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Molecular Characterisation of Extended-Spectrum Beta-lactamase Producing *Escherichia coli* Isolated from Cattle Faeces in Abidjan District, Ivory Coast

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Authors' contributions

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

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ABSTRACT

Aims: To determine the frequency of Extended-Spectrum Beta-lactamase producing *E. coli* strains in cattle farms in Abidjan district (Ivory Coast).
Methodology: A total of 420 bovine faecal samples were collected in five townships in Abidjan district over six (6) month period from April to September 2016. ESBL-producing *E. coli* strains were isolated on Rapid *E. coli* 2 medium supplemented with 2 mg / L ceftazidime (antibiotic). The antibiotic resistance profile was evaluated by the diffusion method in agar media and the detection of strains of Extended-Spectrum Beta-lactamase producing *E. coli* was performed by the double-disk synergy test. Molecular detection of CTX-M, SHV and TEM genes was performed by polymerase chain reaction (PCR).
Results: Out of the 85 strains of *E. coli* isolated (20,2%), 45 strains were confirmed producing ESBL, a prevalence of 52,9%. The $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} genes were detected in 15 strains of

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Extended-Spectrum Beta-lactamase producing *E. coli* resistant to quinolone with respective frequencies of 73,3%, 66,7% and 100%.
Conclusion: This study showed a high prevalence of Extended-Spectrum Beta-lactamase producing *E. coli* strains in cattle faeces in the Abidjan district. A good use of antibiotics would be an alternative to overcome the problem of the emergence of Extended-Spectrum Beta-lactamase producing *E. coli* strains in farms.

Keywords: E. coli; extended-spectrum beta-lactamase; cattle; faeces.

1. INTRODUCTION

Increased introduction of antimicrobial agents into animal production has resulted in selective pressures on bacterial populations [1]. The emergence and spread of multidrug-resistant Gram-negative bacteria (MDR) constitute a major public health problem worldwide. Recently, the bacterial resistance resulting from the production of extended spectrum beta-lactamases (ESBL) has been recognised as a global therapeutic problem [2,3]. This resistance is increasingly reported in enterobacteria isolated in humans and animals in recent years [4,5]. The presence of ESBL in Enterobacteriaceae is of great microbiological and clinical importance [6]. Among these ESBL-producing Enterobacteriaceae, *Escherichia coli* and *Klebsiella pneumoniae* are the most frequent and can be the cause of many infections that can lead to an increase in morbidity, mortality and the cost of treatment [7,8].

Extended-spectrum Beta-Lactamases (ESBLs) are a group of enzymes that promote resistance to most beta-lactams used in human and veterinary medicine, including extended-spectrum cephalosporins, but excluding carbapenems and cephamycins [9]. They are also generally inhibited by beta-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam [9,10].

Discovered in the early 1980s, these ESBL enzymes were initially detected in clinical *E. coli* isolates [11] and mainly derived from TEM, SHV and CTX-M [12]. However, ESBL-producing *E. coli* strains are increasingly observed in an increasing number of food animals. Thus, it is well established that antibiotic-resistant bacteria that are selected in animals may be transmitted to the human intestine via the food chain as well as in environmental settings [13]. These animals are recognised as potential reservoirs of ESBL-producing *E. coli* [14] because their role in the dissemination of antibiotic resistance genes has been documented [15].

In Ivory Coast, there is little information on ESBL-producing *E. coli* strains from food producing animals and the possible contribution of these animals in propagating resistance genes in the environment. The overall objective of this study was to determine the prevalence of ESBL-producing *E. coli* strains isolated in cattle faeces in the district of Abidjan, Ivory Coast.

2. MATERIALS AND METHODS

2.1 Sample Collection

The sample collection was done in five township of Abidjan district that are Port-Bouët, Abobo, Adjamé, Yopougon and Bingerville. A total of 420 apparently healthy cow faecal samples were collected just after emissions over a six (6) month period from April to September 2016. A quantity of two hundred (200) grams of fresh faeces randomly selected from the different sites was collected. The samples were immediately transported in coolers containing cold accumulators to the microbiology laboratory of the Institut Pasteur for analysis.

2.2 Isolation and Identification of ESBL-producing *E. coli*

All samples collected were analysed for the research of extended-spectrum beta-lactamase-producing *E. coli*. Twenty-five (25) grams of fresh cow faeces sample diluted in 225 mL of buffered peptone water (PEF) (Liofilchem®, Italy) were streak seeded directly onto Rapid' *E. coli* 2 agar (Bio- Rad, France) supplemented with 2 mg / L of ceftazidime, then incubated at 37°C for 24 hours. Three to five characteristic colonies of *E. coli due to their morphological appearance on the agar (purple color)* were randomly selected and then identified using the Leminor's reduced rack. Strains of *E. coli* identified were subsequently confirmed at MALDI-TOF MS (BioMérieux, France). The reference strains *E. coli* ATCC 25922 were used as a control to verify the

efficacity of Rapid' *E. coli* 2 media supplemented with 2 mg / L ceftazidime. *E. coli* ATCC 8739 (Biomérieux, France) was used as a MALDI-TOF calibrating strain (Table 1).

2.3 The Susceptibility of ESBL-producing *E. coli* Strains to Antibiotics

Antibiotic susceptibility tests were carried out on all *E. coli* strains (identified and confirmed) by the diffusion method of disks in an agar medium and the results were interpreted according to the standard of the Antibiogram Committee of the French Society. Microbiology (EUCAST / CA-SFM, 2016). The reference Strain of *E. coli* ATCC25922 was used as a quality control for susceptibility testing.The following antibiotic discs (Bio-Rad France) were used: ampicillin (10 µg), amoxicillin + clavulanic acid (30 µg), cefalotine (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).

Extended spectrum beta-lactamase (ESBL) production was detected by a synergism between amoxicillin / clavulanic acid, placed in the center of the Petri dish containing Müller-Hinton agar, and third-generation cephalosporins (ceftazidime, Ceftriaxone, cefotaxime), aztreonam and cefepime [16].

2.4 Detection of Beta-lactam Resistance Genes

The search for beta-lactam resistance genes was performed by simplex PCRs on 15 strains of *E. coli* phenotypically identified ESBL-producing and resistant to quinolone. The extraction of the bacterial DNA was carried out on 24-hour colonies, by boiling method [17] followed by purification with phenol-chloroform-alcohol-iso-amyl (v / v / v) [18]. The primers used in this study are shown in Table 2. The genomic amplification was carried out in a final reaction volume of 50 µl and contained a colored 5X buffer (Promega, USA), a non-colored 5X buffer (Promega, USA), 25 mM $MgCl_2$ (Promega, USA), 10 mM of each dNTPs (Bio-Rad, France), 5 U / µl of Go taq DNA polymerase (Promega, USA) and 10 µM of specific primers for each target (Table 3). Amplification was performed in a thermocycler (Applied Biosystems, USA). The reference strains provided by the National Food Institute's collection (DTU Food) were used as positive controls for PCR (Table 1) and a reaction mixture without DNA extract served as a negative control. The amplification conditions are reported in Table 4. The amplification products were analysed by electrophoresis on agarose gel at 1,5% prepared from a TAE 10X buffer (Tri-Acetate-EDTA) and 5 µL of a solution of EZ-vision® (InqabaBiotec, West Africa) at 120 volts / cm for 1 hour.

Table 1. Reference strains

Identity	Strains	Beta-lactamase gene	Origin of the reference strain
TEM-104	*Salmonella* bredneney	TEM	DUT
58.67 Holland	*Salmonella* virchou	CTX	DUT
DAK2	*Salmonella* keurmassar	SHV	DUT
ATCC 25922	*E. coli*	-	IPCI
ATCC 8739	*E. coli*	-	BioMérieux, France

DUT= Technical University of Denmark; IPCI= Pasteur Institut of Ivory Coast

Table 2. Primer used for amplification of resistance genes by monoplex PCR

Target	Primers	Sequence (5'-3')	Fragment size (pb)	References
bla_{TEM}	TEM front P1	GCGGAACCCCTATTTG	964	[19]
	TEM-C-R-ny	ACCAATGCTTAATCAGTGAG		
$bla_{CTX\text{-}M}$	CTX-M F	TTTGCGATGTGCAGTACCAGTAA	544	[20]
	CTX-M R	CGATATCGTTGGTGGTGCCATA		
bla_{SHV}	SHV F	TTTATGGCGTTACCTTTGACC	1051	[21]
	SHV R	ATTTGTCGCTTCTTTACTCGC		

Table 3. Reaction mixture

	Reactional medium (µl) Gene		
Reagents and DNA extract	***bla*$_{CTX-M}$**	***bla*$_{SHV}$**	***bla*$_{TEM}$**
Colored buffer (5X)	5	5	2,5
non-colored buffer (5X)	5	5	2,5
$MgCl_2$ (25 mM)	3,5	3,5	3,5
dNTPs (10 mM)	2	2	1
Go taq DNA polymérase (5 U/µl)	0,2	0,2	0,25
Primers Forward (10 µM)	0,5	0,5	0,75
Primers Reverse (10 µM)	0,5	0,5	0,75
Nucléase Free Water	28,3	28,3	33,75
DNA extract	5	5	5

Table 4. Amplification program

Amplification step	Temperature C ° / time / cycle
Initial denaturation	94°C/5 min
Cyclic denaturation	94°C/1 min
Hybridisation	60°C/1 min
Cyclic elongation	72°C/1 min
Final elongation	72°C/7 min
Number of cycles	30 cycles

3. RESULTS AND DISCUSSION

3.1 Frequency of Isolation of ESBL-producing *E. coli*

A total of 85 strains of resistant *E. coli* were isolated on Rapid' *E coli* 2 supplemented with 2mg / L of ceftazidime, a prevalence of 20,2%. All isolated strains were tested for susceptibility to antibiotics. The analysis revealed that all isolated strains showed a high level of resistance to beta-lactams with levels ranging from 31,8% to 96,5%. Only imipenem was effective on all strains tested. The results showed a low level of resistance to classical quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin and norfloxacin) with resistance rates ranging from 16,5% to 17,6%. A very high resistance was observed for cyclins with rates of 69,4% (minocycline) and 97,6% (tetracycline). Concerning the aminoglycosides (gentamicin, tobramycin, amikacin) relatively lower levels ranging from 10,6% to 34,1% were obtained. High resistance levels were obtained with colistin (58,8%) and trimethoprim / sulfamethoxazole (69,4%). However, chloramphenicol showed a low resistance level of 7,1% (Table 5). All isolated strains showed a multidrug resistance phenotype to at least two (2) families of antibiotics. Thus, of the 85 strains of *E. coli* isolated, 45 strains were confirmed to produce Extended-Spectrum Beta-lactamase (ESBL) (double synergistic tests), a prevalence of 52,9% (Fig. 1).

Table 5. Antibiotic resistance profile

Antibiotic family	Resistance rate (%)
	R (n=85)
Ampicillin (AMP)	92,9
Amoxicillin + clavulanic acid (AMC)	61,2
Cefalotine (CEF)	96,5
Cefuroxine (CXM)	85,9
Cefoxitin (FOX)	31,8
Ceftriaxone (CRO)	89,4
Ceftazidime (CAZ)	84,7
Cefepime (FEP)	87,1
Aztreonam (ATM)	95,3
Imipenem (IMP)	0
Nalidixic acid (NAL)	17,6
Ciprofloxacin (CIP)	17,6
Norfloxacin (NOR)	16,5
Tétracycline (TET)	97,6
Minocycline (MNO)	69,4
Amikacin (AKN)	10,6
Tobramycin (TMN)	22,4
Gentamycin (GEN)	34.1
Colistin (CST)	58,8
Chloramphénicol (CHL)	7,1
Triméthoprim/sulfamethoxazole (SXT)	69,4

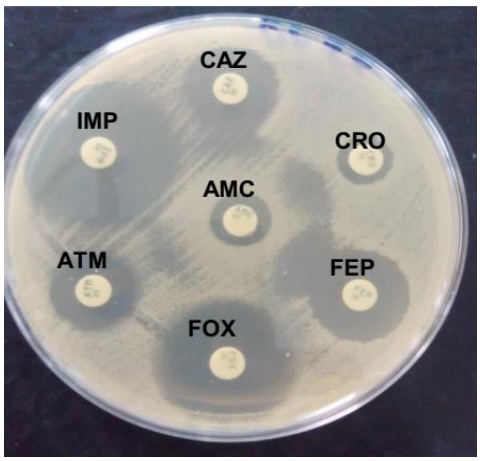

Fig. 1. Image showing the keyhole phenomenon of Double Disc synergy Test (DDS)

AMC = amoxicillin + clavulanic acid; FOX = cefoxitin; CRO = ceftriaxone; CAZ = ceftazidime; FEP = cefepime; ATM = aztreonam; IMP = Imipenem

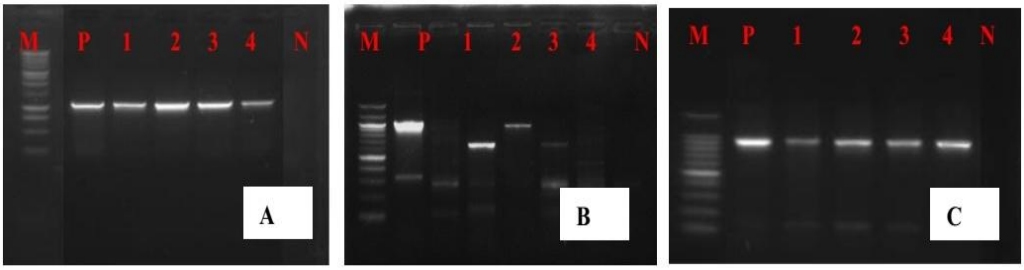

Fig. 2. Electrophoretic profile of the beta-lactam resistance genes on agarose gel at 1.5%
A: detection of the bla_{CTX-M} gene (544 bp); B: detection of the bla_{SHV} gene (1051bp); C: detection of the gene bla_{TEM} (964bp); M: Molecular weight marker (100 bp DNA Ladder); P: Positive control; Number 1 to 5: Bacterial strains; N: Negative control

3.2 Detection of Beta-lactamase Genes in ESBL-producing *E. coli* Isolates

Overall, PCR results showed a high level of detection of beta-lactamase genes (Fig. 2). Out of 15 strains of *E. coli* producing ESBL and resistant to quinolone tested, the bla_{CTX} (544pb), bla_{SHV} (1051pb) and bla_{TEM} (964pb) genes were detected with respective frequencies of 73,3%, 66,7% and 100% (Table 6). The associations of beta-lactam resistance genes have also been observed (Table 7).

Table 6. Detection of beta-lactam resistance genes

Strains	Number of genes detected			Total
	bla_{CTX-M}	bla_{SHV}	bla_{TEM}	
E. coli	11	10	15	15
Frequency (%)	73,3	66,7%	100%	100%

Table 7. Genotypic profiles of extended-spectrum beta-lactamase producing *E. coli* strains

Profiles	Genotypes	Fréquency (%)
P1	$bla_{CTX-M}/bla_{SHV}/bla_{TEM}$	60%
P2	bla_{CTM-M}/bla_{TEM}	13,3%
P3	bla_{SHV}/bla_{TEM}	6,7%
P4	bla_{TEM}	20%

3.3 Discussion

The isolation of resistant *E. coli* strains on Rapid'*E. coli* 2 medium supplemented with ceftazidime revealed a prevalence of 20,2%. Several studies have been conducted to evaluate the prevalence of resistant *E. coli* in cattle faeces. Some studies have reported prevalence lower than that found in this study. This is the case of studies conducted by Sudarwanto et al. [22] in Indonesia and Medhanie et al. [23] in the United States, which revealed prevalence rates of 8,6% and 13,4%, respectively. Other studies have, however, reported higher prevalence. In France, Haenni et al. [24] detected a prevalence rate of 29,4%. In addition, Hille et al. [25] in Germany, reported a prevalence twice as high as that obtained in this study (43%). This difference in prevalence could be attributed to the isolation methodology used. Indeed, all of the studies cited above applied isolation protocols different to those used in this study, including a non-selective pre-enrichment step followed by a selective agar culture, containing 2 mg / L of cefotaxime.

However, a study conducted in Switzerland by Endimiani et al. [26] on surveillance of third-generation cephalosporin-resistant *E. coli* in bovine faeces, using selective agar containing 2 mg / L ceftazidime reported a prevalence of 3,9%. In addition, the comparability of these cited studies may be hindered by the level of antibiotic consumption. Moreover, the detection of resistant *E. coli* strains in cattle faeces reflects the poor use of antibiotics during the practice of breeding [27]. The antibiotic resistance is therefore the evolutionary response of bacteria to the high selective pressure resulting from antibiotic exposure [28].

The *E. coli* strains isolated showed as whole a high level of resistance to beta-lactams. The beta-lactam antibiotics, mainly cephalosporins, are commonly used in veterinary medicine because they are effective in treating environmental mastitis largely caused by strains of *E. coli* [29]. This high prevalence of beta-lactam resistance would be due to the use often

abusive and uncontrolled of beta-lactam antibiotics in livestock. Many previous studies have also reported this high resistance to beta-lactam antibiotics in *E. coli* strains on farms [30, 14]. Among the beta-lactam antibiotics tested, only imipenem was more active on all isolated strains. Of this fact, imipenem constitutes an antibiotic of choice for the treatment of the infections due to multidrug-resistant bacteria [31, 32]. Concerning resistance to quinolones and fluoroquinolones, resistance rates close to those observed in our study were reported by Yassin et al. [33] in China (nalidixic acid (13,1%) and ciprofloxacin (21,3%)). However, lower results than those obtained in our work were reported by Abbassi et al. [34] with rates of 6,7% for nalidixic acid and 3,3% for ciprofloxacin. Very high levels of resistance to nalidixic acid and ciprofloxacin have been observed in Nigeria by Ogunleye et al. [35] with respective rates of 61,7% and 51,3%. It is important to underline the upsurge of the resistance of *E. coli* strains to the cyclin family, in particular, the tetracyclines which are old molecules widely used in first intention. The tetracyclines are one of the most widely used classes of antimicrobial agents in human and veterinary medicine because they present several advantages, including a broad spectrum of activity, a weak cost, oral administration and few secondary effects [36]. This high use of tetracycline in farms has also been reported in Ivory Coast by Ouattara et al. [37]. Therefore, the high resistance to tetracycline in *E. coli* observed in this study would likely be due to the horizontal transfer of resistance genes between *E. coli* populations that have survived the selective pressure caused by the uncontrolled use of this antimicrobial agent. The resistance to colistin was quite important in this work with a frequency of 58,8%. The rates obtained in this work are worrying because they are much higher than the rates reported in several studies [38,39,40,41]. This high prevalence of colistin resistance may be due to the increasing use of colistin on farms. The mechanism of resistance to colistin was known as chromosomal mutations and its spread was therefore limited to vertical transmission [42]. In recent years plasmid-mediated resistance involving the mrc-1 gene has been discovered [43]. The ESBL-producing strains of *E. coli* isolated in cattle faeces have been reported by several authors [44,45]. In our study, this frequency was 52,94%. This frequency is higher than that observed by Schmid et al. [5] which was 32%. Lower frequencies were detected by Faruk et al. [46]. The frequency obtained in our study is close to that reported by Dahms et al. [47] which is 54,4%. However, Stefani et al. [48] detected higher frequencies compared to the one detected in our study. This strong presence of the ESBL-producing *E. coli* strains may be due to misuse of third- and fourth-generation cephalosporins in farms. In this study, the results revealed a high prevalence of the bla_{TEM} gene among isolated ESBL-producing *E. coli* strains. This result is similar to other studies [49,50]. However, other results showed that the $bla_{CTX\text{-}M}$ gene was most detected in the ESBL- producing *E. coli* strains [16,14]. According to studies conducted by Sudarwanto et al. [22] in Indonesia, $bla_{CTX\text{-}M}$ genes were the most widespread type of ESBL in most regions of the world, where the significant increase in ESBL incidence in enterobacteria was attributed to the spread of the members of CTX-M family. The rapid proliferation and worldwide spread of CTX-M in *E. coli* constitute a topic of preoccupation in both human and veterinary medicine [51]. In addition, Njage and Buys, [52] have reported that plasmids carrying CTX-M can transfer these determinants to other commensal enterobacteria. The enzymes of TEM and SHV type are able to hydrolyse cephalosporins but are unable to hydrolyse carbapenems. However, there are some variants that are not considered ESBL because they do not present activity on cephalosporins [53]. Although the subclasses of these enzymes can be differentiated, the double synergy test has shown the production of ESBL. It can be supposed that these classes of enzymes are responsible for the observed phenotypic profile, or that other classes of enzymes are produced by the bacteria. The importance of the bla_{TEM} and bla_{SHV} genes has been significantly reduced because of the rapid increase of $bla_{CTX\text{-}M}$ genes [53]. Several studies have highlighted the alarming emergence of these beta-lactamase genes in cattle faeces. The high levels of ESBL-producing *E. coli* strains carrying the $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} genes obtained in our study were also reported by Geser et al. [54].

4. CONCLUSION

This study showed that cattle are reservoirs of ESBL-producing *E. coli* strains. Indeed, strains of ESBL-producing *E. coli* with multiple resistance to other antibiotic families have been isolated with a high prevalence in cattle faeces in the Abidjan district, which testifies the misuse of antibiotics in farms. However, all ESBL-producing *E. coli* strains remained sensitive to imipenem, which is considered an antibiotic of

choice for serious infections. Molecular determination of resistance markers also revealed a high level of *bla* genes (CTX-M, SHV and TEM). These highly antibiotic-resistant strains may contribute to resistance acquired by transfer of resistance genes to other bacterial strains other than *E. coli*. These antibiotic resistance genes can be easily disseminated in the environment, in foods, in animals and in human. This spread can constitute a public health risk. In addition, it would be necessary to evaluate the mode of propagation of strains of *E. coli* producing ESBL in farms.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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