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RESEARCH ARTICLE

Antimicrobial resistance of *Salmonella enterica* Typhi in the Western and Southern Regions of the Democratic Republic of the Congo: Phenotypic profile and molecular characterization of isolates associated with epidemics of Typhoid Fever

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Abstract: Background: This study has its foundation following the emergence of the phenomenon of antimicrobial resistance of *Salmonella enterica enterica* Typhi associated with severe complications, such as intestinal perforations with a significant lethality. Objectives: Of this antimicrobial resistance, to determine the phenotypic profile, to detect the chromosomal molecular markers (CMMs) such as the class 1 integrons (*intl*-1) and ESBLs (bla_{TEM-1}, $bla_{OXA-1}$and $bla_{CTX-M-1}$) and to measure the association between the phenotypic profile and CMMs of typhoid isolates in concerned areas. Methods: *Salmonella* Typhi strains of typhoid epidemic areas were confirmed by serotyping tests. The antimicrobial susceptibility testing was conducted by disc diffusion method using the following commercial antimicrobials: Chloramphenicol-C, Ampicillin-AMP, Sulfamethoxazole-RL and Trimethoprim-W (former first-line antimicrobials), Ciprofloxacin-CIP or Cefotaxime-CTX, Ceftriaxone-CRO, Ceftazidime-CAZ (first-line antimicrobials), Tetracycline-TE, Amoxicillin-Potassium clavulanate-AMC, Nalidixic acid-NA, Cefoxitine-FOX, Gentamicin-CN (varied antimicrobials) and FEP-Cefepime (4GC). The phenotypic antimicrobial resistance profile was determined by Kirby-Bauer diffusion method on Mueller-Hinton agar. To perform the molecular characterization, the *Salmonella* Typhi isolates DNA has been extracted by Sigma Aldrich kit and the CMMs detection was performed by DNA Engine for PCR test. The association between phenotypic profile and CMMs has been measured by Pearson's chi-square test. Results: Out of 320 *Salmonella* Typhi isolates, 50 were identified conform. The phenotypic profile of antimicrobial resistance was 59.5% in all the Western and Southern regions and 61% in the provinces of Kinshasa City and Bas-Congo to the former antimicrobials of first intention and the mean of CMMs rates were 22.5% and 27.4%, respectively. Those isolates showed a significant resistance profile to AMP, C and RL in those last two provinces. Conclusion: The rate of phenotypic multidrug-resistance of *Salmonella* Typhi isolates was more than 50% with the predominance of CMMs in Kinshasa and Bas-Congo. This study suggests to give up the use of AMP, C and RL in those two provinces. This may also indicate that the antimicrobial resistance surveillance system would be one strategy to manage food borne pathogens.

Keywords: multidrug-resistance, *Salmonella* Typhi, phenotypic profile, chromosomal markers

1 Introduction

Today, serovar Typhi, the causative agent of typhoid fever which is an endemic in developing countries is becoming increasingly multidrug-resistant to the antimicrobials recommended by the WHO [1,2]. To fight this endemic, Chloramphenicol was introduced in 1948, as an antibiotic of choice which has led to satisfactory results against serovar Typhi [3].

However, despite the therapeutic progress brought by this antibiotic, the persistence of the faecal hazard and the misuse of the latter had increased bacterial infections and their spread by strains resistant to this molecule [4]. From the resurgence of epidemic episodes of

the disease followed by excessive intake of Chloramphenicol, the first cases of resistance of *Salmonella* Typhi strains to this phenicol were notified at the end of the 1960s [2,5]. In order to cope with this resistance to Chloramphenicol and on the basis of the triad of efficacy, cost and availability of antimicrobials on the market, the WHO had combined with this antibiotic Sulfamethoxazole-Trimethoprim and Ampicillin as first-line treatment against Typhoid fever in the 1970s [2,3]. These antimicrobials thus instituted have brought a significant improvement in the management of endemic regions. In the mid-1980s and early 1990s, a few strains of *Salmonella* Typhi were declared multidrug-resistant in some parts of Asia and Africa to this first-line treatment [3,6]. A few years later, with the dramatic increase in multidrug-resistant typhoid strains, WHO has been led to change its recommendation for first-line treatment. For this purpose, it was the use of Ciprofloxacin or third generation cephalosporins [6]. After a period of increased use of Ciprofloxacin, decreased susceptibility of a large proportion of *Salmonella* Typhi strains to this fluoroquinolone has been reported [7]. This decrease in susceptibility has also been observed in some countries in South East Asia and sub-Saharan Africa [8,9]. Third-generation cephalosporins, for their part, are until now the molecules of choice in infectious pathology [2,10]. In the same regions of Asia and Africa as well as in the Maghreb countries, a few cases of resistance of *Salmonella* to third and fourth generation cephalosporins have however been reported [11–13]. As for former first-line antimicrobials, their use continues in low-income regions because of their accessibility and affordability despite the decline in their pharmacological activities. Compared to the phenotypic multidrug resistance of *Salmonella* Typhi strains to former first-line antimicrobials, the results of some studies carried out in the DRC have been reported. In this case 100% to those three molecules including Tetracycline in some outlying districts of East of Kinshasa City, from October 2004 to January 2005 with a case fatality rate of 53% [14] followed by 31.1% and 54.5%, respectively in some administrative areas of the DRC and the provinces of Bas-Congo as well as Kinshasa [15] and 49.0% to those same molecules in some provinces of the DRC [16].

The phenotypic profile of antimicrobial resistance is the genotypic expression of molecular markers harbored by the chromosome, plasmid, transposon, integron and free gene [17]. The molecular markers concerned in this study are in fact the integron (*intI*-l) and beta-lactamase (*bla*) genes, hosted by the bacterial chromosome and which code respectively for Intl and β-lactamases which are usually the proteins inactivating the majority of antimicrobials [18, 19]. Integrons as genetic elements ensure the capture, conservation and dissemination of antimicrobial resistance genes by recombination mechanisms specific to the *attIl* site [20]. Faced with antimicrobial stress, bacteria regulate the cassette genes associated with multidrug resistance by the integrons they host and activate the SOS system which spares their DNA [21].

In the functional platform of an integron, the SOS system of bacteria exposed to antimicrobial stress acting on its double-stranded DNA activates the *recA* regulatory gene which encodes a RecA protein. Once the DNA has unwound, this RecA protein recombines with one of the single strands of DNA to form a nucleoprotein filament called "single stranded DNA and RecA" which will degrade the LexA protein by autoproteolysis from the promoter integrase. This repressor gene codes for the repair proteins of the damaged DNA molecule by returning the other strand to it based on the genetic code. The cassette gene capable of encoding the inhibitory protein of the stressor antimicrobial is rearranged in the integron in a useful position in order to continue the transcription and translation, respectively of the mRNA and of the inhibitory protein of this antimicrobial [22,23]. β-lactams are commonly used in clinical biology in the treatment of infections due to Gram-negative bacilli and during which resistance to this family of antibacterials is increasingly observed [24–26]. For more than 340 β-lactamases described, the most active genes of this family hosted by the bacillus of *Salmonella enterica*, in particular bla_{TEM-1}, bla_{OXA-1}, $bla_{SHV-1}$and $bla_{CTX-M-1}$, respectively encode the proteins TEM-1, OXA-1, SHV-1 and CTX-M-1 which are involved in these resistances [27,28]. Many countries have notified outbreaks of nosocomial infections due to strains of *Salmonella enterica* producing β-lactamases [27,29] including Tunisia by the serovar Mbandaka in which the bla_{TEM}- gene 4 was predominant [30,31]. The general objective of this study was to contribute to improving the management of antibiotic resistance in the DRC and specifically, to determine the phenotypic profile, to detect chromosomal molecular markers (MMC), to measure the association between the phenotypic profile and CMMs of typhoid isolates in the Western and Southern regions of the DRC in general and in the provinces of Kinshasa City and Bas-Congo in particular and to support the start-up of antimicrobial resistance surveillance strategy in the DRC.

2 Material and methods

This cross-sectional study was focused on the antimicrobial resistance of the *Salmonella* Typhi isolates in the Western and Southern regions of the DRC. It was performed by determining

the phenotypic profile and the molecular characterization of the antimicrobial resistance of typhoid isolates associated with the epidemics of Typhoid Fever from 2003 to 2014 and to measure the association between these objectives. The strains of *Salmonella* Typhi isolated from blood cultures of inpatients of concerned administrative entities were stored in nutrient agar and refrigerated (Panasonic, MPR-311-H) at 2 to 8°C.

2.1 Sample collection and selection of the *Salmonella* Typhi isolates

All *Salmonella* Typhi isolates were collected from administrative zones concerned by the outbreaks of Typhoid Fever in pre-sterilized nutrient agar tube provided by the National Institute for Biomedical Research (INRB). From those isolation entities, these specimens packaged in secure insulated boxes were sent to INRB for subsequent analyzes. To undertake the present work, these *Salmonella* Typhi isolates were shipped to the Center for Microbiology Research at Kenya Medical Research Institute (CMR-KEMRI) in Nairobi.

All the 320 *Salmonella* Typhi isolates were submitted to the physical integrity examination according to their containers and nutrient agar by certain requirements of conformity. Selection of those isolates was pursued by testing their viability as bacterial cells by streaking each one on the MacConkey plate agar (Oxoid, UK) and incubated (Memmert D, model, 600) at 37°C for 24 h. These presumptive serovar Typhi isolates have been submitted on serotyping test (Remel Dartford, UK) that has conducted to the agglutination between bacterial colonies and commercial serovar Typhi antiserum on slide test for identification. Out of 95 identified *Salmonella* Typhi isolates, 50 were known of the administrative isolation areas of the Western and Southern regions of the DRC. It is about Kingasani and Lukaya in Kinshasa province, Kisantu and Matadi in Bas-Congo, Bandundu-Ville and Inongo in Bandundu, Bwamanda in Equateur, Ilebo in Kasai Occidental, Mweneditu in Kasai Oriental and Lubumbashi in Katanga.

2.2 Determination of antimicrobial resistance of the *Salmonella* Typhi isolates

2.2.1 Antimicrobial susceptibility testing of the phenotypic profile

The selected *Salmonella* Typhi isolates were submitted for antimicrobial susceptibility testing to a range of fourteen antimicrobial molecules (Oxoid Basingstoke, UK, CLSI) subdivided into four categories including, former first line antimicrobials, first line antimicrobials, varied antimicrobials and a Cephalosporin of fourth generation.

2.2.1.1. Preparation of the inoculum

Three to five identical bacterial colonies taken from the surface of the agar of Mueller-Hinton previously prepared and inoculated with typhoid isolates were homogenized in about 5 ml of physiological solution (9 g/L of NaCl). The turbidity obtained from the suspension of the diluted bacteria was compared, under good light, and reduced to that of the MacFarland standard 0.5. The prepared inoculum was thus left for about 30 minutes on the bench.

2.2.1.2. Inoculation, deposit of antimicrobial discs, incubation and reading interpretative

After bringing the 9 to 10 cm diameter Petri dishes (Oxoid, UK) back to the bench, they were identified by codes relating to the quality control strain of *Escherichia coli*, ATCC No 25922 and to each of the studied *Salmonella* Typhi isolates. For the fourteen antimicrobials tested, due to seven discs per box of Petri dishes, the quality control strain like every typhoid isolate used two plates of Mueller-Hinton agar (Oxoid, UK). Next to the Bunsen burner (YOP2) flame, a volume of inoculum taken using a sterile cotton swab (MW-108-112) wrung against the wall of the tube was inoculated by rubbing over the entire surface of the agar. After 10 to 15 minutes of exposure of those dishes to room temperature, using the automatic dispenser (Disp-FG), 6 discs of β-lactam antimicrobials (Oxoid Basingstoke, UK, CLSI) were placed in a circle in the first agar dish. They are Ampicillin (AMP): 10 μg, Cefoxitin (FOX): 30 μg, Ceftriaxone (CRO): 30 μg, Cefotaxime (CTX): 30 μg, Ceftazidime (CAZ): 30 μg, and Cefepime (FEP): 30 μg with Amoxicillin + Potassium Clavulanate (AMC): 20/10 μg, manually deposited in the center by using an anatomical forceps (Steel-6 AC). On the bacterial mat of the second box, the 6 other but non-β-antimicrobials lactams (Oxoid Basingstoke, UK, CLSI) were also deposited in a circle. Those are Nalidixic Acid (NA): 30 μg, Ciprofloxacin (CIP): 5 μg, Gentamicin (CN): 30 μg, Chloramphenicol (C): 30 μg, Trimethoprim (W): 5 μg, and Sulfamethoxazole (RL): 25 μg with the Tetracycline (TE) 30 μg in the center. These inoculated petri dishes loaded with antimicrobial discs were incubated for 24 hours at 37°C. The next day, the diameters of the zones of bacterial inhibition were measured on the agar plates using a millimeter slat. The interpretative reading made on all the diameter measurements taken led to the following results: Resistant (R), Susceptible (S) and Intermediate (I).

2.2.2 Molecular characterization of antimicrobial resistance in *Salmonella* Typhi isolates

This genotypic approach was carried out by the detection of CMMs which are the class 1 integron (*intI*-1) and β-lactamase genes of antibiotic resistance, namely: bla_{TEM-1}, $bla_{OXA-1}$and $bla_{CTX-M-1}$. Indeed, this molecular characterization was carried out on pure and fresh *Salmonella* Typhi isolates, from which the chromosomal DNA likely to contain these markers was extracted.

2.2.2.1. Extraction of the DNA of the *Salmonella* Typhi isolates

This technique was applied by using the Sigma Aldrich kit under heat shock. To perform it, these serovar Typhi isolates were thawed in an ice tray on the bench at room temperature and were re-cultured on Mueller-Hinton agar and incubated at 37°C for 24 hours. The next day, approximately 5 colonies of the typhoid bacillus were collected and emulsified therein in an Eppendorf 250 μL cryotube of sterile distilled water and boiled for 15 minutes during which, those lysed isolates were centrifuged (Micro 240 A, Spintron inc.) at 15,000 revolutions per minute for 5 minutes. The extracted DNA supernatant was collected in an Eppendorf cryotube and stored in freezer (Serial N°15087 U 0214, USA) at minus 20°C.

2.2.2.2. Detection of the chromosomal molecular markers (class 1 integron genes: *intl*-1 and ESBL genes: bla_{TEM-1}, bla_{OXA-1} and $bla_{CTX-M-1}$)

Polymerase Chain Reaction (PCR) technique:

(1) Preparation of control samples

The samples of class 1 (*intl*-1) and bla_{TEM-1}, bla_{OXA-1}, $bla_{CTX-M-1}$, validated by Sigma Aldrich laboratories are marketed in lyophilized form and served as positive control. To do this, each one was reconstituted with an appropriate volume of the PCR distilled water then subdivided into aliquots and labelled and stored in freezer at minus 20°C. The negative control, on the other hand, was only the PCR distilled water.

(2) Preparation of Master Mix

To those 50 typhoid isolates of this study, were added the two positive and negative control samples. A total of 52 wells were provided for each gene to be detected and the volume of the reaction mixture was prepared therefrom. In a clean and dry sterile tube free of any contamination of organic matter (DNA), the Master Mix was prepared in multiplying the unit volume of each of its constituents by 52.

Table 1 Preparation of Master Mix (Reaction Mix)

No.	Reagent	Unit volume	Number of wells	Total volume
1	Distilled PCR water	12.0 µl	Multiplied by 52	624 µl
2	Betaine 5M	1.0 µl	Multiplied by 52	52 µl
3	Primer Forward (F) 10 µM	0.5 µl	Multiplied by 52	26 µl
4	Primer Reverse (R) 10 µM	0.5 µl	Multiplied by 52	26 µl
5	FIREPol	4.0 µl	Multiplied by 52	208 µl
	Total	18.0 µl	Multiplied by 52	**936 µl**

Table 1 shows different reagents which made up the mixture reaction that reacted with all the PCR products in the microplate wells.

Oligonucleotide primers Once prepared, these primers identified in F and R were distributed in aliquots and stored at minus 20°C.

FIREPoL is a ready-to-use mix composed of 0.2 mM dNTP, Buffer (Tris 10 mM HCl, 50 mM KCl, 0.001% gelatin and stabilizers), 7.5 mM $MgCl_2$, 1.5 Unit Taq DNA Polymerase.

The required volumes of Master Mix and DNA Matrix (test sample), for each well were, respectively, 18 μl and 2 μl, or 20 μl in total.

(3) Titration of the Master Mix followed by the DNA Matrix in each well

Each microplate well was distributed with 18 μl of Master Mix to which were added 2 μl of the positive and negative control and DNA Matrix. After pipetting, all of the microplate wells were covered with tape and mixed briefly by vortexing. This microplate wells with working solutions was placed in the thermal cycler turned on some time before.

(4) Starting the thermal cycler

The thermal cycler (DNA Engine) was manually set according to the type of molecular marker to be detected, in particular the amplification time, the appropriate temperature at each polymerization step and the number of cycles are variables. Thus, the different amplification steps took place over 3 hours for 35 cycles.

(5) Features specific to the selected amplification of each marker

A. *intI*-1: nucleotide primers (F: GTTCGGTCAAGGTTCTG and R: GCCAACTTTCAGC ACATG), program: 1155, hybridization temperature: 50°C and expected CMM size: 923 bp.

B. bla_{TEM-1}: nucleotide primers (F: GCGGAACCCCTATTTG and R: TCTAAAGT ATAT ATGAGTAAACTTGGTCTGAC), program: 20158, hybridization temperature: 50°C and expected CMM size: 964 bp.

C. bla_{OXA-1}: nucleotide primers (F: ATGAAAAACACAATACATATCAACTTCGC and R: GTGTGTTTAGAATGGTGATCGCATT), program: 20158, hybridization temperature: 62°C and expected CMM size: 820 bp.

D. $bla_{CTX-M-1}$: nucleotide primers (F: GACGATGTCACTGGCTGAGC and R: AG CCG C CGACGCTAATACA), program: 1144, hybridization temperature: 55°C and expected CMM size: 499 bp.

(6) Electrophoretic migration of the PCR products on agarose gel

At the end of the polymerization, the PCR amplicons were submitted to the electrophoretic migration (Thermo EC, Australia) on the agarose gel and were identified by revealing themselves under ultraviolet rays (Transilluminator, UVP,USA) using the reference marks of the bands of the marker of DNA in base pair size. The freshly prepared agarose gel stained with Ethidium bromide was poured into the tank with a comb and transferred from its mold to the electrophoretic migration tank.

The PCR products were loaded onto the wells followed by electrophoretic migration for 60 minutes at 120 volts regulated with voltage regulator (Capital Equipment, USA).

(7) Revelation of DNA molecular mass marker and PCR products of isolates studied and the positive control

The *intI*-1, bla_{TEM-1} and bla_{OXA-1} except $bla_{CTX-M-1}$have been detected in some *Salmonella* Typhi isolates.

2.3 Entering the obtained data

On the Excel 2013 matrix, the phenotypic and CMMs results have been reported respectively as coded. To measure the statistical association between the phenotypic profile and CMMs data entered on the Excel 2013 matrix, these were taken on SPSS software, IBM SPSS version, Statistics 23.0 [32].

2.4 Measurement of association between the phenotypic profile and CMMs of the antimicrobial resistance of the *Salmonella* Typhi isolates

To measure this association, the results of intermediate susceptibility were assimilated to those of the resistant parameter and their combination was crossed to the susceptible results.

This measure was applied on these dichotomous variables (R and S) to Karl Pearson Chi^2, at the 95% confidence interval of the Odds Ratio [(95% CI (OR)] at the value of $P \leq 0.05$.

2.5 Approval of the ethics committee

The approval of the Ethics Committee N ° ESP / CE / 060/2014, UNIKIN, DRC, granted for the period from December 30, 2014 to December 29, 2015, was renewed by the one bearing the N°ESP / CE / 060B / 2016 , UNIKIN, DRC, from January 20, 2016 to January 19, 2017, following the extension of the study.

3 Results

The results of antimicrobial susceptibility with phenotypic profile testing and molecular characterization of the selected *Salmonella* Typhi isolates in the Western and Southern Regions of the DRC are registered in the tables below.

3.1 Selected *Salmonella* Typhi isolates of the epidemic administrative zones and year of collection

Table 2 shows the administrative zones and the years of isolation of the *Salmonella* Typhi strains selected from specimens collected from the typhoid fever epidemics in the western and southern regions of the DRC.

Table 2 Administrative zones and years of isolation of the selected *Salmonella* Typhi strains collection

No.	Administrative zones	Province	Year
1	Kisantu	Bas-Congo	2003
2	Kisantu	Bas-Congo	2004
3	Matadi	Bas-Congo	2005
4	Inongo	Bandundu	2005
5	Bandundu-Ville	Bandundu	2005
6	Ilebo	Kasaï Occidental	2008
7	Lukaya	Kinshasa	2008
8	Inongo	Bandundu	2008
9	Bandundu-Ville	Bandundu	2008
10	Kingasani	Kinshasa	2009
11	Mweneditu	Kasaï Oriental	2009
12	Lubumbashi	Katanga	2009
13	Bwamanda	Equateur	2009
14	Kingasani	Kinshasa	2013
15	Kingasani	Kinshasa	2014

3.2 Antimicrobial resistance of *Salmonella* Typhi

3.2.1 Phenotypic profile of antimicrobial resistance of *Salmonella* Typhi isolates

3.2.1.1. Phenotypic profile of antimicrobial resistance of the *Salmonella* Typhi isolates

The results of the phenotypic resistance profile reflected the behavior of typhoid isolates with respect to former first-line antimicrobials, first-line antimicrobials, other diverse antimicrobials, and a fourth-generation cephalosporin.

A. Resistance profile of *Salmonella* Typhi isolates to former first-line antimicrobials: RL, W, AMP and C.

The behavior of typhoid isolates to these molecules is presented in Table 3.

Table 3 Average rate of the phenotypic profile of resistance of *Salmonella* Typhi isolates to former First-line antimicrobials in the Western and Southern regions of the DRC (n = 50)

Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
RL	46	92.0	1	2.0	3	6.0
W	25	50.0	20	40.0	5	10.0
AMP	25	50.0	12	24.0	13	26.0
C	23	46.0	23	46.0	4	8.0
Average rate		59.5		28.0		12.5

Note: RL: Sulfamethoxazole; W: Trimethoprim; AMP: Ampicillin; C: Chloramphenicol.

Table 3 reveals the average rate of the phenotypic profile of resistance of *Salmonella* Typhi isolates to former antimicrobials at 59.5% in the Western and Southern regions of the DRC.

B. Resistance profile of *Salmonella* Typhi isolates to first-line antimicrobials: CIP or CTX, CRO and CAZ

The behavior of the isolates of this enteric bacillus to these antimicrobials is presented in Table 4.

Table 4 Average rate of phenotypic resistance profile of *Salmonella* Typhi isolates to first-line antimicrobials (CIP or CTX, CRO and CAZ) in the Western and Southern regions of the DRC (n = 50)

Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
CIP	0	0.0	50	100.0	0	0.0
CTX	1	2.0	49	98.0	0	0.0
CRO	1	2.0	49	98.0	0	0.0
CAZ	0	0.0	50	100.0	0	0.0
Average rate		1.0		99.0		0.0

Note: CIP: Ciprofloxacin; CTX: Cefotaxime; CRO: Ceftriaxone; CAZ: Ceftazidime.

Table 4 shows the average resistance rate of typhoid isolates to first-line antimicrobials at 1.0%.

C. Resistance profile of *Salmonella* Typhi isolates to other diverse antimicrobials: TE, AMC, NA, FOX, CN.

The behavior of these typhoid isolates to other diverse antimicrobials is shown in Table 5.

Table 5 Average rate of the phenotypic resistance profile of *Salmonella* isolates Typhi to other diverse antimicrobials (TE, AMC, NA, FOX, CN) (n = 50)

Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
TE	12	24.0	33	66.0	5	10.0
AMC	6	12.0	41	82.0	3	6.0
NA	4	8.0	44	88.0	2	4.0
FOX	3	6.0	47	94.0	0	0.0
CN	1	2.0	47	94.0	2	4.0
Average rate		10.4		84.8		4.8

Note: TE: Tetracycline; AMC: Amoxicillin + Potassium Clavulanate; NA: Nalidixic Acid; FOX: Cefoxitin; CN: Gentamicin.

Table 5 shows 10.4% of the average resistance rate of typhoid isolates to other diverse antimicrobials.

D. Resistance profile of *Salmonella* Typhi isolates to a cephalosporin from fourth generation.

Table 6 Behavior of typhoid isolates to 4GC (FEP) (n = 50)

Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
FEP	0	0.0	50	100.0	0	0.0
Rate		0.0		100.0		0.0

Note: 4GC: Fourth Generation Cephalosporin; FEP: Cefepime.

Table 6 shows no *Salmonella* Typhi isolates were resistant to FEP.

3.2.1.2. Phenotypic profile of resistance of the *Salmonella* Typhi isolates to former first-line antimicrobials

The results of the phenotypic profile of typhoid isolates to the former first-line antimicrobials in the provinces of Kinshasa City and Bas-Congo.

Table 7 Average rate of the phenotypic resistance profile of typhoid isolates to former first line antimicrobials (RL, W, AMP and C) in provinces of Kinshasa City and Bas-Congo (n = 32)

Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
RL	30	93.8	0	0.0	2	6.3
W	16	50.0	12	37.5	4	12.5
AMP	16	50.0	7	21.8	9	28.1
C	16	50.0	13	40.6	3	9.3
Average rate		61.0		25.0		14.0

Table 7 presents 61.0% of the average rate of phenotypic resistance profile of *Salmonella* Typhi isolates to former first-line antimicrobials in Kinshasa City and Bas-Congo.

3.2.2 Molecular characterization of antimicrobial resistance of *Salmonella* Typhi isolates in all of the Western and Southern regions of the DRC

On a genotypic basis, this molecular characterization of antimicrobial resistance of the *Salmonella* Typhi isolates was determined in the Western and Southern regions of the DRC in general and in the provinces of Kinshasa City and Bas-Congo in particular by the detection of CMMs.

3.2.2.1. Molecular characterization of antimicrobial resistance of *Salmonella* Typhi isolates of urban areas of the Western and Southern regions of the DRC

Table 8 Average rate of CMMs detected in *Salmonella* Typhi isolates of urban areas of the provinces of the Western and Southern regions of the DRC (n = 50)

CMM	Presence of Resistant genes		Absence of Resistant genes		Total	%
	Yes	%	No	%		
intI-1	24	48.0	26	52.0	50	100
$bla_{\text{TEM-1}}$	20	40.0	30	60.0	50	100
$bla_{\text{OXA-1}}$	1	2.0	49	98.0	50	100
$bla_{\text{CTX-M-1}}$	0	0.0	50	100.0	50	100
Average rate		22.5				

The CMMs detected in these typhoid isolates are listed in Table 8.

The average rate of four chromosomal markers detected was 22.5%. This table shows also the rates of these CMMs in order of importance. *Intl*-1 predominated with 48.0% followed by bla_{TEM-1} (40.0%) and bla_{OXA-1} (2.0%). No $bla_{CTX-M-1}$ was detected.

3.2.2.2. Molecular characterization of antimicrobial resistance in *Salmonella* Typhi isolates urban areas in the provinces of Kinshasa City and Bas-Congo

The MMCs detected in these typhoid isolates are listed in Table 9.

Table 9 Average rate of CMMs detected in *Salmonella* Typhi isolates urban areas in the provinces of Kinshasa City and Bas-Congo (n = 32)

CMM	Presence of Resistant genes		Absence of Resistant genes		Total	%
	Yes	%	No	%		
intI-1	18	56.3	14	43.7	32	100
bla TEM-1	16	50.0	16	50.0	32	100
bla OXA-1	1	3.1	31	96.8	32	100
bla CTX-M-1	0	0.0	32	100.0	32	100
Average rate		27.4				

Note: *intl*-1: integron gene; bla_{TEM-1}: betalactamase Temoniera gene; bla_{OXA-1}: betalactamase oxacillinase gene; $bla_{CTX-M-1}$: betalactamase Cefotaximase Munich gene.

The average rate of four CMMs investigated has been 27.4% from 56.3% of *intl*-1, 50% of bla_{TEM-1}, 3.1% of bla_{OXA-1} and 0% of $bla_{CTX-M-1}$.

3.2.3 Measurement of association between the determined phenotypic profile and the CMMs of antimicrobial resistance detected in *Salmonella* Typhi isolates

This measurement was performed using Pearson's chi-square, 95% confidence interval of the Odds Ratio [(95% CI (OR)] at a value of $P \leq 0.05$.

3.2.3.1. Association between the phenotypic profile and CMMs of antimicrobial resistance of *Salmonella* Typhi isolates

The results of the measurement of the association between the phenotypic profile and CMMs, in particular *intl*-1 and $bla_{TEM-1}$of typhoid isolates from the provinces of the Western and Southern regions of the DRC are respectively shown in Table 10 and 11.

Table 10 Association between the phenotypic profile and the *intl*-1 ofresistance of the typhoid isolates to former first-line antimicrobials

Phenotype of the resistance	*intl*- 1		Total	OR	95%CI (OR)		Chi^2	p
	Positive	Negative			Lim<	Lim>		
AMP								
R+I	17	21	38	0.57	0.155	2.151	0.675	0.411
S	7	5	12					
W								
R+I	15	15	30	1.20	0.393	3.803	0.120	0.729
S	9	11	20					
RL								
R+I	23	26	49	0.47	0.349	0.632	1105.000	0.293
S	1	0	1					
C								
R+I	14	13	27	1.40	0.458	4.281	0.349	0.555
S	10	13	23					
Total	24	26	50					

Table 11 Association between the phenotypic profile and the bla_{TEM-1}gene of resistance of Typhoid isolates to former first-line antimicrobials

Phenotype of the resistance	*bla* TEM-1		Total	OR	95%CI (OR)		Chi^2	p
	Positive	Negative			Lim<	Lim>		
AMP								
R+I	15	23	38	0.91	0.244	3.415	0.018	0.892
S	5	7	12					
W								
R+I	13	17	30	1.40	0.441	4.570	0.347	0.556
S	7	13	20					
RL								
R+I	23	26	49	0.60	0.469	0.747	0.680	0.409
S	1	0	1					
C								
R+I	11	16	27	1.07	0.343	3.331	0.013	0.908
S	9	14	23					
Total	24	26	50					

There is no significant association between phenotypic results and the *intl*-1 of resistance of *Salmonella* Typhi isolates to former first-line antimicrobials ($P > 0.05$) (Table 10). And there is no significant association either between the phenotypic results and the bla_{TEM-1} gene for resistance of *Salmonella* Typhi isolates to former first-line antimicrobials ($P > 0.05$) (Table 11).

3.2.3.2. Association between phenotypic profile and CMMs of antimicrobial resistance of the *Salmonella* Typhi isolates in the provinces of Kinshasa City and Bas-Congo

The results of the measurement of the association between the phenotypic profile and CMMs, in particular *intl*-1 and the bla_{TEM-1}gene of typhoid isolates from the provinces of Kinshasa City and Bas-Congo are respectively shown in Table 12 and 13.

Table 12 Association between phenotypic profile and the *intl*-1 of resistance of isolates typhoid to former first-line antimicrobials in the provinces of Kinshasa City and Bas-Congo

Phenotype of the resistance	*intl-1*		Total	OR	95%CI (OR)		Chi^2	p
	Positive	Negative			Lim<	Lim>		
AMP								
R+I	12	13	25	2.30	2.375	14.210	8.839	0.043
S	2	5	7					
W								
R+I	7	13	20	0.38	0.088	1.673	1.659	0.198
S	7	5	12					
C								
R+I	6	13	19	3.90	1.066	3.265	5.880	0.003
S	8	5	13					
Total	14	18	32					

No association was measured with RL as a statistical constant because all *Salmonella* Typhi isolates were resistant and of intermediate susceptibility to this antimicrobial but statistical significance was established between the phenotypic profile of AMP and C and the *intl*-1, respectively P = 0.043 and P = 0.003.

Table 13 Association between the phenotypic profile and the resistance bla_{TEM-1} gene from typhoid isolates to former first-line antimicrobials in Kinshasa City and Bas-Congo

Phenotype of the resistance	*bla TEM-1*		Total	OR	95%CI (OR)		Chi^2	p
	Positive	Negative			Lim<	Lim>		
AMP								
R+I	14	11	25	3.20	2.516	19.630	7.680	0.005
S	2	5	7					
W								
R+I	9	11	20	0.60	0.138	2.483	0.530	0.465
S	7	5	12					
C								
R+I	9	10	19	4.80	1.188	3.173	8.890	0.024
S	7	6	13					
Total	16	16	32					

No association was measured with RL as a statistical constant because all *Salmonella* Typhi isolates were resistant and of intermediate susceptibility to this antimicrobial but statistical significance was established between the phenotypic profile of AMP and C and the bla_{TEM-1}, respectively P = 0.005 and P = 0.034.

4 Discussion

4.1 Antimicrobial resistance phenotypic profile

4.1.1 Average rates resistance of *Salmonella* Typhi to all antimicrobials tested

Those phenotypic profile results of antimicrobial resistance of typhoid isolates concerned the urban administrative entities of the provinces of the Western and Southern regions in DRC and those of the provinces of Kinshasa and Bas-Congo particularly.

4.1.1.1 Former first-line antimicrobials: RL, W, AMP and C

Selected according to the algorithm "effectiveness, affordable cost and availability on the drug market", this first category of antimicrobials recommended by the WHO for the benefit of the poor communities, has shown the decline in effectiveness of those molecules [5, 6]. In front of those former first-line antimicrobials including RL, W, AMP and C, the *Salmonella* Typhi isolates of Western and Southern regions of the DRC and those of Kinshasa City and Bas-Congo particularly presented multidrug resistance rates of 59.5% (Table 3) and 61% (Table 7) respectively. Those results remain slightly above the 54% reported from an evaluation of the antimicrobial resistance surveillance system in the administrative area of Ahmedabad City

in India [33]. Furthermore, our results confirm those reported in some Kenyan administrative entities during the period form 1988 to 2008 and from 2001 to 2008, with respective values of 60.4% and 60.0% [34].

4.1.1.2 First-line antimicrobials: CIP or Third generation Cephalosporins (3GC)

Before the first-line antimicrobials tested, *Salmonella* Typhi isolates generally behaved weakly, that is 1% of the average rate. It is about CIP and 3GC including CTX, CAZ and CRO (Table 4). This result confirms the result which has been reported from a study carried out on a typhoid fever epidemic in Kinshasa City, from October 2004 to January 2005, where no resistance of typhoid strains to those molecules was recorded [14]. The result of the behavior of typhoid isolates at CIP (Table 4) is close to that reported from a study carried out in Côte d'Ivoire with a rate of 1.6% [35]. This result differs from that reported by Kariuki's team in Kenya from 2001-2008, where the decreased susceptibility to the CIP was high at 64% [7]. Compared with third generation cephalosporins, our results confirm those of a study undertaken in Kinshasa where no *Salmonella* Typhi strain was resistant to CTX [15]. However, those results differ from those obtained in Bukavu in DRC, where the resistance of Eberth's bacillus to CAZ and CRO was observed at rates of 18.3% and 16.7% respectively [36].

The reasons for this significant resistance developed by typhoid strains to these two 3GCs in Bukavu remain unknown to us.

4.1.1.3 Diverse antimicrobials: TE, AMC, NA, FOX and CN

In order of importance, the resistance profile of typhoid isolates to those antimicrobials was respectively 24%, 12%, 8%, 6% and 2% (Table 5). The result of these typhoid isolates to NA does not agree with that reported in a study conducted in Kenya, from 2001 to 2008 with 18.4% of rate [7]. However, it confirms that of Koffi's team which noted 5.6% in Abidjan [35].

4.1.1.4 Fourth generation cephalosporin (4GC): FEP

Of all the typhoid isolates submitted to this work, none was resistant to FEP (Table 6).

To secure this molecule, the WHO regulations and those of many countries for the judicious use of 4GC and 5GC seem to be followed in human medicine and are not unanimously observed in veterinary medicine [37]. Some authors have then raised some evidence of that breach by reporting resistance of *Salmonella* serovars namely Idikan, Anatum, Mbandaka and *Salmonella* spp to FEP in animals at farms of N'Djamena with the rates of 14.3%, 25%, 40% and 90% respectively [13].

4.2 Chromosomal molecular markers (CMM) of the *Salmonella* Typhi isolates

The average rate of the CMMs of antimicrobial resistance of *Salmonella* Typhi isolates in those whole areas of the DRC and that of the provinces of Kinshasa City and Bas-Congo particularly have been 22.5% (Table 8) and 27.4% (Table 9) respectively. These results are close to those reported in another study in proportions of 10 to 20% in enterobacterial chromosome and by contrast with about 80% in the plasmid [38].

4.3 Measurement of association between phenotypic profile and CMMs of antimicrobial resistance of *Salmonella* Typhi isolates

The association made between different results was presented at two levels according to the distribution and the importance of antimicrobial resistance in all provinces of the Western and Southern regions of the DRC and particularly in the provinces of Kinshasa City and Bas-Congo

4.3.1 Association between the phenotypic profile and the MMC of antimicrobial resistance of the *Salmonella* Typhi isolates

Those results concerned the urban administrative entities of all provinces located in the Western and Southern regions of the DRC.

4.3.1.1 Association between phenotypic behavior and the *intl*-1 resistance of the *Salmonella* Typhi isolates to former first-line antimicrobials (AMP, W, C and RL)

The measurement of association between the phenotypic profile and the *intl*-1 resistance of typhoid isolates to former first-line antimicrobials of which each has been coupled to its corresponding P value, including AMP: 0.441; W: 0.729; RL: 0.293; C: 0.555 (Table 10) did not lead to any statistical significance ($P > 0.05$).

Contrary to those results, with the measurement of the same parameters, those reported from a study carried out in *Enterobacteriaceae* isolates from the urine of patients at some clinics in

Sydney were of statistical significance with P $<$ 0.0001, $<$ 0.0001, $<$ 0.0001, 0.0002 of AMP, W, RL, C respectively and *intl*-1, 2, 3 [38]. The results of this work also do not differ from those performed between the AMP/Sulbactam complex of phenotypic resistance of *Acinetobacter calcoaceticus-baumannii* and the *intl*-1 and the RL/W complex of the same bacillus and the same *intl*-1 where the two measurements were significant. These are respectively P = 0.019 and P $<$ 0.001 [39].

4.3.1.2 Association between phenotypic behavior and the bla_{TEM-1} resistance of the *Salmonella* Typhi isolates to former first-line antimicrobials (AMP, W, C and RL)

The association between the phenotypic profile and the $bla_{TEM-1}$of the *Salmonella* Typhi isolates of resistance to former first-line antimicrobials of which, each one was coupled to its corresponding P value, including AMP: 0.892, W: 0.556, RL : 0.409, C: 0.908 (Table 11) was not statistically significant (P $>$ 0.05). The difference between our results and those of the Koczura team would testify to the absence of the bla_{TEM-1}variant associated with *intl*-1 detected in one or the other replicon of *Acinetobacter calcoaceticus-baumannii* [39].

4.3.2 Association between the phenotypic profile and CMMs of antimicrobial resistance of the *Salmonella* Typhi isolates in the provinces of Kinshasa City and Bas-Congo

4.3.2.1 Association between phenotypic behavior and the *intl*-1 resistance of the *Salmonella* Typhi isolates to former first-line antimicrobials (AMP, W, C and RL)

The measures of association between the phenotypic profile and the *intl*-1 resistance of *Salmonella* Typhi isolates to former first-line antimicrobials namely to calculated P: 0.043, 0.003 and 0.198 respectively AMP, C and W (Table 12) were statistically significant with the two first molecules (P $<$ 0.05). The RL having appeared as a constant, did not allow any measure of association with the CMMs detected. These isolates were all found to be resistant and of intermediate susceptibility to this former, first-line antimicrobial. Those statistically significant associations between the phenotypic behavior of *Salmonella* Typhi isolates with AMP (P=0.043) and C (P=0.003) and *intl*-1 corroborate the results reported by White, Mciver and Rawlinson [38] including AMP (P = 0.0001), C (P = 0.0002) and RL (P $<$ 0.0001). On the other hand, the measurement of association between the behavior of the isolates of *Salmonella* Typhi with W and the *intl*-1 of the present work, not only that it was not significant (P=0.198), but especially it differs with the results from the hospitals of Sydney (P $<$ 0.0001) [38].

4.3.2.2 Association between phenotypic behavior and the resistance bla_{TEM-1} of *Salmonella* Typhi isolates to former first-line antimicrobials (AMP, W, C and RL)

The association between the phenotypic profile and the bla_{TEM-1} resistance of typhoid isolates to former first-line antimicrobials, namely AMP, C and W with calculated P, 0.005, 0.024 and 0.465 respectively (Table 13) were statistically significant with the two first antimicrobials (P $<$ 0.05). The RL having appeared as a constant, cannot allow any association with the CMMs with the same conditions as the preceding one. The significant associations between the behavior of the *Salmonella* Typhi isolates with AMP and C and bla_{TEM-1}would testify the importance of these CMMs with the emergence of antimicrobial resistance in the geographic concerned areas. In contrast with the significant associations in the two concerned provinces, the absence of links with the other genes would be attributed to the phenotypic resistance observed to the others factors including the plasmid [38, 40].

5 Conclusion

This study was carried out on the antimicrobial resistance of *Salmonella* Typhi in the Western and Southern regions of the DRC, from 2003 to 2014. It has shown that the phenotypic profile was more than half of the multidrug resistant isolates to former first-line antimicrobials in all provinces in general and in the provinces of Kinshasa City and Bas-Congo in particular. Almost all typhoid isolates have presented low resistance to the three other categories of antimicrobials. Concerning molecular characterization, the rates of the detected *intl*-1 and bla_{TEM-1} have predominated in Kinshasa and Bas-Congo compared to those of all other administrative areas. The association measured between the phenotypic profile and CMMs of the antimicrobial resistance of *Salmonella* Typhi isolates in the Western and Southern regions of the DRC has shown no statistically significance but has been however established in those of Kinshasa and Bas-Congo provinces. Among all those detected genes, *intl*-1 and bla_{TEM-1}have strongly revealed their resistance impact on AMP, C and RL with a significant association. Thus, the

study suggests to give up the use of AMP, C and RL in those two provinces and to operationalize the surveillance of antimicrobial resistance system in the DRC.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author Contributions

Conceptualization: K.K.K, J.K., J.J.M., C.N.M.;
Investigation: K.K.K., B.N.M., R.N., S.N., J.K.;
Methodology: K.K.K., B.N.M., R.N., S.N., J.K., J.J.M.;
Supervision: J.K., S.K., C.N.M., J.J.T.M;
Visualization: K.K.K. and all other have read and approved the final version of the manuscript.

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