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Characterization of the Genetic Supports for Betalactam Resistance in *Escherichia coli* Strains of Porcine Origin Producing Extended-spectrum Beta-Lactamase (ESBL)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IKK and NG designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors IKK and AK managed the analyses of the study. Authors IKK and AD managed the literature searches. Authors IKK and SK performed the laboratory microorganism culture. Authors VG, BT, MBO, FK and AA contributed to the writing and editing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was to evaluate the genetic potential of beta-lactam resistance in *Escherichia coli* strains producing extended-spectrum beta-lactamase (ESBL) isolated from the faecal flora of piglets.

Place and Duration of Study: National Reference Center for Antibiotics and Molecular Biology Platform of Pasteur Institute of Côte d'Ivoire, between June 2017 at July 2017.

Methodology: A detection of *E. coli* strains were carried out from 30 samples of faeces of piglets after five-day of treatment with amoxicillin was studied. The isolation of *E. coli* was performed on MacConkey agar supplemented with amoxicillin and identification using biochemical test. The Antibiotic susceptibility test was carried out according to diffusion method in agar medium.

Results: The genetic supports for betalactam resistance, in particular the *bla* $_{TEM}$, *bla* $_{SHV}$ and *bla* $_{CTX-M}$ genes, have been detected by PCR in strains producing extended-spectrum beta-lactamase (ESBL). A total of thirty five (35) *E. coli* isolates (35%) showed ESBL production. The *bla* $_{TEM}$, *bla* $_{SHV}$ and *bla*$_{CTX-M}$ genes were detected with frequency of 51%, 40% and 31% respectively. The strains were resistant to antibiotics from other families with the most common resistance profile consisting of tetracycline (100%), trimethoprim / sulfamethoxazole (80%), gentaminicin (70%), kanamycin (70%) and streptomycin (90%).

Conclusion: The detection of *bla* genes which are plasmid borne, therefore potentially horizontally transmissible to other strains, constitute a risk to public health and requires a monitoring of antibiotic-resistant bacteria in the animal production sector.

Keywords: Piglets; E. coli; ESBL; antibiotic; bla $_{TEM}$ *genes; bla* $_{SHV}$*; bla* $_{CTX-M}$.

1. INTRODUCTION

Antibiotics use in piggery constitutes now, one of the largest markets for the sale of veterinary medicines. In 2006, 51% of worldwide sales of antibiotics in the animal sector were attributed to the pig sector alone [1]. It is currently a known fact that the excessive use of antibiotics during pig treatment favors a significant selection of multi-resistant bacteria in their digestive flora [2, 3]. The selected antibiotics resistant bacteria of the digestive tract constitute commensal or zoonotic enterobacteria [4-6]. They can transfer their resistance genes to bacteria that are part of the human intestinal flora via the food chain [7, 8]. The most commonly detected antibiotic resistance genes among fecal isolates from pigs are *bla* $_{TEM-1}$, *bla* $_{SHV-1}$, *bla* $_{CTX-M-1}$ genes and *tet (A)* genes encoding resistance to tetracycline [9,10]. The digestive tract of pigs therefore represents a real reservoir of resistance genes where mobile genetic elements are easily transferred to other bacteria by horizontal transfer [11]. These exchanges may occur between resident strains of the intestinal flora from identical, different or even remote species [2]. Thus, commensal bacteria of animal origin can potentially end up in the human digestive tract and even if they are only transient, they may eventually transfer genes to the human commensal bacteria. The human intestinal ecosystem would thus be enriched with genetic determinants of resistance of animal origin, potentially transmissible to pathogenic bacteria [4]. The study of these potential gene flows between the animal and human world is encouraged by AFSSA (French Food Safety Agency) and EFSA (European Food Safety Authority) in order to better understand its importance [12]. Bacteria isolated from animals and humans share the same mechanisms of resistance, which is an argument in favor of the lack of sealing between bacterial populations of human and animal origin [13]. In fact, a recent study showed that human populations and livestock shared genetically identical strains of *E. coli* [13]. Given the real and potential consequences of the transfer of resistance genes from bacteria of porcine origin to those of humans via food in public health, it appeared relevant to this research to study the genotypic characterization of porcine-derived *Escherichia coli* producing Extended-Spectrum Beta-Lactamase type *TEM, CTX-M* and *SHV* is necessary.

2. MATERIALS AND METHODS

2.1 Isolation and Bacterial Identification

In the month of February 2016, one hundred (100) *E. coli* isolates from the faeces of piglets.

"Large White" (*Sus scrofa domesticus*) collected after treatment with amoxicillin (Vetrimoxin®, France) were obtained for the study. The faeces came from a farm located in Abadjin-Doumé, a village located on the western outskirts of Abidjan (Côte d'Ivoire). The *E. coli* isolates were isolated with MacConkey agar (Becton, Dickinson and Company, USA). They were identified using biochemical test.

2.2 Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed by disc diffusion method on Mueller-Hinton agar (Bio-rad, Marne-la-coquette, France) according to the recommendations of the Antibiogram Committee of the French Society of Microbiology (CASFM, 2014). Fourteen (14) antibiotics discs (Bio-rad, Marne-la-coquette, France) were used in the study: Amoxicillin (25 µg), Amoxicillin + clavulanic acid (20 / 10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Nalidixic Acid (30 µg), Tetracycline (30 µg), Trimethoprim / sulfamethoxazole (1.25 / 23.75 µg), Chloramphenicol (30 µg), Kanamycin (30 µg), Streptomycin (10 µg), Colistin (50 µg). The reference strain *E. coli* ATCC 25922 was used as a control.

2.3 ESBL Production

The *E. coli* isolates detected ESBL by antibiotic susceptibility test were confirmed by the double disc synergy test using cefotaxime (30 g), ceftazidime (30 g), ceftriaxone (30) and aztreonam placed 30 mm center to center from an amoxicillin / clavulanic acid disk (20/10 µg). ESBL production was detected when synergy was observed between the inhibition zones of cephalosporins and amoxicillin / clavulanic.

2.4 Detection of *bla* $_{TEM}$, *bla* $_{CTX-M}$ and *bla* $_{SHV}$ Genes Coding for ESBL Production

The *E. coli* isolates producing ESBL were chosen for the genotypic study. Detection of *bla* $_{TEM}$, *bla* $_{CTX-M}$ and *bla* $_{SHV}$ resistance genes using the classical PCR method was performed after extraction of the total DNA by boiling method. The primers used are shown in Table 1. The reaction mixture consisted of 5 µL of colored buffer (Green GoTaq®, Promega, USA), 5 µL of uncolored buffer (Colorless GoTaq®, Promega, USA), 30,3 µL ultrapure water (Nuclease-Free Water, Promega, USA), 3 µL of $MgCl_2$ (Promega, Madison, USA) (25 mM), 0.5 µL of DNTP (10 mM), 0.5 µL of each primer (20 mM) (Inqaba biotec, South Africa) and 0.2 µL of Taq polymerase (GoTaq®, Promega, USA). Amplification was carried out in thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with amplification conditions shown in Table 2. The PCR products were analyzed by electrophoresis with 1.5% agarose (Invitrogen, USA). Three reference strains were used as positive controls (Table 3) and a reaction mixture without DNA was considered negative control.

Table 1. Primers for PCR reaction

Genes	Primers	Sequences (5'->3')	Size of amplicon (bp)	N° Accession Genbank
bla$_{TEM}$	a 216 (+)	ATAAAATTCTTGAAGACGAAA	1079	AB282997
	a 217 (-)	GACAGTTACCAATGCTTAATCA		
bla$_{SHV}$	os-6 (-)	TTATCTCCCTGTTAGCCACC	795	X98098
	os-6 (-)	GATTTGCTGATTTCGCTCGG		
bla$_{CTX-M}$	ctx M1(+)	GGTTAAAAAATCACTGCGTC	863	X 92506
	ctx M1(-)	TTGGTGACGATTTTAGCCGC		

Table 2. PCR amplification conditions for *bla* $_{TEM}$, *bla* $_{SHV}$ and *bla* $_{CTX-M}$ genes

Amplification steps	Temperature conditions / duration	
	bla $_{TEM}$	*bla* $_{SHV}$, *bla* $_{CTX-M}$
Initial denaturation	94℃ / 5 min	94℃ / 5 min
Cyclic denaturation	94℃ / 1 min	94℃ / 1 min
Annealing	50℃ / 1 min	60℃ / 1 min
Cyclic elongation	72℃ / 1 min	72℃ / 1min
Final elongation	72℃ / 7 min	72℃ / 7 min
Number of cycles	30	30

Table 3. Bacteria isolate used as positive control for PCR reactions

Bacteria	Number	Characteristics	Positive controls	Types of analysis
Salmonella sp	U2A1446	*TEM-1* et *SHV12*	*bla* $_{TEM}$ et *bla* $_{SHV}$	Simplex
E. coli	U2A1790	*CTX-M* groupe 1	*bla* $_{CTX-M}$	Simplex

3. RESULTS

3.1 Detection of *E. coli* Isolates Producing ESBL

One hundred (100) Amoxicillin-resistant *E. coli* isolates isolated on MacConkey agar were used for analyze. Thirty five (35) of these isolates producing ESBL were detected after antibiotic susceptibility test and by the double disc synergy test. The frequency of ESBL isolates was 35% (35/100).

3.2 Cross-resistance to Beta-lactams and Other Antibiotics

These *E. coli* isolates producing ESBL also exhibited cross-resistance with betalactamins. The resistance of the isolate to other associated antibiotics was tetracycline (100%), trimethoprim / sulfamethoxazole (80%), gentaminicin (70%), kanamycin (70%) and streptomycin (90%). None strain of *E. coli* producing ESBL showed resistance to colistin. The antibiotic resistance profiles associated with beta-lactams have been shown in Table 4.

3.3 Electrophoretic Profile of Beta-Lactam Resistance Genes

The electrophoretic profile of products of amplification of the *bla* $_{TEM}$ gene is shown in Fig. 1. It reveals characteristic signals of this gene, in particular expected bands, of 1079 base pair size; which shows the detection of the gene at strains 4, 5, 6 and 7.

The electrophoretic profile of products of amplification of the *bla* $_{CTX-M}$ gene is shown in Fig. 2. It reveals characteristic signals of this gene, in particular expected bands, of 863 base pair size; which shows the detection of the gene at strains 10 and 14.

The electrophoretic profile of products of amplification of the *bla* $_{SHV}$ gene is shown in Fig. 3. It reveals characteristic signals of this gene, in particular expected bands, of 795 base pair size; which shows the detection of the gene at strains 3, 4, 5 and 6.

3.4 Frequency of Detection of Genes Encoding ESBL Production

Frequency of detection of genes encoding production was presented in Table 5.

4. DISCUSSION

The results show that more than one-third (35%) of the strains of *Escherichia coli* analyzed are resistant to β-lactams by the production of Extended-Spectrum Beta-Lactamase. This occurrence rate of ESBL isolates is in line with the reported by Dierikx et al. [14] and Randall et al. [15]. In Belgium, a study also reported 36% ESBL producing *E. coli* of avian origin [16]. However in this study occurrence rate was lower in relation to results obtained in Tunisia, Spain and Italy. In Tunisia, this rate varied from 42 to 45.5% [17,18]; in Spain and Italy, the frequency of *E. coli* isolates producing ESBL was 52.3% and 54.8% respectively [19]. In Denmark, a study carried out on pig farms indicated a very high level of *E. coli* producing ESBL estimated at 95% [20]. The rate of 34% of *E. coli* producing ESBL in our study was higher than those determined by [21] in France, which was 24.5% and by [22] in Japan with isolates of porcine origin and avian which was 0.15%. In Taiwan, the rate was 19.7% [23]. Studies carried out in other parts of Africa also reported lower rates than our result. In Cameroon was 18.8% and in South Africa 28.1% [24]. This low frequency could be explained by the fact that the hyperproduction of a cephalosporinase would mask a possible ESBL [25].

In this study *E. coli* isolates producing ESBL showed at least two antibiotic resistance associated with beta-lactams and other antibiotics such as tetracycline (100%), trimethoprim / sulfamethoxazole (80%), gentaminicin (70%), kanamycin (70%), and streptomycin (90%). This finding is similar to a study in France where *bla* $_{TEM}$ genes detected displayed resistance to tetracycline (90%), streptomycin (82%) chloramphenicol (32%), trimethoprim (58%), sulfonamide (63%) [26]. In another study reported a similar result from 24 isolates of *E. coli* of bovine and porcine origin

[27]. The most common beta-lactam resistance profile was tetracycline (88%), streptomycin (96%), trimethoprim / sulfamethoxazole (70%) and gentamicin (54%). *bla* $_{TEM}$, *bla* $_{SHV}$ and *bla* $_{CTX-M}$ genes was detected in some of the ESBL *E. coli* strains obtained. *bla* $_{TEM}$ genes with a frequency of 51% was dominant followed by *bla* $_{SHV}$ genes (40%) and *bla* $_{CTX-M}$ genes (31%). This is in consonant with the study in Nigeria [28]. They reported a predominance of *bla* $_{TEM}$ gene 81.5%, *bla* $_{SHV}$ gene with 16.7% occurrence and *bla* $_{CTX-M}$ gene with 31.5% in strains of *E. coli* producing ESBL.

The high occurrence of *bla* $_{TEM}$ genes in ESBL producing *E. coli* of food animal origin tends to be wide spread. In Canada and Germany with frequency has been reported. In *E. coli* isolated from cattle, pigs and chickens Germany [29,30]. Also in Denmark *bla* $_{TEM}$ genes was reported to have a frequency of 69% in *E. coli* isolated from faeces of healthy and diseased animals [31]. A higher frequency 83% was obtained in Spain compared to the occurrence of other *bla* genes (*bla* $_{CTX-M}$ and *bla* $_{SHV}$) [32]. However, in France or Netherlands and other countries the *bla* $_{CTX-M1}$ tend to have a higher frequency in recent times [33,34]. In Algeria, the *bla* $_{CTX-M}$ gene had a frequence of 95% compared to *bla* $_{TEM}$ (92.5%) and *bla* $_{SHV}$ (91.25%) [35]. Also in domestic pests such as cats and dogs *bla* $_{CTX-M}$ and *bla* $_{SHV}$ genes has been reported. In Italy, the prevalence of of the *bla* $_{CTX-M}$ gene in *E. coli* isolated from 204 dogs and 61 cats was 76% and 23% respective [36]. In Côte d'Ivoire, a study in Abidjan on strains of *E. coli* producing ESBL isolated from community patients showed that

Table 4. Cross-resistance profile to antibiotics other that beta-lactams of *E. coli* producing ESBL

Antibiotic resistance associated	Number of resistant *E. coli* isolates ESBL	Rate of resistance (%)
Tetracycline	35	100
Trimethoprim / Sulfamethoxazole	28	80
Gentaminicin	25	70
Kanamycin	25	70
Streptomycin	32	90
Ciprofloxacin	7	20
Nalidixic acid	7	20
Chloramphenicol	3	10
Colistin	0	0

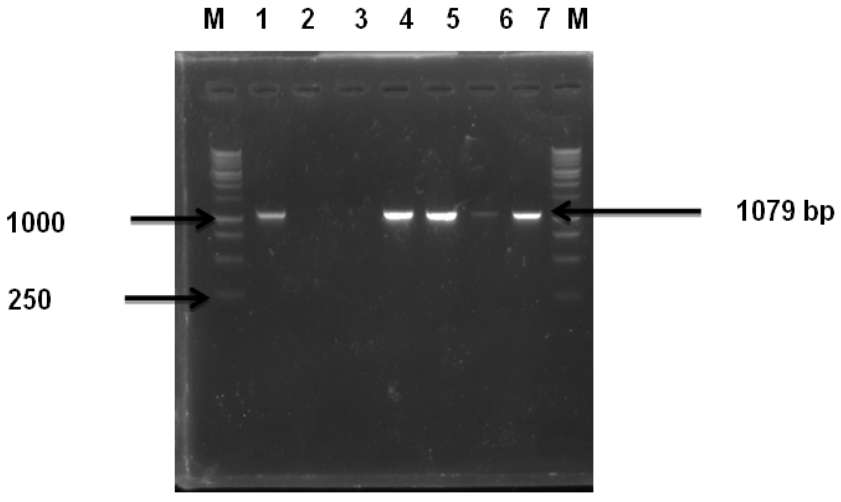

Fig. 1. Electrophoretic profile of *bla* $_{TEM}$ gene amplification product

Line M: Molecular weight marker (Benchtop, 1kb DNA Ladder, USA); Line 4, 5, 6 and 7: Reference of strains analyzed harboring bla $_{TEM}$ *genes,*
Line 1: Positive control strain; Line 2: Negative control

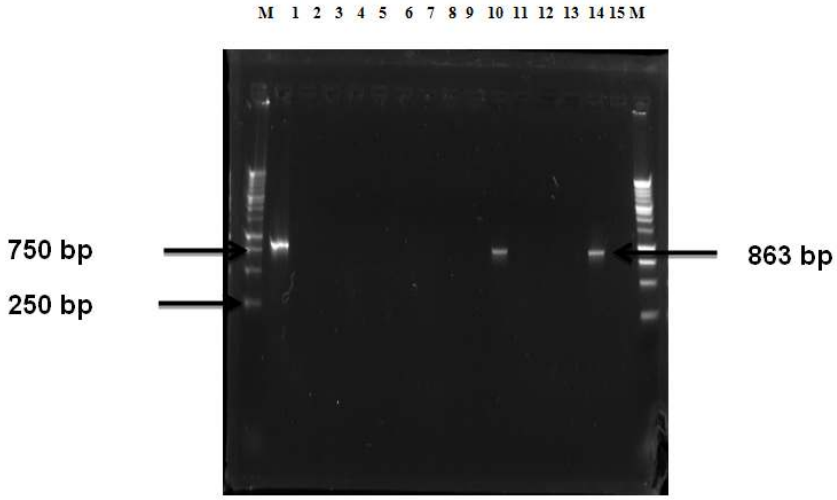

Fig. 2. Electrophoretic profile of the *bla* $_{CTX-M}$ gene amplification product
Line M: Molecular weight marker (Benchtop, 1kb DNA Ladder, USA)
Line 10 and 14: Reference of strains analyzed harboring bla $_{CTX-M}$ *genes*
Line 1: Positive control strain; Line 2: Negative control

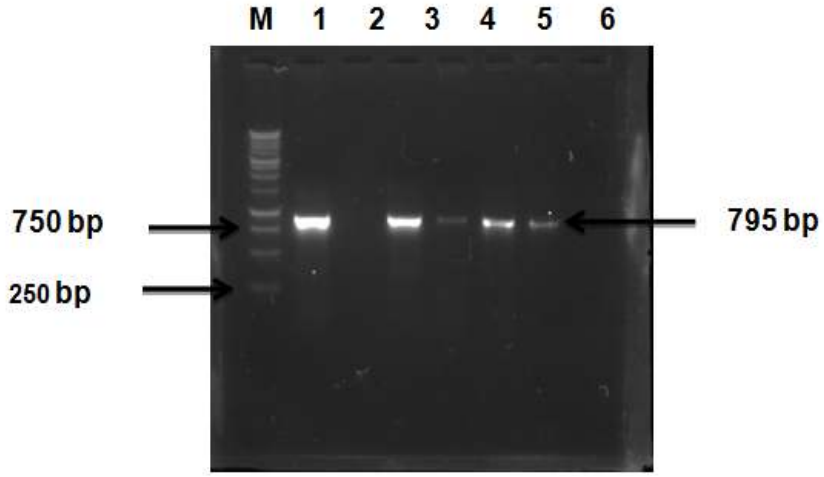

Fig. 3. Electrophoretic profile of the *bla* $_{SHV}$ gene amplification product
Line M: Molecular weight marker (Benchtop, 1kb DNA Ladder, USA)
Line 3, 4, 5, 6: Reference of analyzed strains harboring the bla $_{SHV}$ *genes*
Line 1: Positive control strain; Line 2: Negative control

Table 5. Frequency of detection of genes encoding ESBL production

Resistance genes encoding ESBL production	Frequency of resistance genes N (%)
bla $_{TEM}$	18 / 35 (51%)
bla $_{CTX-M}$	11 / 35 (31%)
bla $_{SHV}$	14 / 35 (40%)

bla $_{CTX-M}$ genes were the most numerous with 65.6% followed by *bla* $_{TEM}$ genes with 64.9% and *bla* $_{SHV}$ genes with 48.3% [37]. The low number of the *bla* $_{CTX-M}$ gene observed in our *E. coli* isolates could be attributed to the low use of third generation cephalosporins compared to other antibiotics in pig farms in Côte d'Ivoire.

5. CONCLUSION

The emerging increase of ESBL producing *E. coli* resistant to beta-lactams group of antibiotics is on the raise in most food animals especially in the porcine sector. This is of public health significance since these bacteria find their way into the food chain coupled with a rapid spread due to easy exchange of foodstuff of animal origin across borders of numerous countries. The genotypic characterization of these strains of *E. coli* producing ESBL from porcine origin armed with genes such as the *bla* $_{TEM,}$ *bla* $_{CTX-M}$ and *bla* $_{SHV}$ in Côte d'Ivoire is an issue of great concern. It is therefore imperative for appropriate authorities to formulate policies geared towards monitoring the administration of antibiotics in animal husbandry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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