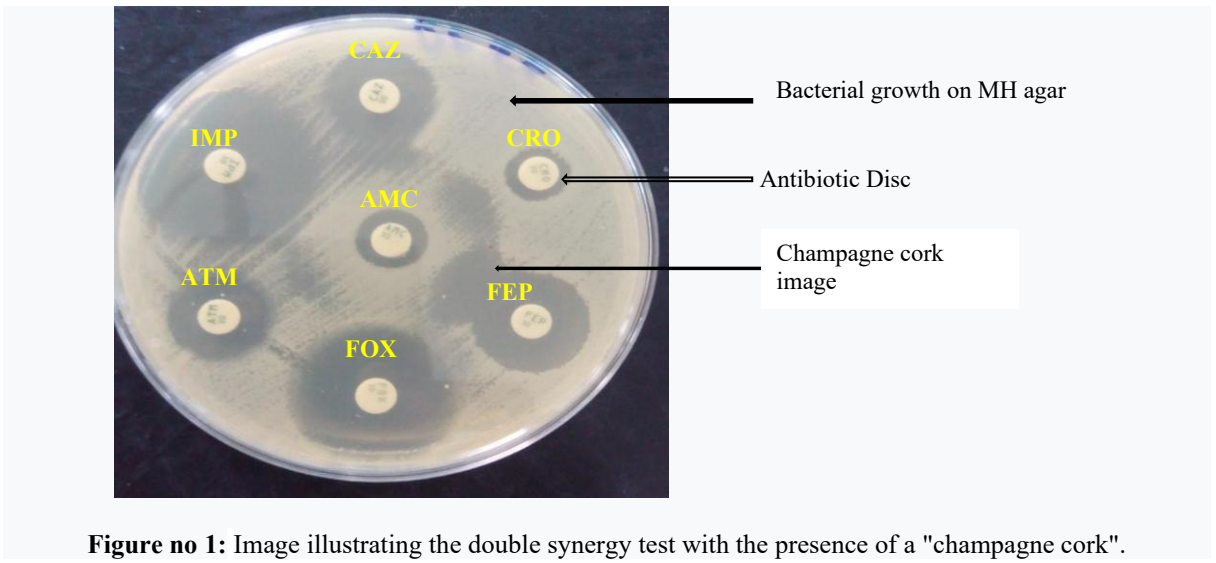

Figure no 1: Image illustrating the double synergy test with the presence of a "champagne cork". AMC = amoxicillin + clavulanic acid**; FOX =** cefoxitin**; CRO =** ceftriaxone**; CAZ =** ceftazidime**; FEP =** cefepime**; ATM =** aztreonam**; IMP =** imipenem. MH = Mueller-Hinton

Pathogenicity genes detected in isolated *Escherichia coli* strains

In order to study virulence genes, 15 quinolone-resistant ESBL E. coli strains were selected from among the 45 strains detected and were used to search for virulence genes.

The search for virulence genes in ESBL-producing *E. coli* strains isolated from bovine faeces was performed by multiplex PCR (*stx1/stx2* and *eae/sta*) and single-plex PCR (*lt* and *ehxA*).

The results showed that 100% of the 15 quinolone-resistant ESBL *E. coli* strains possessed at least one virulence gene. Analysis of the electrophoretic profiles revealed the presence of the stx1 (555 bp), stx2 (118 bp), eae (425 bp), sta (190 bp) and lt (450 bp) genes (Figure 2).

The most frequently observed gene was eae (86.7%), followed by stx2 (60%). The stx1, sta and lt genes were detected with frequencies of 40%, 2% and 13.3%, respectively. However, no strains possessing the *ehxA* gene were observed. Four (4) groups of pathovars were identified, namely Shigatoxin-producing *E. coli* (STEC, 46.7%), Enterotoxigenic *E. coli* (ETEC, 13.3%), enteropathogenic *E. coli* (EPEC, 13.3%) and hybrids (STEC+/ETEC, 26.7%) (Table 5).

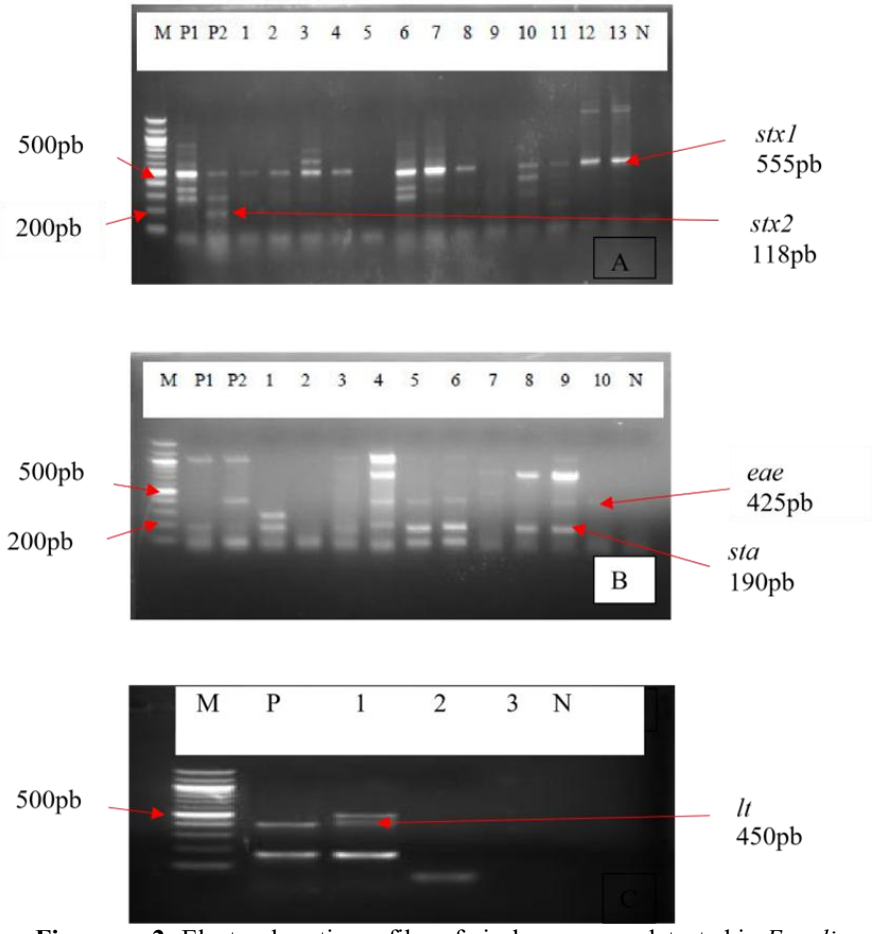

Figure no 2: Electrophoretic profiles of virulence genes detected in *E. coli*
C : detection of *stx1 and stx2* genes by multiplex PCR
D : detection of eae and sta genes by multiplex PCR
E *:* detection of the *lt* gene by simplex PCR
M: molecular weight marker (100 to 2000 bp)
P1: positive control (Shigella sonei strain
P2: positive control (Shigella flexeni strain)
N : negative control (contains ppi water instead of bacterial DNA).
Numbers 1 to 6: bacterial strains

Table no 5: *E. coli* pathovars and distribution of virulence genes

	Virulence genes						
Strains	Number/Frequency (%)						
	Stx1	*Stx2*	*eae*	*sta*	*ehxA*	*lt*	Number of strains
STEC+ETEC	+	+	+	-	-	+	4 (26.7%)
STEC+ETEC	+	-	+	-	-	+	

STEC+ETEC	-	+	+	+	-	-	
STEC	+	+	+	-	-	-	7 (46.7%)
STEC	-	+	+	-	-	-	
STEC	+	+	-	-	-	-	
ETEC	-	-	+	+	-	-	2(13.3%)
ETEC	-	-	-	+	-	-	
EPEC	-	-	+	-	-	-	2(13.3%)
TOTAL (%)	6 (40%)	9 (60%)	13 (86.7%)	3 (20%)	0	2 (13.3%)	15 (100%)

+: positive, -: negative**, ETEC**: enterotoxigenic *E. coli*, **STEC**: Shiga toxin-producing *E. coli*, **EPEC**: enteropathogenic *E. coli*.

IV. Discussion

In this study, the results revealed an average prevalence of 20.2% of resistant *E. coli* strains isolated on Rapid *E. coli* 2 medium supplemented with 2 mg/L ceftazidime from fresh bovine faecal samples (Yao *et al*., 2018). The presence of resistant *E. coli* in faeces reflects the misuse of antibiotics (Sanders *et al*., 2017). In livestock farming, it has been established that there may be a correlation between the amount of antibiotics used and the prevalence of resistance in the faecal flora of animals. Therefore, the prevalence of resistant *E. coli* strains observed in this study could be linked to the misuse of antibiotics in the treatment of animals.

However, ESBL-producing *E. coli* strains isolated from cattle faeces have been reported by several authors (Hartmann *et al*., 2012; Nguyen *et al*., 2016). In this study, the frequency was 52.9%. This frequency is higher than that observed in Germany by Schmid et al. (2013), which is 32%. Lower frequencies were detected by Faruk et al. (2016). The frequency obtained in this study is close to that reported by Dahms *et al*. (2015), which is 54.4%. However, Stefani *et al*. (2014) detected higher frequencies than those detected in our study. This high presence of extended-spectrum beta-lactamase-producing *E. coli* strains could be due to the misuse of third- and fourth-generation cephalosporins in livestock farming.

The frequency of detection of strains with at least one virulence gene corroborates that of Shahrani *et al*. (2014) in Iran, who also detected a frequency of 100%. This result is significantly higher than that of Rigobelo *et al*. (2006), who detected a frequency of 49.1%. The high detection frequency observed in our study could be linked to the acquisition of virulence. Indeed, during colonization by commensal E. coli bacteria, ESBLs can spread and transfer genes coding for intestinal pathogen s through horizontal transfers.

Shigatoxin-producing *E. coli* (STEC) strains were the most commonly detected in this study. This result corroborates that of Leomil *et al*. (2003) on the study of the frequency of Shigatoxin-producing *E. coli* (STEC) isolates in diarrhoeic and non-diarrhoeic calves in Brazil. Studies conducted by several authors have indicated that ruminants are the main natural reservoir of STEC infection (Cookson *et al*., 1996; Martikainen *et al*., 2012). The STEC rate (46.7%) detected in our study is higher than that detected by several authors (Salvadori *et al*., 2003; Rigobelo *et al*., 2006). In addition, studies conducted in different countries have shown that 10 to 80% of cattle may be carriers of STEC (Cobbold and Desmarchelier, 2001). The different STEC prevalence rates could be related to STEC excretion patterns, which are influenced by several factors, such as geographical differences, sampling and detection methods, cattle age and seasonal variations (Menrath *et al*., 2010). Analysis of STEC virulence genes revealed a high detection rate for *stx2* genes (60%) compared to *stx1* genes (40%). Similar results have been obtained by other authors (Shin *et al*., 2014). Based on epidemiological data indicating the importance of the *stx2* toxin in the development of haemolytic uraemic syndrome (HUS), the predominance of STEC strains carrying the *stx2* gene in cattle could pose a serious risk to public health *(*Shin *et al.*, 2014). Furthermore, the detection of STEC carrying the chromosomal *eae* gene may be necessary in the development of virulence in STEC by providing them with attachment and effacement activity. The detection of STEC carrying *eae* genes in cattle has also been reported in several studies (Nguyen *et al*., 2011).

ETEC strains can produce thermostable (*sta*) and/or thermolabile (*lt*) enterotoxins (Sjoling *et al.* 2007). In this study, these ETEC-coding genes were detected with frequencies of 20% and 13.3% for the *sta* and *lt* genes*,* respectively. These detection frequencies are lower than those reported by Mahanti *et al*. (2014), who detected frequencies of 72.2% (*sta*) and 66.7% (*lt*) in their work. They are higher than those obtained by Shin *et al*. (2014), which were 4.1% (*sta*) and 0% (*lt*). These strains are the causative agents of diarrhoea in cattle (Nagy and Fekete, 1999). Consequently, the spread of these strains in the environment through cattle faeces could pose a risk to human health.

The gene encoding the chromosomal intimin *eae* may be necessary for the development of virulence in certain strains of *E. coli* (enteropathogenic *E. coli,* EPEC) by providing them with attachment and effacement activity. However, several authors have described significant associations between the presence of the *eae* gene and the pathogenicity of *E. coli* strains (Shin et al., 2014). The presence of the *eae* gene was detected in 86.7% of strains. In addition, the STEC/ETEC association was detected with a frequency of 26.7%. The combination of multiple virulence genes hosted by STEC/ETEC associations showed that the strains were a mixture of

several different *E.* coli with properties associated with the pathogenic group. The presence of *sta* and *lt* virulence genes associated with *E. coli* STEC strains confirms their hybrid nature.

V. Conclusion

Ultimately, this study on the molecular characterization of virulence genes in ESBL-producing *E. coli* strains isolated from cattle faeces in the Abidjan district highlights the importance of monitoring the spread of these resistant and virulent strains in the animal environment. The results obtained showed the presence of virulence factors in ESBL-producing *E. coli* strains isolated from cattle faeces in the Abidjan district, highlighting the potential role of cattle as a reservoir for these bacteria and underscoring the need to implement rigorous control measures to limit the transmission of these strains to humans, thereby reducing the risks to public health. Therefore, in order to better understand the risks associated with their presence in the environment, it would be interesting to study the direct impact of these strains on human and animal health in order to develop more effective prevention and control strategies.

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