



## Phenotype and Molecular Characterization of Antibiotic Resistance of *Salmonella* spp. from Cattle in Abidjan District, Côte d'Ivoire

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### Authors' contributions

This work was carried out in collaboration among all authors. Author CKJ designed the study, wrote a part of the protocol and the first draft of the manuscript. Authors YKR, DK, TKB and AA collected the samples and managed the analyses of the study. Author KKE managed the breeders, the sellers and wrote a part of the protocol. Authors DAJ and DM corrected the protocol and the article. Author YHF supervised the study. All authors read and approved the final manuscript.

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### ABSTRACT

**Aims:** The overall objective of this study was to study antibiotic-resistant strains of *Salmonella* in livestock sales sites in the Abidjan district.

**Place and Duration of Study:** Researchers team from Institute Pasteur of Ivory Coast and their students collected samples of cattle feces in five townships of the district of Abidjan from April to September 2016.

**Methodology:** Fresh cow dung has been collected from sales outlets and livestock pens in five municipalities in Abidjan. The prevalence of *Salmonella* carrying has been studied by classical

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microbiological techniques. These included strain isolation by culture on the Hektoen medium, biochemical identification using Leminor's reduced rack and strain confirmation by MALDI-TOF MS. Phenotypic determination of antibiotic resistance and detection their genes were carried out respectively by the method of discs diffusion on the Muller-Hinton agar and using PCR simplex.

**Results:** In this study, we collected 420 samples.

The results showed that the overall prevalence of *Salmonella* isolated from cattle feces was 20% (84/420). Twenty-six (26) strains were resistant to at least one antibiotic. The *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *TetA* resistance genes were detected with the respective frequencies of 7.7%, 57.7% and 7.7%.

**Conclusion:** The isolation of antibiotic-resistant *Salmonella* strains in these healthy cattle poses a significant threat to public health. However, a good use of antibiotics in farms could help limit the phenomenon of resistance of *Salmonella* to antibiotics.

**Keywords:** *Salmonella* spp.; antibiotics; resistance; cattle; Abidjan; Côte d'Ivoire.

## 1. INTRODUCTION

The possibility of transmission of antimicrobial resistant bacteria or their determinants of resistance from food animals to humans has been a public health concern for several decades [1]. This situation is generally due to the misuse of antibiotics in human or veterinary medicine. Indeed, the use of antimicrobials in animal production systems has long been suspected of being responsible for the emergence and spread of resistant bacteria [2].

In addition, resistant bacteria can act as a donor of a determinant of resistance to an optional pathogen of the commensal flora, which could be associated with a disease and in turn provide the resistance gene to another one [3]. Among the animals in the production system, cattle have been implicated as a potential source of human infection by antimicrobial-resistant bacteria through direct contact with livestock and by the consumption of food contaminated with these Bacteria.

Antibiotic resistant and multidrug-resistant bacteria are actually increasing on farms because of the use of antibiotics in the treatment of animals, at prophylactic or metaphylactic doses likely to favor the selection of resistant strains on farms. This situation greatly increases the risks to human health through the consumption of contaminated food products [4].

Recently, the emergence and spread of antimicrobial-resistant *Salmonella* from animal foods has been recognized as a serious danger in the world, particularly in developing countries [5,6]. According to the Centers for Disease Control and Prevention (CDC), non-typhoid *Salmonella* is one of the leading threats to antibiotic resistance in the United States,

associated with about 1.2 million infections, 100,000 antimicrobial-resistant infections and 300 million of dollars in annual medical expenses [7].

In Europe, multi-antibiotic resistant *Salmonella* continues to spread in all health sectors according to the latest available data on the resistance of bacteria in humans, food and animals, provided by ECDC and EFSA [8]. In Côte d'Ivoire, several studies on *Salmonella* have shown an expansion of antibiotic-resistant strains [9,10,11,13,14]. However, data on antibiotic resistance of *Salmonella* in cattle farms are almost nonexistent to our knowledge in Côte d'Ivoire.

The general objective of this study is to characterize by phenotypic and molecular methods, antibiotic resistance in strains of *Salmonella* isolated from cattle feces in the district of Abidjan (Côte d'Ivoire).

## 2. MATERIALS AND METHODS

### 2.1 Isolation of *Salmonella* Strains in Cattle Feces

Fresh cattle feces samples were collected in five townships in the Abidjan district of Côte d'Ivoire over a six-month period from April to September 2016. A quantity of about two hundred (200) grams of randomly selected fresh feces was collected from the various sites.

The samples were taken from the surface and inside of feces freshly emitted with wooden spatulas and put in sterile Zip sachets. These in turn, have been placed in coolers containing ice pack and immediately sent to the microbiology laboratory of the Pasteur Institute about to 2

hours after collection. *Salmonella* isolation and identification was performed using the conventional method for the detection of *Salmonella* spp. according to ISO 6579:2002(E)). Briefly, 25 g of fresh feces were pre-enriched in 225 ml of buffered peptone water (EPT) (Bio-Rad) and incubated for 24 hours at 37°C. A suspension of 0.1 ml of the pre-enriched medium was added to 10 ml of Rappaport-Vassiliadis (RV10) enrichment broth (Bio-Rad) and incubated at 44°C for 24 hours. Then, each enrichment broth was streaked onto the Hektoen selective medium (Bio-Rad) and incubated for 24 hours at 37°C. Three to five characteristic *Salmonella* colonies were randomly selected and then identified using the Leminor reduced rack. The *Salmonella* strains identified were subsequently confirmed at MALDI-TOF MS (BioMérieux, France). The strain of *Salmonella* typhimurium (IPCI) was used as a positive control to verify the effectiveness of isolation on Hektoen agar. The strain of *E. coli* ATCC 8739 (Biomérieux, France) was used as a calibration strain of MALDI-TOF.

## 2.2 The Susceptibility of *Salmonella* Strains to Antibiotics

Antibiotic susceptibility tests were performed on all *Salmonella* strains. A standardized amount

was inoculated (standard 0.5 of McFarland) and antimicrobial susceptibility testing was performed by the disk diffusion method on Mueller–Hinton (MH) agar using interpretative criteria of the Antibigram Committee of the French Society of Microbiology (EUCAST/ CA-SFM, 2016). Quality control was carried out using standard strains of *Escherichia coli* (ATCC 25922). Intermediate susceptibility to each antibiotic was considered to be resistant. The following antibiotic discs (Bio-Rad France) were used: ampicillin (10 µg), amoxicillin + clavulanic acid (30 µg), cefalotin (30 µg), cefepime (30 µg), aztreonam (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), colistine (50 µg) and trimethoprim/sulfamethoxazole (25 µg).

## 2.3 Detection of Antibiotic Resistance Genes

Detection of antibiotic resistance genes was performed by simplex PCRs on all strains of *Salmonella* phenotypically resistant to at least one antibiotic. Extraction of the bacterial DNA was carried out on 24-hour colonies by heat

**Table 1. Specific primer**

Gene	Primers	Sequence (5'-3')	Size of fragment (pb)	References
<i>bla<sub>TEM</sub></i>	TEM front P1	GCGGAACCCCTATTTG	964	[16]
	TEM-C-R-ny	ACCAATGCTTAATCAGTGAG		[17]
<i>bla<sub>CTX-M</sub></i>	CTX-M F	TTTGCGATGTGCAGTACCAGTAA	544	[18]
	CTX-M R	CGATATCGTTGGTGGTGCCATA		
<i>bla<sub>SHV</sub></i>	SHV F	TTTATGGCGTTACCTTTGACC	1051	[19]
	SHV R	ATTTGTCGCTTCTTTACTCGC		
<i>tetA</i>	TetA primer1	GTAATTCTGAGCACTGTTCGC	956	[20]
	TetA primer2	CTGCCTGGACAACATTGCTT		

**Table 2. Reference strains**

Strains	Usage	Origin
<i>E. coli</i> ATCC 25922	Quality controls of the antibiogram	DTU Food (Denmark)
<i>Salmonella typhimurium</i>	Quality control of Hektoen agar	IPCI (Côte d'Ivoire)
<i>E. coli</i> ATCC 8739	Souche calibrante du MALDI-TOF	Biomérieux
<i>Salmonella bredeney TEM-104</i>	Positive control for <i>bla<sub>TEM</sub></i> gene detection	DTU Food (Denmark)
<i>Salmonella virchow 58.67 Holland</i>	Positive control for <i>bla<sub>CTX-M</sub></i> gene detection	DTU Food (Denmark)
<i>Salmonella keurmassar DAK2</i>	Positive control for <i>bla<sub>SHV</sub></i> gene detection	DTU Food (Denmark)
<i>Escherichia coli</i> NCTC50078	Positive control for <i>tetA</i> gene detection	DTU Food (Denmark)

DTU: Technical University of Denmark; IPCI: Pasteur Institute of Côte d'Ivoire

**Table 3. Amplification program**

Amplification step	Temperature C°/Time/cycle	
	CTX-M, TEM, SHV	TetA
Initial denaturation	94°C/5min	95°C/5min
Cyclical denaturation	94°C/1min	95°C/30s
Hybridization	60°C/1min	56°C/30s
Cyclization	72°C/1min	72°C/10min
Final elongation	72°C/7min	72°C/10min
Number of cycles	30 cycles	35

shock [14] followed by phenol-chloroform-alcohol-isoamyl (v / v / v) purification [15]. The study of the different resistance genes was carried out by multiplexed PCRs using specific primers recorded in Table 1. The genomic amplification was carried out in a final reaction volume of 50 µl. This reaction mix contained a 5X color buffer (Promega, USA), a 5X non-stained buffer (Promega, USA), MgCl<sub>2</sub> concentrated at 25 mM (Promega, USA), dNTPs to 10 mM (Bio-Rad, France), Go taq DNA polymerase to 5U/µl (Promega, USA) and specific primers to 10 µM. The DNA extracted from reference strains provided by the National Food Institute collection (DTU Food) were used as positive controls for PCR (Table 2) and a reaction mixture without DNA extract served as a negative control. The amplification conditions are shown in Table 3. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel prepared from a 10X TAE buffer (Tri-Acetate-EDTA) and 5 µL of a solution of EZ-vision® (Inqababiotec, West Africa) at 120 volts / cm for 1 hour.

### 3. RESULTS AND DISCUSSION

#### 3.1 Results

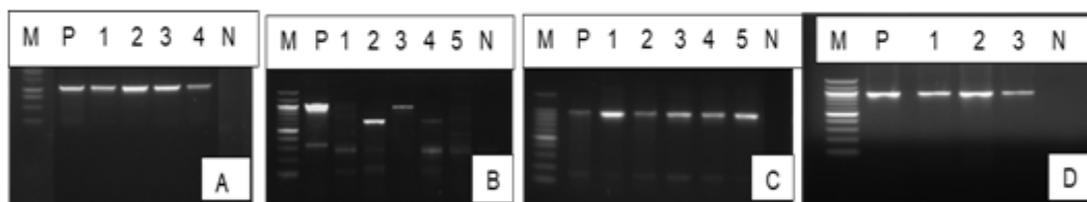
##### 3.1.1 Prevalence of *Salmonella* spp. strains

The use of the MALDI-TOF (vitek MS) automated system allowed the effective identification of isolated *Salmonella* strains in cattle feces. Thus,

out of a total of 420 samples of bovine feces collected and analysed, 84 (20%) *Salmonella* strains were isolated. Also, the proportion of isolated *Salmonella* strains varied according to the township. Thus, the communes of Port-Bouët (9.8%) and Adjamé (7%) had high proportions. On the other hand, they were 1.2%, 1% and 1% respectively in the communes of Abobo, Yopougon and Bingerville.

##### 3.1.2 Resistance profile of strains of *Salmonella* spp.

The determination of the resistance profile of the strains tested revealed that 26 strains of *Salmonella* spp. were resistant to at least one antibiotic. However, overall, a low level of resistance to beta-lactam antibiotics with resistance rates ranging from 1.2 to 6% was observed. As for quinolones, a low level of resistance was generally observed mainly with nalidixic acid (3.6%), ciprofloxacin (3.6%) and norfloxacin (3.6%). Concerning the level of resistance to cyclins, aminoglycosides and other antibiotics, it appears that the resistance rate was higher at colistin (33.3%) followed by cyclins including tetracycline (20.2%) and minocycline (20.2%). Low levels of resistance were observed with gentamicin (1.2%), tobramycin (3.6%) and trimethoprim/sulfamethoxazole (6%). However, no resistance was observed with cefoxitin, imipenem, amikacin and chloramphenicol Table 4.



**Fig. 1. Electrophoretic profile of antibiotic resistance genes**

A: detection of the *bla*<sub>CTX-M</sub> gene (544 bp); B: detection of the *bla*<sub>SHV</sub> gene (1051bp); C: detection of the *bla*<sub>TEM</sub> gene (964bp); D: detection of the *TetA* gene (956 bp); M: Molecular Weight marker (100 bp DNA Ladder); P: Positive control; Number 1 to 5: Bacterial strain; N: Negative control

**Table 4. Antibiotic resistance of *Salmonella* strains**

Antibiotic	Resistance rate (%) R (n=84)
<b>Betalactams</b>	
Ampicillin (AMP)	2.4
Amoxicillin/Clavulanicacid (AMC)	1.2
Cefalotine (CEF)	6
Cefuroxime (CXM)	2.4
Cefoxitine (FOX)	0
Ceftriaxone (CRO)	1.2
Ceftazidime (CAZ)	1.2
Cefepime (FEP)	1.2
Aztreonam (ATM)	1.2
Imipem (IMP)	0
<b>Quinolones</b>	
Nalidixicacid (NAL)	3.6
Ciprofloxacin (CIP)	3.6
Norfloxacin (NOR)	3.6
<b>Cyclins</b>	
Tetracycline (TET)	20.2
Minocycline (MNO)	20.2
<b>Aminosides</b>	
Amikacin (AKN)	0
Tobramycin (TMN)	3.6
Gentamycin (GEN)	1.2
<b>Other</b>	
Colistin (CST)	33.3
Chloramphenicol (CHL)	0
Trimethoprim/Sulfamethoxazole (SXT)	6

**Table 5. Detection of antibiotic resistance genes**

Strains (n=26)	Number of genes detected (%)			
	<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> <sub>SHV</sub>	<i>bla</i> <sub>TEM</sub>	<i>TetA</i>
<i>Salmonella</i> spp.	0	2	15	2
Frequency (%)	0	7.7	57.7	7.7

### 3.1.3 Molecular detection of antibiotic resistance

In total, the 26 strains of antibiotic-resistant *Salmonella* were selected for antibiotic resistance gene research (Fig. 1). PCR results for *bla*<sub>CTX-M</sub> (544pb), *bla*<sub>SHV</sub> (1051pb), *bla*<sub>TEM</sub> (964bp) and *TetA* (956bp) showed detection rates of 7.7%, 57.7% and 7, respectively. 7% (Table 5). However, no *bla*<sub>CTX-M</sub> genes were detected.

### 3.2 Discussion

The results of this study showed a Prevalence of *Salmonella* strains isolated from cattle feces was 20%. Also, sectoral prevalence in the five townships have been determined and gave relatively high rates in the townships of Port-

Bouët (9.8%) and Adjamé (7%), while in other townships (Abobo, Yopougon and Bingerville) a low rate ranging from 1% to 1.2% has been observed. This prevalence of 20% of *Salmonella* strains determined in our study is higher than that determined by Alemayehu et al. [21] who reported a prevalence of 7.1%. Studies in Ethiopia (2.3%) and Nairobi (2.6%) reported lower prevalences than those obtained in this study [22,23]. Significantly lower carriage of *Salmonella* has been reported in cattle in Japan and Great Britain with prevalence of 0.5% and 1.4% respectively [24,25]. The high prevalence observed in this study may be due to the different methods used for isolating strains. However, it is comparable to that obtained by Akoachere et al. [26] in Cameroon. These authors reported a prevalence of 28.7% in cattle during their work. The report of a study by Kagambèga et al. [27] in

Burkina Faso reported a very high prevalence of *Salmonella* (52%) in cattle feces. These fluctuations in results could be explained by the fact that these works were not carried out in the same environments and the difference in the good practice of hygiene during the breeding. Indeed, during the practice of breeding the feces and waste due to livestock feed are not removed regularly. Under these conditions, there may be enough nutrients in moist soils to keep *Salmonella* viable for several years [28].

Isolated *Salmonella* strains showed a low level of resistance to beta-lactam. Also, resistance to beta-lactams mainly to C3G / C4G in animals appears more occasional. Indeed, this bacterium does not seem to constitute a major reservoir of resistance genes [29]. Moreover, the cumulative data from surveillance networks for *Salmonella* strains in France confirm this very small proportion of *Salmonella* strains of animal origin with C3G / C4G. In our study, the low prevalence of the resistance observed with beta-lactam could be explained by the low pressure exerted by beta-lactams on these *Salmonella* strains. Several authors have also reported similar rates of resistance [30,31,32,33]. Variable resistance rates to third generation cephalosporins have been reported in China (1.6%), Romania (11.4%) and the United States (16%) [27,28,29].

In terms of resistance to quinolones and fluoroquinolones, our results showed that *Salmonella* strains are still largely sensitive to quinolones. Similar results have been reported by Gorman and Adley. [34] with nalidixic acid (2.6%) in the Republic of Ireland, ciprofloxacin (5.7%) in India [35]. Lower rates compared to those obtained in our study (less than 1%) were reported by Smith et al. [36]. In addition, high rates of resistance to nalidixic acid have been reported in Kenya (12%) [37], India (17.9% and 24.5%) [38], Romania (65.1%) [39]. However, the low rate observed in our study could be explained by the fact that fluoroquinolones are not widely used in cattle breeding in Côte d'Ivoire. Also, a high rate of resistance to cyclins was observed (20.2% tetracycline, 20.2% minocycline). This increase in cyclin resistance has also been observed by Edrington et al. [40] who reported a resistance rate of 20.9%. High resistance rates compared to the rates obtained in our study were observed in Nigeria [41], Ethiopia [22], the United States [42]. In addition, high resistance rates to tetracycline (62.2%) and minocycline (46.3%) were observed in South Africa [43]. However, low resistance rates have

been reported in several studies [38,40]. Tetracycline resistance is usually caused by genes associated with primary resistance mechanisms involving active efflux pumps, ribosomal protection and enzymatic inactivation. Therefore, the high tetracycline resistance observed in this study would probably be due to selection pressure caused by uncontrolled use of cyclins. In terms of resistance to aminoglycosides, there is a general decrease of the *Salmonella* resistance to them. Aminoglycosides generally contained molecules for hospital use like amikacin and gentamicin. According to Ouattara et al. [44] their use in veterinary medicine remains limited.

In addition, the strains showed a fairly high resistance to colistin in this work and involved 33.3% of *Salmonella*. The rate obtained in this work is worrying. Indeed, this rate is much higher than the rates reported in several studies [45,46,47,48]. This high prevalence of colistin resistance may be due to the increasing use of colistin in livestock farming.

Three different beta-lactam resistance genes belonging to Ambler's molecular classes a (*bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>* and *bla<sub>SHV</sub>*) were investigated in this study. These genes have recently been reported in cattle, but in varying proportions. In this study, the *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>* genes were detected with frequencies of 7.69% and 57.69% respectively. However, no *bla<sub>CTX-M</sub>* genes were detected. This study showed a high frequency of *bla<sub>TEM</sub>* genes. Nevertheless, phenotypic tests revealed a low rate of resistance to beta-lactam antibiotics. This result could be explained by the detection of beta-lactamase TEM which has the ability to hydrolyze only early penicillins and/or cephalosporins but not third or fourth generation cephalosporins, which could not be detected phenotypically. As for the beta-lactamase SHV, our results revealed a low frequency of detection. This observed rate is higher than those of Lertworapreecha et al. [49] who reported a negative result for beta-lactamase SHV. This could explain an acquisition of the *bla<sub>SHV</sub>* gene. About tetracycline resistance, our results revealed the detection of the *tetA* gene with a frequency of 7.6% in *Salmonella* strains. Tetracycline resistance is usually caused by the acquisition of a tetracycline resistance gene (*tet*). These genes are associated with primary resistance mechanisms, which involve active efflux pumps, ribosomal protection and enzymatic inactivation [50]. The difference between the resistance of *Salmonella* to

tetracycline evaluated by the phenotypic method and the resistance profile obtained by PCR could be due to other tetracycline resistance genes that were not evaluated in this study.

#### 4. CONCLUSION

This study showed a high prevalence of *Salmonella* isolated in cattle feces in the Abidjan district. It has also generally shown a high level of resistance of these strains to antibiotics commonly used in human and veterinary medicine. The isolation of antibiotic-resistant *Salmonella* strains from these healthy cattle is a very significant health threat given *Salmonella*'s place in the causes of diseases with bacterial etiology. In addition, the determination of this resistance by the molecular epidemiology method using resistance markers (PCR) showed the presence of resistance genes such as *bla<sub>SHV</sub>*, *bla<sub>TEM</sub>* and *TetA*. The emergence and spread of antibiotic-resistant *Salmonella* strains in food-producing animals is a significant public health problem. However, continuous surveillance of antibiotic therapy in farms must be put in place to limit the phenomenon of antibiotic resistance.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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