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Detection of genetic determinants of extended-spectrum beta-lactam resistance in *Klebsiella pneumoniae* and *Escherichia coli* strains from cattle feces in the Haut-Sassandra region, central-west of Côte d'Ivoire

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Abstract

As in human health, beta-lactams are widely used molecules in veterinary medicine for the treatment of bacterial infections. This study conducted in the Haut-Sassandra region of Côte d'Ivoire aims to investigate the extended-spectrum beta-lactamase resistance of *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from cattle excrement. Fecal samples from cattle were collected in the Daloa and Zoukougbeu departments. Fifty cattle fecal samples were collected across the two departments, including thirty-six in Daloa and fourteen in Zoukougbeu. Bacterial cultures were performed on these fecal samples, followed by molecular detection of ESBL resistance genes using PCR. In total, 19 strains of *Escherichia coli* and 31 strains of *Klebsiella pneumoniae* were isolated from these fecal samples. The results showed that 15.79 % of *E. coli* isolates and 41.93 % of *K. pneumoniae* presented ESBL resistance genes, primarily the blaCTX-M and blaTEM genes. Electrophoretic analysis confirmed the presence of these genes, with a predominance of the blaCTX-M gene. The irrational use of antibiotics in cattle farming is a key factor contributing to the emergence of resistant bacteria. The study highlights the importance of increased monitoring of antibiotic resistance and calls for urgent actions to counter this growing threat to public and animal health. The results also suggest that integrated strategies based on the "*One Health*" concept are necessary to effectively combat antibiotic resistance in this region. In conclusion, this research sheds light on the challenges posed by antibiotic resistance in the livestock sector and the need for a collaborative approach to improve health outcomes.

Keywords: *Klebsiella pneumoniae; Escherichia coli*; cattle; Resistance gene; ESBL; Côte d'Ivoire

1. Introduction

Antimicrobial resistance (AMR) poses a major challenge to humanity today, comparable in scale to climate change. According to the World Health Organization (WHO), this scourge has become a growing threat to global health and must be managed with extreme urgency [1]. Since their introduction in the 1940s, antimicrobials have revolutionized modern medicine in the treatment of infectious diseases in animals and humans [2]. Additionally, their application has enabled more intensive production of animals food to meet the growing global demand for animal protein [3]. In recent decades, antimicrobials as a whole have experienced a drastic decrease in their effectiveness in treating infections, particularly antibacterials. This ineffectiveness of antimicrobials primarily results from their excessive and disproportionate use in both human and veterinary medicine and in our livestock systems where they are often used as growth promoters [4,5]. This situation, coupled with the lack of development of new antibacterial molecules, increases the number of resistant

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and multi-resistant bacteria and their spread in all ecosystems. The consequences are severe and constitute a threat of returning to a therapeutic impasse where infections, which were treated until now, could again worsen the vital prognosis of infected patients or animals. In view of this situation, a genuine awareness of the health, economic, and social issues at local, national, and international levels is essential and must lead to the implementation of effective strategies to combat antimicrobial resistance by integrating all concerned actors. At its 2019 session, the FAO Conference emphasized the importance of urgently addressing the growing global threat of antimicrobial resistance in all countries through a "*One Health*" approach applied within the framework of the 2030 Agenda for Sustainable Development [6]. Thus, this research project is part of this concept of integrated approaches to developing innovative strategies aimed at effectively and sustainably combating antimicrobial resistance.

In the Haut-Sassandra region, cattle farming is booming and uses a large number of antibiotics for its development. The protocols for treating infectious diseases employed do not follow any therapeutic logic. Antibiotics are used hastily and irrationally by untrained farmers, which could consequently constitute a risk of emergence of resistant bacteria. The objective of this study is to investigate ESBL antibiotic resistance in cattle herds in Haut-Sassandra.

2. Material and methods

2.1. Study sites

This prospective study was conducted in the Haut-Sassandra region, specifically in the departments of Daloa and Zoukougbeu located in the central-west of Côte d'Ivoire. In this region, cattle farming is growing rapidly and constitutes the main source of beef supply for the area. In Daloa, five (05) sites were surveyed: Kenedy, N'Drikro, Tagoura, Seria, and Toroghue. In Zoukougbeu, only one site was visited. The number of sites depended on the number of farmers who gave their consent for this study.

2.2. Sample collection

The statistical unit of this study consisted of fecal samples collected from cattle. Under the supervision of a veterinary agent, farmers in the visited localities were informed about the implementation of a screening campaign for their herd. During the awareness or information phase, the veterinarian explained the rationale for this study and the potential risks involved. For farmers who gave their consent, freshly emitted fecal samples were collected from certain animals. It is important to note that only cattle that freely emitted their feces were considered for this study.

2.3. Isolation and identification of bacterial strains

The bacterial strains were isolated on CHROMAgar Orientation (Becton Dickinson, Cockeysville, MD), chromogenic medium [7]. The identification of *E. coli* and *K. pneumoniae* species was performed using the identification catalog of CHROMAgar Orientation (https://www.chromagar.com/en/product/chromagar-orientation/).

This identification was confirmed by performing classical biochemical tests.

2.4. Molecular characterization of strains collected and *genotypic detection of ESBLs*

The extraction of genomic DNA from *E. coli* and *K. pneumoniae* strains was performed using the phenol-chloroform method described by Chan and Goodwin [8]. Specific DNA sequences involved in resistance to broad-spectrum beta-lactam antibiotics were amplified by PCR using specific primers blaCTX-M, blaTEM, and blaSHV (Table 1). To do this, a total reaction volume of 50 µL containing 1 µL of each primer at 10 pmol/µL (Eurogentec, Belgium), 5 µL of PCR buffer (10x) with MgCl2, 2.5 µL of deoxyribonucleoside triphosphates (dNTPs, 200 µM), 0.1 µL of Taq polymerase (Qiagen), 37.4 µL of ultrapure water was prepared, to which 3 µL of bacterial genomic DNA was added. PCR was performed in a thermal cycler under the following conditions: initial denaturation for 5 minutes at 94 °C followed by 30 cycles comprising denaturation at 94 °C for 45 seconds, annealing at 60 °C for 1 minute, and elongation at 72 °C for 1 minute, followed by a final elongation at 72 °C for 10 minutes. The products of this PCR amplification were migrated on 2 % agarose gel for 45 minutes under an electric voltage of 100 volts, then the bands were visualized in an ultraviolet transilluminator.

Table 1 Primers used for the screening of genes coding for the production of broad-spectrum beta-lactamases

Genes	Primers Sequences (5'- 3')		References	Amplicon size (bp)
*bla*TEM	R	CTCAAGGATCTTACCGCTGTTG	[9]	112
	F	TTCCTGTTTTTGCTCACCCAG		
*bla*CTX-M	R	TTTATCCCCCACAACCCAG	[9]	701
	F	AATCACTGCGTCAGTTCAC		
*bla*SHV	R	CGCAGATAAATCACCACAATG	[9]	768
	F	TCGCCTGTGTATTATCTCCC		

R: Reverse primer, F: Forward primer

2.5. Statistical analysis of data

The experimental data were subjected to statistical analyses. The occurrence of ESBL resistance genes was calculated. A logistic regression of the occurrence of ESBL resistance genes on the variables Site, bacterial species, sex, and age of the animal was calculated using R software version 4.4.2 [10], followed by a binomial test. Significant differences are observed when the probability value (p) associated with the statistical tests is strictly less than 0.05

3. Results

3.1. Description of the studied cattle population

This prospective study was conducted in two departments of the Haut-Sassandra region, namely Daloa and Zoukougbeu. Table 2 presents the characteristics of the studied cattle population. A total of 11 farmers, including 8 in the Daloa department and 3 in Zoukougbeu, gave their consent for the collection of cattle fecal samples. In Daloa, 36 fecal samples were collected from five sites : Kenedy (4 samples), N'Drikro (2 samples), Seria (5 samples), Tagoura (6 samples), and Toroghue (19 samples). The majority of fecal samples came from cows (88.9 %), whose average age ranged from approximately 20 to 94 months (Table 2). In the Zoukougbeu department, 14 fecal samples were collected, of which 9 were from females (64.3 %) and 5 from male cattle (35.7 %). The female cattle had an average age of about 51 months, or approximately 4 years, and the males about 20 months.

Table 2 Characteristics of the studied cattle population in the Haut Sassandra region

Departments	Sites	N. Farmer	N.Samples	N.Female (%)	Mean.Age_F (month)	N. Male (%)	Mean.Age_M (month)
Daloa	Kenedy	1	4	3 (75)	84 ± 48	1 (25)	24 ± 0
	N'Drikro	1	2	2 (100)	20 ± 16	0	0
	Seria	2	5	5 (100)	93.6 ± 22.08	0	0
	Tagoura	3	6	6 (100)	28.5 ± 17	0	0
	Toroghue	1	19	16 (84.2)	56.6 ± 25.3	3 (15.8)	64 ± 24.2
	Total	8	36	32 (88.9)	57.4 ± 16	4 (11.1)	54 ± 32.4
Zoukougbeu	Zouk	3	14	9 (64.3)	51.4 ± 28.9	5 (35.7)	20.4 ± 16
	Total	11	50	41 (82)	56.10 ± 32.4	9 (18)	35.3 ± 25.2

N. Farmer : number of farmers ; N. Samples : number of fecal samples ; N.Female : number of female cattles ; Mean.Age_F : Mean age of female cattle ; N. Male : number of male cattles ; Mean.Age_M : Mean age of male cattle. Zouk : Zoukougbeu locality.

3.2. Identification of Escherichia coli and *Klebsiella pneumoniae* Isolates

Bacterial culture of feces was performed on a chromogenic medium. The results obtained are generally polycultures, meaning that on the culture medium, several bacterial colonies distinguishable by their color and shape appear. The selected bacteria are those that dominate on the isolation medium. Thus, *Escherichia coli* and *Klebsiella pneumoniae* were the predominant species in the isolation media. These two bacterial species were identified based on their

characteristics on the chromogenic medium, referring to the species identification catalog of CHROMAgar medium. Accordingly, culture on CHROMAgar chromogenic medium shows that E. coli isolates are distinguished by large dark pink colonies with or without a halo, while *K. pneumoniae* is characterized by medium-sized colonies colored in metallic green (Figure 1).

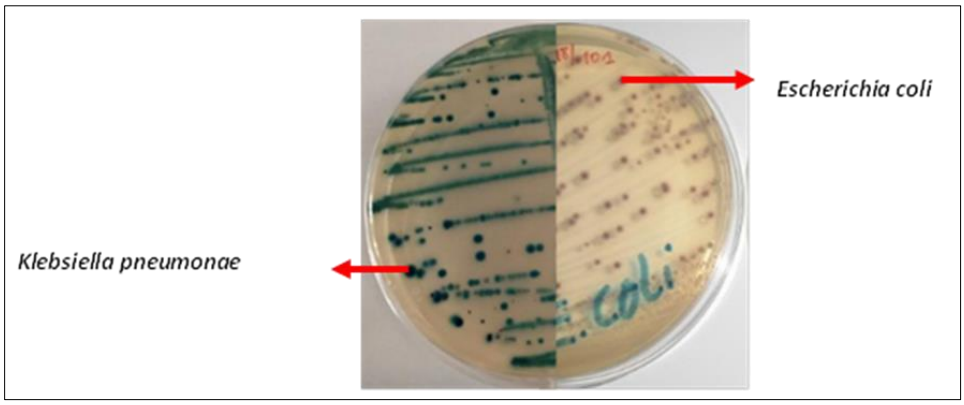

Figure 1 Differential characteristics of *Klebsiella pneumoniae* isolates (metallic green colonies) and *Escherichia coli* (dark pink colonies) on CHROMAgar medium

3.3. Molecular detection of ESBLs resistance genes

Molecular resistance to ESBLs is generally due to the expression of three main genes, namely blaCTX-M, blaTEM, and blaSHV. These genes were investigated in *Escherichia coli* and *Klebsiella pneumoniae* bacterial strains isolated from cattle feces.

Figure 2 Shows the electrophoretic profiles of ESBL genes detected in the DNA of these isolates. Indeed, molecular typing revealed the presence of blaCTX-M genes encoding cefotaximases-München (CTX-M) and the blaTEM gene encoding TEM penicillinases in the tested DNA samples

On the electrophoretic profile, these genes are characterized by DNA fragments of 112 bases pairs for blaTEM and 701 bases pairs for blaCTX-M genes (Figure 2)

A total of 50 bacterial isolates were tested. These isolates consist of 19 *Escherichia coli* strains and 31 *Klebsiella pneumoniae* strains (Table 3). In the *E. coli* population, only three isolates representing 15.79 % of the isolates showed the presence of ESBL genes in their DNA, including one isolate from Daloa and 2 isolates from Zoukougbeu. For *K. pneumoniae*, the number of isolates presenting resistance genes in their DNA is slightly higher. Indeed, 13 isolates showed the presence of ESBL genes in their genomes, representing 41.93 % of the isolates. In the Daloa department, 50 % of the isolated *K. pneumoniae* strains showed the presence of ESBL genes, while in Zoukougbeu, this rate is 27.27 % (Table 3).

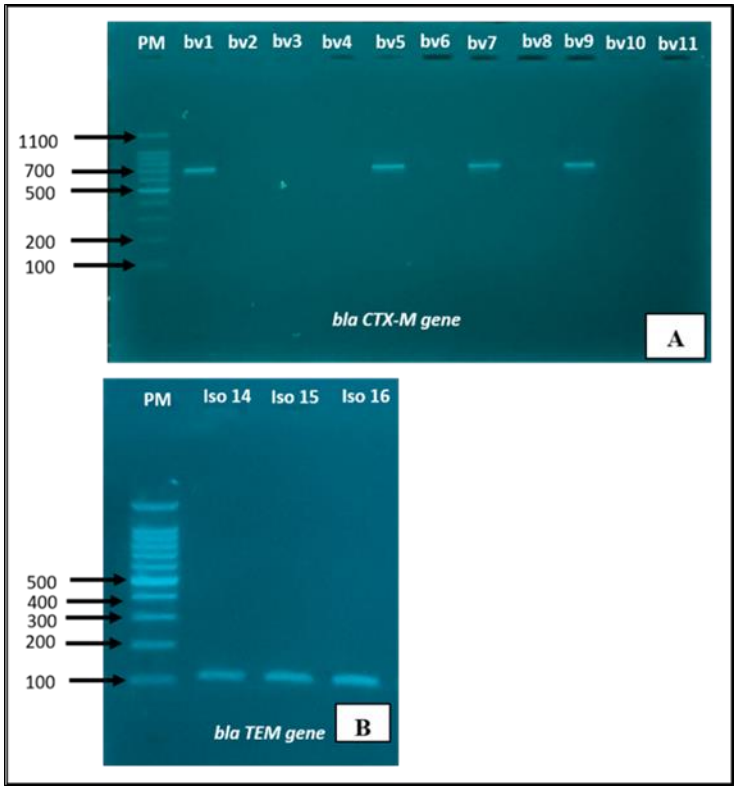

Figure 2 Electrophoretic profile on 2 % agarose gel of blaCTX-M (A) and blaTEM (B) genes. PM: Molecular weight marker (100 bp, Invitrogen); Iso notations followed by numbers indicate the tested samples.

Table 3 Characteristics of predominant bacterial species isolated from cattle feces in the surveyed departments

	Escherichia coli		***Klebsiella pneumoniae***	
Departments	Nº.Isolates	Nº. AMR (%)	Nº.Isolates	Nº. AMR (%)
Daloa	16	1 (6.25)	20	10 (50)
Zoukougbeu	3	2 (67)	11	3 (27.27)
Total	19	3 (15.79)	31	13 (41.93)

No. Isolates : Number of Isolates ; No. AMR : number of isolates possessing at least one resistance gene

3.4. Distribution of different types of ESBL resistance genes

In *Escherichia coli*, only three isolates showed the presence of ESBL genes in their genomes, including one (01) isolate from Daloa and two (02) from Zoukougbeu. The detected genes were all of the blaCTX-M type in both departments (Figure 3 A). In *Klebsiella pneumoniae,* 10 isolates from the Daloa samples showed the presence of ESBL genes, with 6 of the blaCTX-M type (60 %) and 4 of the blaTEM type (40 %) (Figure 4). In Zoukougbeu, the ESBL genes detected in the genomes of the three *Klebsiella pneumoniae* isolates were all of the blaCTX-M type (Figure 3 B).

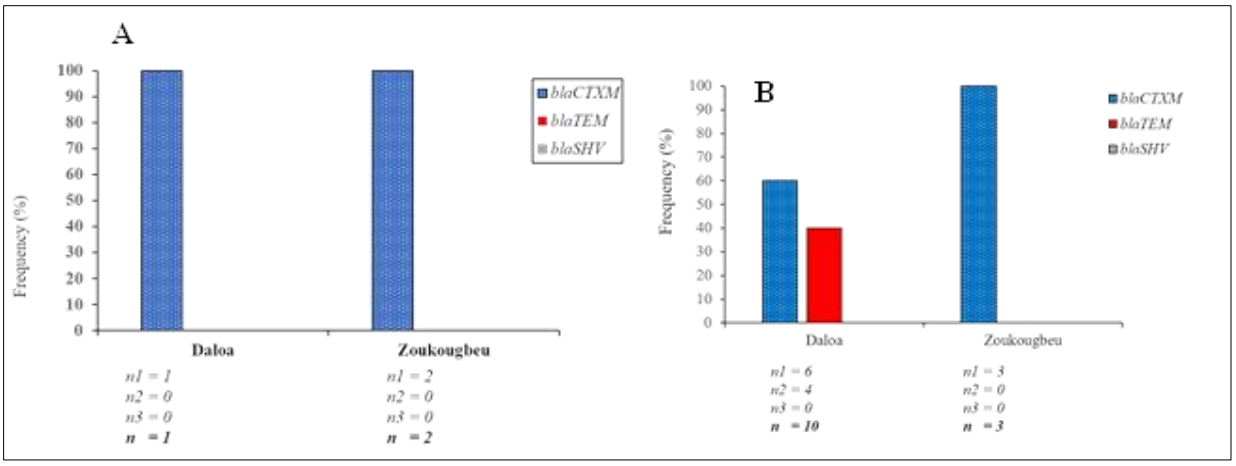

Figure 3 Distribution of ESBL resistance genes detected in *Escherichia coli* (A) and *Klebsiella pneumoniae* (B). n1, n2, n3 and n indicate the number of isolates presenting the bla CTX-M, bla TEM and bla SHV genes, as well as for all ESBL genes respectively

3.5. Factors Likely to Influence the Distribution of ESBL Genes

In order to identify factors that may influence the distribution of ESBL genes, a logistic regression analysis was performed on the occurrence of ESBL genes based on the bacterial species isolated, the gene type, the animal's sex, and the sample collection site. It was calculated using the 'quasibinomial' function. The results showed that only the Species variable is significant (Table 4). This leads us to calculate, among the bacterial species presenting ESBL resistance genes, the proportion of *K. pneumoniae* and that of *E. coli* and to compare this proportion to 0.5. The binomial test conducted (binom.test(13, 16, p = 0.5, alternative = "greater", conf.level = 0.95); p = 0.01064) indicates that the proportion of *Klebsiella pneumoniae* isolates possessing at least one ESBL resistance gene (81.25 %) is significantly higher than that of *Escherichia coli* (18.75 %).

Table 4 Logistic regression of the occurrence of ESBL resistance genes on the variables Sites, Isolate Species, sex, and age of the animal calculated using the "quasibinomial" function

NULL	Df	Deviance	Resid. Df	Resid. Dev	F	Pr (>F)
			49	62.687		
Sites	5	10.2561	44	52.431	1.5593	0.19372
Species	1	8.0854	43	44.345	6.1464	0.01748 *
Sex	2	4.9851	41	39.360	1.8948	0.16360
Age	1	0.0458	40	39.315	0.0348	0.85286

Df: Degrees of freedom, Deviance: model deviance, Resid. Df: residual degrees of freedom, Resid. Dev: residual deviance; F: F statistic, Pr: probability associated with the F statistic.

4. Discussion

Bacterial cultures of cattle fecal samples in this study showed that the species dominating the intestinal flora of cattle are Enterobacteriaceae, notably *Klebsiella pneumoniae* and *Escherichia coli*. This predominance can be explained by several reasons. Indeed, *Escherichia coli* is part of the natural intestinal flora of ruminants. This bacterium is a normal inhabitant of the intestinal tract of cattle [11]. *Klebsiella pneumoniae*, being an environmental bacterium, is encountered by cattle during feeding, particularly through manure and contaminated water [12]. Additionally, confined environments and variations in abiotic factors such as temperature and humidity in livestock buildings can promote the proliferation of these bacteria. Lin et al. [13] showed that certain bacterial strains possess specific virulence factors, such as adhesins, which facilitate their colonization of the intestinal tract. This combination of factors explains why *E. coli* and *K. pneumoniae* are frequently isolated from cattle feces, reflecting their adaptation to the intestinal environment of ruminants. The presence of *Klebsiella pneumoniae* and *Escherichia coli* in cattle feces attests to the zoonotic nature of these bacteria, which merits particular surveillance.

Molecular typing of the ESBL resistance genes of *E. coli* and *K. pneumoniae* strains isolated from cattle feces in the Haut-Sassandra region showed that the resistance profile is encoded by the blaCTX-M and blaTEM genes, with a high occurrence rate of the blaCTX-M gene. The dominance of this resistance profile by these genes can be explained by the fact that blaCTX-M and blaTEM genes are the most widespread among ESBL-producing Enterobacteriaceae. Indeed, these genes are often carried by conjugative plasmids, facilitating their spread among different bacterial strains [14,15]. Additionally, following our survey conducted among farmers, the injectable penicillins and cefotaxime antibiotics frequently used in treating cattle herds are β-lactam-based, which promotes the selection and persistence of these resistance genes [16].

The high occurrence rate of the blaCTX-M gene is likely due to its significant genetic diversity and rapid dissemination capability [15,17]. The absence of the blaTEM gene in the tested *Escherichia coli* isolates does not exclude its presence in *E. coli*. However, this absence may be attributed to the small sample size of *E. coli* tested. Indeed, the small number of *E. coli* samples possessing ESBL genes (only three isolates in this preliminary study) does not provide the opportunity to detect the blaTEM gene in the *E. coli* sample. This result suggests that sampling efforts need to be increased to allow for the detection of the blaTEM gene and even the blaSHV gene in the genomes of these bacterial strains. In contrast to this study, several studies have shown the presence of the blaTEM gene in both *E. coli* and *K. pneumoniae* [18,19,20].

Moreover, our results revealed that the occurrence of ESBL genes is related to the bacterial species isolated from cattle feces, and the proportion of *Klebsiella pneumoniae* isolates possessing at least one ESBL resistance gene is significantly higher than that of *Escherichia coli*. These results may be attributed to the high presence of *Klebsiella pneumoniae* in cattle feces at the visited sites. Furthermore, this bacterial species is likely exposed to strong selection pressure from β-lactam antibiotics, thereby promoting the emergence of multidrug-resistant strains to extended-spectrum β-lactams (ESBLs). These findings are corroborated by the work of Yasmeen et al. [21], who reported a high occurrence rate of *K. pneumoniae* possessing ESBL genes. The high occurrence rate of *K. pneumoniae* carrying an ESBL among cattle is alarming, as it may spread in the environment and pose a risk to human health.

5. Conclusion

This prospective study conducted in the departments of Daloa and Zoukougbeu, in the Haut-Sassandra region, highlighted the presence of *Escherichia coli* and *Klebsiella pneumoniae* strains in cattle feces. The presence of these bacteria, commonly found in human biological samples, indicates the zoonotic nature of *E. coli* and *K. pneumoniae*. Additionally, the detection of *E. coli* and *K. pneumoniae* strains possessing ESBL resistance genes confirms the dynamic spread of ESBL resistance genes across various ecosystems, including human, animal, and environmental contexts. These observations underscore the importance of adopting integrated methods such as the One Health approach for effective monitoring of antimicrobial resistance, which is currently a major global health challenge. This study sheds light on the challenges posed by antibiotic resistance in the livestock sector and the necessity for a collaborative approach to improve health outcomes.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interests.

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