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Phenotypic and Molecular Characterization of Antibiotic resistance of Isolated Salmonella Strains from Chickens in Côte D'ivoire

Bonny Aya Carole* and Karou Tago Germain

Laboratory of Biotechnology, Agriculture and Development of Biological Resources, of the Biosciences Training and Research Unit, Félix Houphouët-Boigny University, Abidjan, Côte d'Ivoire, 22 BP 582 Abidjan 22.

**Corresponding author*

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A B S T R A C T

Poultry consumption in Côte d'Ivoire is booming, however it is the main reservoir of antibiotic resistant strains of *Salmonella*. The objective of this work is to assess the level of resistance to *Salmonella* antibiotics isolated from chickens. *Salmonella* strains (104) isolated from 51 batches of raw chicken gedisers were subjected to phenotypic and molecular characterization. Derby (18.9%), Budapest (17%), Essen and Kentucky (11.3%) represent the predominant serotypes. The antibiogram carried out showed resistance: high to cotrimoxazole (93.37%) and to tetracycline (73.08%); relatively moderate for ticarcillin (46.15%) and ciprofloxacin (28.85%) and lower for cefotaxime (0.96%). The resistance genes *tet* (A), *bla* CTX-M-1, *bla* CTX-Mconsensus, *sul* 1, *qnr* (A, B and S), sought by molecular tests (PCR and sequencing) revealed the presence of genes *tet* (A) (40%), *sul* 1 (40%), *bla* CTX-M-1 (65%) and the presumption of a diversity of *bla* genes including: CTX-M-2, -5, -44, -59, -92, -97, OXY and NDM-1. Therefore, monitoring the use of antibiotics in poultry farming remains an essential precaution to guarantee the safety of food intended for human consumption.

Introduction

Foodborne illnesses are a major cause of morbidity and mortality across the world. The World Health Organization (WHO) estimates that 2 million people die each year from infectious diarrhea (Anonymous 1, 2006). Of these, salmonellosis is a real problem in all parts of the world. Indeed, they have a considerable importance in the veterinary and medical fields, as much by the economic losses linked to the reduction in production, as by the high incidence of collective food poisoning, in a current context where absolute sanitary safety is required by the consumer. Salmonellosis is one of the main causes of

foodborne gastroenteritis in humans (Anonymous 2, 2002). They cause symptoms of a wide range of severity, from mild abdominal pain and varying degrees of enteritis, to sepsis and in extreme cases, death. *Salmonella* enterica, through its ubiquitous serotypes, represents the main pathogenic agent in the contamination of agro-food products intended for human consumption (Fablet *et al.,* 2003).

Salmonella infection is also very commonly associated with the consumption of meat and meat products, especially those made from poultry. In fact, poultry play a major role as vectors of transmission in human cases of salmonellosis (Anonymous 2, 2002). Otherwise, the consumption of poultry meat has grown considerably on all continents with an increase in volumes sold worldwide, by 10% per year (Prin *et al.,* 2001). However, in Côte d'Ivoire, farming and slaughtering practices are lagging far behind in industrialized countries, not only with regard to the productivity of poultry workshops, but also and above all with regard to public health.

The increase and accumulation of resistance to antibiotics by *Salmonella* is another aspect of the public health problem, because it is accepted that some of the multidrug-resistant strains found in humans are of animal origin and have acquired their genes from resistance in farms before being transmitted to humans through food (Ungemach *et al.,* 2006).

In fact, the continued use of antibiotics has led to the selection of resistant germs (Anonymous 3, 2009) with the consequences of an increase in infections in chickens, an increase in the mortality rate and a reduction in the productivity of an animal. go; and the possible transfer of this resistance from chicken to humans on the other hand (Jianhua *et al.,* 2002; Bourgeois *et al.,* 2003; Moubareck *et al.,* 2003).

Materials and Methods

The animal material consists of raw chicken gizzards taken from poultry slaughtering sites in the District of Abidjan. Reference bacterial strains (*Salmonella* ATCC 14028 and IPCI 8297) were used as a positive control for carrying out the various biochemical tests, as well as to validate the tests for studying resistance to antibiotics. Six strains of *Escherichia coli* (*E. coli* PSL 18X61367- *E. coli* Y10278- *E. coli* X92506- *E. coli* DJ21-15- *E. Coli* J53 PMG252 and *E. coli* 57) served as positive control for detection respectively *tet*(A), *bla* CTX-M consensus, *bla* CTX-M-1 (group 1), *sul* 1, *qnr* (A) and *qnr* (S) genes. *Klebsiella pneumoniae* B1 served as a positive control for the *qnr* (B) gene. Six pairs of specific primers and a pair of universal primers (Eurogentec, France) were used for the search for antibiotic resistance genes (Table 1).

Sampling and microbiological analysis for the detection of *Salmonella*

Batches (66) of raw gizzards were taken from slaughtering sites in 11 communes of the district of Abidjan (Abobo, Adjamé, Anyama, Attécoubé, Bingerville, Cocody, Koumassi, Marcory, Port-Bouët, Treichville and Yopougon), from April to September 2012.

The microbiological analysis of the different batches of raw gizzards was carried out according to standard NF EN ISO 6579 (ISO-6579, 2002) comprising 4 stages: pre-enrichment, enrichment, isolation and biochemical identification.

Serotyping of isolated *Salmonella* strains

The serotyping of *Salmonella* was carried out according to the method described by Kauffmann and White (1934), consisting in successively detecting somatic (Ag O),

flagellar H (Ag H) or capsule Ag (Vi) antigens, by agglutination on slide using antigenic sera.

Determination of antibiotic resistance

The antibiogram carried out on all the strains isolated was carried out by diffusion in agar medium according to the CLSI standard (Clinical Laboratory Standard Institute) on Müller-Hinton agar (CLSI, 2005). Antibiotic discs: amoxicillin (AMX, 10µg), amoxicillin / clavulanic acid combination (AMC, 10 / 20µg), ticarcillin (TIC, 75 µg), cefalotin (CF, 10µg), cefoxitin (FOX, 10µg), cefotaxime (CTX, 10µg), gentamicin (GM, 10µg), nalidixic acid (Nal, 10µg), ciprofloxacin (Cip, 10µg), cotrimoxazole (SXT, 10 / 20µg), tetracycline (TE, 10µg) and chloramphenicol (C, 10µg), were tested.

Detection and amplification of resistance genes by PCR

The detection of genetic carriers of antibiotic resistance was carried out by the polymerase chain reaction (PCR) technique on 20 strains of *Salmonella* exhibiting a profile of multidrug resistance. The search for certain resistance markers including: the *bla* CTX-M-1 (group 1) and *bla* CTX-Mconsensus genes encoding resistance to β-lactams (ticarcillin, cefotaxime); the tet(A) gene encoding resistance to cyclins (tetracycline), the *sul*1 gene encoding resistance to sulfonamides (cotrimoxazole) and the *qnr* genes (A, B and S) encoding resistance to fluoroquinolones (ciprofloxacin), a been carried out. The genetic material (plasmid DNA) was extracted according to the method described by Rozilla *et al.,* (2007), then amplified using primers (Table 1). The amplification products were subjected to electrophoresis on 1% agarose gel (Eurobio, France) and the target genes were revealed under UV. The gene amplification reaction was carried out using a thermocycler (Applied Biosystems Gene Amp PCR 9700), in a reaction mixture of 50 µL. The gene amplification program comprises an initial denaturation of 5 min at 94 ° C, followed by 40 cycles of PCR, each of which consists of a denaturation step of 30 s at 94 ° C; a hybridization step for 1 min at 55 ° C. for the pair of primers *bla* CTX-Mconsensus; at 60 ° C. for the pairs of primers *bla* CTX-M-1 (group 1), *tet* (A) and *qnr* (A, B, S); at 69 ° C for the initiator pair *sul* 1; in a one-minute elongation step at 72 ° C. At the end of the 40 cycles, a final elongation of 10 minute at 72 ° C, completes the amplication reaction.

Sequencing of amplified genes

The DNA amplicons obtained by PCR from the degenerate primer bla CTX-Mconsensus are sequenced at GATC Biotech (Germany). The nucleotide sequences obtained are identified using the NCBI (National Center for Biotechnology Information) database, available on the website www.blast.ncbi.nlm.gov/Blast.cgi.

Results and Discussion

Microbiological analysis revealed the microbiological quality of raw chicken gizzards. Thus, out of the 66 batches of gizzards analyzed, 51 batches were contaminated by *Salmonella*, ie a percentage of contaminated batches of 77.27%. From these contaminated batches, 104 strains of *Salmonella* were isolated. Of all the serotyped strains, 15 serotypes including 11 agglutinating with serum OMA and 4 with serum OMB could be determined. The serotypes derived from strains agglutinating with the OMA serum are: Derby (18.9%), Budapest (17%), Essen (11.3%), Agona (7.5%), Chester (3.8%), Schwarzen ground (3.8%), Ruiru (3.8%), Fortune (1.9%), Elisabethville (1.9%), Aoto (1.9%), and Santiago (1.9%). Those derived from strains

agglutinating with OMB serum are: Kentucky (11.3%), Hadar (9.4%), Bargny (1.9%), and Poeselderf (1.9%).

The study of antibiotic resistance of *Salmonella* strains, carried out on all strains showed resistance to β-lactams (ticarcillin (46.15%)), sulfonamides (cotrimoxazole (93.27%)), quinolones (nalidixic acid (35.76%) and ciprofloxacin (28.85%)) and cyclins (tetracyclines (73.08%)). Cyclins and sulfonamides remain the least active antibiotic families against isolated *Salmonella* strains. The antibiogram also revealed resistance profiles ranging from mono resistance to multiple resistance (3, 4, 5, 6, 8, 9 and 11 molecules). The serotypes involved in multidrug resistance are: Agona (17.39%), Derby (8.69%), Hadar (4.34%), Budapest (21.73%), Ruiru (8.69%), Essen (17.39 %), Kentucky (17.39%) and Chester (4.34%) (Table 2).

Electrophoresis of PCR products revealed the presence of markers implicated in antibiotic resistance of *Salmonella* strains isolated from raw chicken gizzards (Figure 1). None of the targeted *Salmonella* strains possess the fluoroquinolone resistance genes (qnr (A, B, S)).

The sequencing carried out on the amplicons of the degenerate primer *bla* CTX-Mconsensus at the level of two strains (*Salmonella* Kentucky and *Salmonella* O: 3,10), revealed similarities of 96 to 100% with fragments of nucleotide sequences encoding *bla* CTX-M-2, -5, -44, -59, -92, 97 and -131 enzymes; *bla* NDM-1 and *bla* OXY (Table 3). Also sequencing reveals the presence of mobile genetic elements such as *ISE*cp1 type insertion sequences and *ISCR1*.

The isolation of the *Salmonella* strains from the different batches of gizzards analyzed revealed a rate of contaminated batches of 77.27%. The presence of these strains in the chicken lays bare the process of treating slaughtered chickens. Indeed, this process constitutes an important means of diffusion of microorganisms such as *Salmonella*. *Salmonella* strains are isolated from viscera (Gaedirelwe and Sebunya, 2008; Traoré, 2003), and gizzards indirectly contaminated by the intestinal contents of chicken (Chaiba *et al.*, 2008; Karou *et al.*, 2013).

The serotyping carried out on all the strains isolated revealed 15 serotypes. Derby (18.9%), Budapest (17%), Essen (11.3%) and Kentucky (11.3%) represent the most dominant serotypes. Indeed, since 2000, the Derby and Kentucky serotypes have been the main *Salmonella* serotypes most widely distributed in France and Belgium (Weill and Le Hello, 2011; Bertrand *et al.*, 2010). Also, some studies have shown the existence of these serotypes in Salmonella strains isolated from various sources including poultry (Tao *et al.*, 2014; Karraouan *et al.*, 2010; Turki *et al.*, 2011). The Kentucky serotype, in particular, remains an emerging serotype, associated with strains highly resistant to critical molecules such as fluoroquinolones (ciprofloxacin), recommended in cases of severe infections,

Analysis of the resistance profile of multiresistant strains of *Salmonella* to antibiotics revealed 4 levels of multiple resistance, involving several different families of antibiotics. β-lactams, cyclins, sulfonamides and quinolones are the most affected in these multiple resistances. The appearance of these combinations involving these different families would be the direct consequence of their overuse in the Ivorian poultry sector (Ouattara *et al.*, 2013). Indeed, despite the WHO recommendations on the use of antibiotics in farms, molecules similar to those used in clinics are still used in some countries and are undoubtedly at the origin of the appearance of cross-resistance.

Table.1 Primer pairs of antibiotic resistance genes used during the study.

Target genes	Nucleotide sequences (5'-3')	Function	Amplicon (bp)	References
bla CTX-Mc	F: ATGTGCAGYACCAGTAARGTKATGGC R:TGGGTRAARTARGTSACCAGAAYCAGCGG	Search for *bla*CTX-M genes	593	Kiiru et al. (2012)
***bla* CTX-M1**	F: GACGATGTCACTGGCTGAGC R: AGCCGCCGACGCTAATACA	Search for *bla* CTX-M1 genes	499	Kiiru et al. (2012)
***tet* (A)**	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	Search for *tet* genes (A)	210	Ng et al. (2001)
***sul* 1**	F: CTTCGATGAGAGCCGGCGGC R: GCAAGGCGGAAACCCGCGCC	Search for genes *sul* 1	417	Hao-Chang et al. (2012)
***qnr*(A)**	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	Search for *qnr* genes	516	Robicsek et al. (2006)
***qnr* (B)**	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC		469	
***qnr* (S)**	F: ACGACATTCGTCAACTGCAA R: AAATTGGCACCCTGTAGGC		417	

Table.2 ATB resistance profile of multidrug-resistant *Salmonella* serotypes (MDR) isolated from raw chicken gizzards

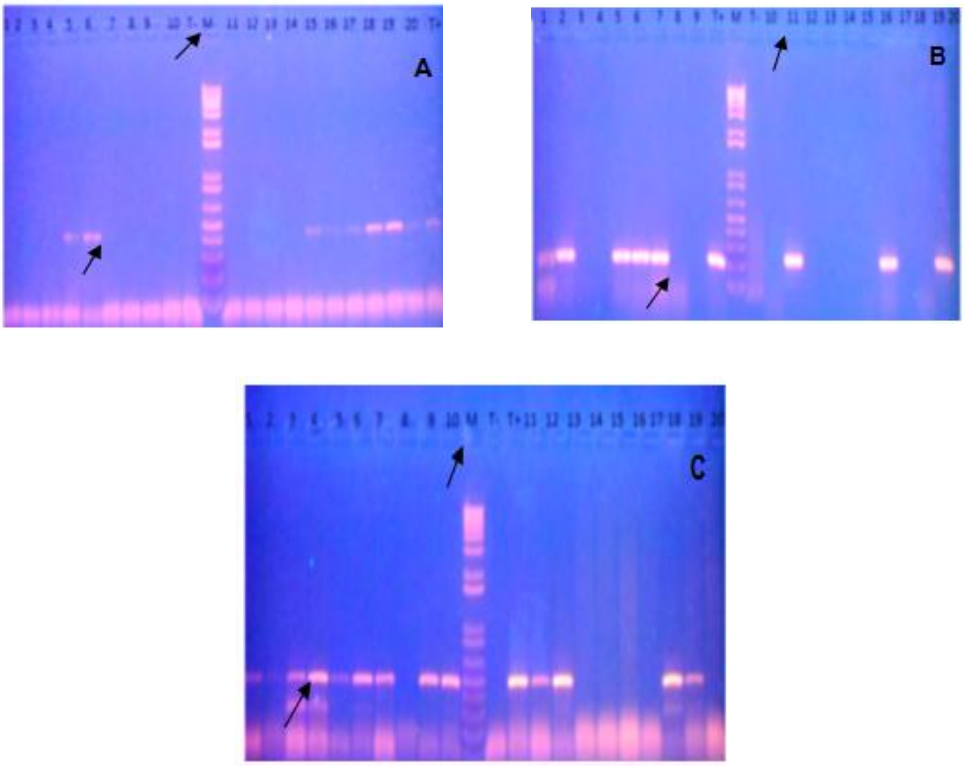
Serotypes	Multidrug-resistant Serotype Profiles	MRS
Agona	TicTeSXT / TicCSXT	4
Derby	TicCSXT / TicCTeSXT	2
Hadar	TicSXTNalCipTe	1
Budapest	TicTeSXT / TicCSXT / TiCTeSXTCTeSXT	5
Riuru	CTeSXTNal / CTeSXT	2
Essen	TicCTeSXT / AAMCTicSXTTe / SXTNalTe / TicGTeSXT	4
Kentucky	GSXTNalCipTe / TicGTeSXT	4
Chester	TicSXTTe	1

MRS: multiresistant strains; A: Amoxicillin; AMC: Amoxicillin / Clavulanic acid, Tic: Tircacillin; C: Chloramphenicol; G: Gentamycin; Nal: Nalidixic acid; Cip: Ciprofloxacin; SXT: Cotrimoxazole; Te: Tetracycline.

Table.3 Strains with *bla* genes similar to strains isolated

***Salmonella* isolated**	**NCBI Strains**	**Gene type *bla***	**% identity**
	Salmonella Schwarzengrund S782	*bla* CTX-M-2	96%
	Salmonella Typhimurium 18-425 -M-5	*bla* CTX-M-5 (ISEcp1)	
	Escherichia coli BR-79	*bla* CTX-M-2 (ISCR1)	
	Escherichia coli KUN-9085	*bla* CTX-M-44	
	Escherichia coli B275	*bla* CTX-M-97	
***Salmonella* Kentucky**	*Escherichia coli* E39 (ESBL)	*bla* CTX-M-92	
	Proteus mirabilis TUM11514	*bla* CTX-M-2	
	Pseudomonas aeruginosa PHB 53	*blaC*TX-M-2	
	Klebsiella pneumoniae K6P	*bla* CTX-M-2	
	Klebsiella pneumoniae HB 99	*bla* CTXM-59	
	Klebsiella oxytoca 76C	*bla* OXY	100%
***Salmonella* O: 3.10**	*Klebsiella pneumoniae*	*bla* NDM-1	100%

Fig.1 Electrophoretic profile of the PCR amplification products of the *sul* 1 (A), *tet* (A) (B) and *bla*CTX-M (1) genes, existing in *Salmonella* strains isolated from raw chicken gizzards.



A: M. 1 kb (+) molecular weight marker (Eurogentec, Smart Ladder); T (-). The negative control. T (+). The positive control (*E. coli* DJ21-15). The amplicons positive for the *sul*1 gene have the expected size of 417 bp; **B**: M. 1 kb (+) molecular weight marker (Eurogentec, Smart Ladder); T (-). The negative control. T (+). The positive control (*E. coli* PSL 18X61367). The positive amplicons *tet* (A) gene have the expected size of 210 bp. *tet* (A): gene involved in resistance to tetracycline; **C**: M. 1 kb (+) molecular weight marker (Eurogentec, Smart Ladder); T (-). The negative control. T (+). The positive control (*E. coli*X92506). Amplicons positive for the *bla* CTX-M gene (1) have the expected size of 499 bp.

Overall, the same problems of resistance to antibiotics are found in strains of *Salmonella* whether they are of animal or human origin. Thus, the *Salmonella* strains isolated from poultry farm products are also affected by multidrug resistance to antibiotics. The direct involvement of β-lactams, sulfonamides, cyclins and fluoroquinolones, as well as the presence of resistance genes in our multidrug-resistant strains could reflect their ability to develop resistance mechanisms, both genetic and biochemical, for the simple purpose of counterbalance their action.

Indeed, the *bla* CTX-M genes are those which mainly confer resistance to third generation cephalosporins such as cefotaxime (Arlet *et al.*, 2006; Hur *et al.*, 2010). The sequencing carried out on all of the amplicons of the degenerate primer *bla* CTX-M consensus revealed similarities varying from 96 to 100% with the *bla* sequences of bacterial strains contained in the NCBI database. These enzyme sequences are of *bla* CTX-M-2, -5, -44, -59, -92, -97, -131, *bla* NDM-1 and *bla* OXY type. These observations reflect the probable existence of a diversity of *bla* genes in the isolated *Salmonella* strains. The different types of *bla* genes obtained, belonging to classes A and B according to the classification of Ambler (1980), reflect the ability of our *Salmonella* strains to resist

antibiotics of the β-lactam family, through the mechanism of enzymatic hydrolysis (enzymatic inactivation). The presence of mobile genetic elements, in particular the insertion sequences of the *bla* CTX-M 44 ISEcp1 and *bla* CTX-M-2 ISCR1 type, reflect the possible mobilization of the *bla* CTX-M genes. Indeed, the mobilization of *bla* CTXM genes is demonstrated experimentally by IS insertion sequences located upstream of the genes, such as ISEcp1 (Latirgue *et al.,* 2006). In addition, the effect of the promoter of these insertion sequences, increasing the expression of *bla* CTX-M genes, suggests that these insertion sequences located upstream of these genes would play a role in the selection and dissemination of genes. *bla* CTX-M. Furthermore, this insertion sequence (ISEcp1) is found associated with the expression of all the groups of β-lactamases of the cefotaximase type with the exception of the *bla* CTX-M-8 group. The ISCR1 insertion sequence, for its part, is linked to several members of the CTX-M-2 group (Barlow *et al.*, 2008).

The presence of the *tet* (A) gene in multiresistant *Salmonella* strains possibly testifies to the involvement of the tet genes, in this case the *tet* (A) gene, in the resistance of *Salmonella* to tetracyclines. Indeed, several authors have put forward the hypothesis according to which bacteria have the capacity to develop a mechanism of resistance to this molecule (tetracycline), thanks to the existence of an efflux pump encoded by the genes tet, which would have the direct consequence of reducing the level of toxicity of this antibiotic within the bacteria (Butaye *et al.,* 2003; Chopra and Roberts, 2001). These genes are also the most prevalent genetic determinants among Gram-negative bacteria including *Salmonella* (Schnabel and Jones, 1999; Carattoli *et al.,* 2001). Resistance to sulfonamides generally arises according to Hendi *et al.,* (2013), the acquisition of sul genes (sul1 and / or sul2) in Gram-negative bacilli. As a result, the acquisition of this resistance marker may reflect the ability of isolated strains to develop a resistance mechanism to sulfonamides by production of enzymes of the dehydropteroate synthetase type, encoded by the sul genes (Huovinen *et al.*, 1995).

Regarding bacterial resistance to fluoroquinolones, phenotypic tests revealed a considerable rate of resistance to ciprofloxacin (30.77%). However, molecular tests did not reveal the presence of any of the *qnr* genes tested for resistance to ciprofloxacin by PCR.

This absence can be explained by the fact that in addition to the *qnr* genes, resistance to fluoroquinolones can also be the plasmid factor such as the variant gene aac6′-Ibcr, which increases the level of resistance of *Salmonella* strains to fluoroquinolones. Indeed, from 2009 to 2011, antibiotic sensitivity studies carried out by the National *Salmonella* Research Center in Paris, on strains of various contamination origins made it possible to confirm the presence of these two types of resistance markers involved in resistance to fluoroquinolones for the serotypes Agona (India), Nima (Mali) and Typhimurium (Unknown) (Weill and Le Hello, 2011). Thus, the resistance to fluoroquinolones of the isolated *Salmonella* strains can be attributed to the *aac6'-Ibcr* gene.

This work showed the presence of strains of *Salmonella* highly resistant to antibiotics in the raw gizzards of chickens analyzed. These multiresistant *Salmonella* strains, carriers of plasmid support resistance genes, give them the power to disseminate in the environment. This could constitute a real risk for the health of the consumer.

The monitoring of the evolution of antibiotic resistance in the Ivorian poultry sector

therefore remains the sine qua non to overcome the appearance of such strains in order to guarantee the safety of food in general and those based on food. poultry in particular.

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