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Antimicrobial Resistance Profiles of *Salmonella enterica* subspecie *enterica* serovar Typhi isolates Associated with Typhoid Fever Epidemics in the Democratic Republic of the Congo, 2002-2014

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Abstract:-

➢ ***Introduction***

The World Health Organization (WHO) estimates 540 cases of Salmonella Typhi infection per 100,000 inhabitants with the majority of deaths attributed to multidrug-resistant strains. The purpose of this study was to determine the antimicrobial resistance profile of *Salmonella* Typhi isolates from typhoid epidemics and to evaluate multidrug resistance in the Democratic Republic of the Congo (DRC).

➢ ***Materials and methods***

The National Institute for Biomedical Research (INRB) in Kinshasa has, from 2002 to 2014, received *Salmonella* Typhi isolates from blood and stool cultures from different geographical areas of the DRC for analytical expertise. From INRB, these isolates were shipped to the Kenya Medical Research Institute (KEMRI) in Nairobi, where this work was performed. Sorted isolates from the lot were streaked on MacConkey agar (Oxoid, UK) and incubated (Memmert) at 37° C for 24 hours for isolation. Lactose-negative colonies were purified on Mueller-Hinton agar (Oxoid, UK). For confirmation, these isolates were agglutinated on a glass slide with *Salmonella* Typhi antisera anti O and anti H (Remel, Dartford, UK). Colonies of 95 isolates selected were diluted in NaCl 0.85g/L in accordance with MacFarland Standard 0.5. These isolates were submitted to antimicrobial susceptibility testing with 14 common antimicrobials (Oxoid), using the Kirby-Bauer diffusion method on Mueller-Hinton agar. The inhibition diameter of each antimicrobial was recorded and interpreted.

➢ ***Results***

The antimicrobial resistance profiles of *Salmonella* Typhi were: Sulfamethoxazole 87%, Chloramphenicol 48%, Trimethoprim 46%, Ampicillin 42%, Tetracycline 22%, Nalidixic acid 8%, Amoxicillin-Clavulanic acid 8%, Cefoxitin 4%, Gentamicin 2%, Ciprofloxacin 1 %, Cefotaxime 1%, Ceftazidime 0%, Ceftriaxone 0% and Cefepime0%. Of the former first-line antimicrobials used in the treatment of typhoid fever, 49% of the isolates were multidrug-resistant.

➢ ***Conclusion***

The increase of the multidrug resistance rate in the DRC is a real Public health threat. The judicious use of antimicrobials and the rigorous exploitation of antimicrobial resistance surveillance systems has to be the concern of all.

Keywords*:* - Antimicrobial resistance profiles, *Salmonella enterica* Typh*i* isolates, evaluation, multidrug resistance, INRB, Kinshasa DRC, KEMRI, Nairobi.

I. INTRODUCTION

Typhoid fever is a real burden in low- and middle-income countries whose supply of safe drinking water is a major challenge. Communities in these areas do not take adequate steps to avert contamination with fecal matter (Steele A.D. et al, 2016, Buckle G.C. *et al*, 2012). The consequence of all these shortcomings inevitably leads to populations exposed to infection by ingestion of *Salmonella enterica* subspecie *enterica* serovar Typhi, a peritrichious bacterium, 2-3 µm long and 0.6 µm wide (Delmont J., & Pichard E., 2012).

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The complication of typhoid fever is certainly peritonitis due to ileal perforation which is a major public health problem (Mongasale V., *et al*, 2014). The discovery and use of antimicrobials has significantly contributed to the fall of the morbidity and mortality rates attributed to infectious diseases, including typhoid fever (Tortora G. J. *et al*, 2012, Pages J.M. 2004).

However, as the use of antimicrobials increased in humans and animals, resistance mechanisms associated with selective pressures also made bacteria evolve (Kariuki S. *et al*, 2004, Anibal by J. Sosa *et al*, 2010). The expression of bacterial resistance to certain antimicrobials has only been expected one to two years after their introduction on the market (Cohen Y. & Jacquot C. 2008; Buller N., Thomas A., Barton M., 2014, Cavallo JD). Typhoid fever remains, like cholera and shigellosis, one of the most impoverished diseases, "Diseases of the Most Impoverished" (DOMI) (Steele A.D., *et al*, 2016). According to the WHO estimations, 22 million of the *Salmonella* Typhi infections were reported in 2000 (Crump J.A. et al, 2004). In endemic-epidemic disease areas, the lack of material resources for bacteriological diagnosis, based on blood culture, has kept communities rely on the empiric use of antimicrobials (Baker S., *et al*, 2016 Lunguya O., *et al*, 2012). This important absorption of antimicrobials against these typhoid infections was a major challenge with increasing resistance to Chloramphenicol, Sulfamethoxazole-Trimethoprim, and Ampicillin which were used as first-line antimicrobials in the treatment of typhoid fever with the associated use of Tetracycline. This rapid spread of resistance to more than one antimicrobial was widespread in communities in Southeast Asia and sub-Saharan Africa from the 1990s, and hence the term "multidrug-resistant". Faced with the emergence of these multidrug-resistant strains, the WHO in 2003 recommended first-line treatment with Ciprofloxacin or Cefixime (C3G) (World Health Organization, 2003). However, first line antimicrobials continue to be used in many areas of developing countries. Despite the new WHO recommendation for quinolones and cephalosporins, second- and third-generation, respectively in the first-line treatment of typhoid fever, new epidemics of typhoid fever are increasingly reported in the affected areas. These epidemic outbreaks are due to the use, without success, of the former first line antimicrobials. In regions with inefficient disease surveillance systems, WHO estimates between 60% and 90% of cases of undiagnosed typhoid infections have an incidence of between 100 and 600 cases per 100,000 inhabitants per year. Children aged 1 to 5 and young adults up to 25 years of age pay the highest price of their life (Tomas A & Strobel M. 2005). Resistance to the former first-line antimicrobials was also observed in the susceptibility testing of *Salmonella* Typhi isolates from the Kikwit epidemic in DRC between 2011 and 2012 (Aubry P. 2013). In the world and over the years, typhoid fever has marked history with major devastation in Southeast Asia, Latin America, and South-Sahara Africa (Fagherazzi-Pagel H., 2012; Tortora GJ *et al*, 2012).

According to the recent documented estimates, 11.9 to 26.9 million new cases and 129,000 to 161,000 deaths attributed to typhoid fever are reported annually (Steele AD, *et al*, 2016, Buckle G.C. *et al*, 2012) in South East Asia where the disease is endemic and with multidrug-resistant strains (Tomas A & Strobel M. 2005, Aubry P. 2013). In the Maghreb, typhoid fever is classified as re-emerging disease (Anibal J. Sosa et al, 2010). In industrialized countries, however, the incidence is estimated at 0.2 cases of typhoid fever per 100,000 inhabitants, the majority of documented typhic attacks being, mainly, imported and acquired, in 91% of cases, during a stay in an endemic area (Crump JA *et al*, 2004, Aubry P. 2013).

At the stage of worsening of the disease, only early diagnosis and appropriate treatment with antibiotic therapy and surgery can essentially be the factor in reducing the morbidity and mortality rate (Togola *et al*, 2013). One study showed that out of every 10,000 people who each year die in hospitals as a result of a nosocomial infection, 75% would be infected with multidrug-resistant bacteria (Institut Veille sanitaire, 2006). This phenomenon of bacterial multidrug resistance, having no geographical limits, affects the whole world. This growing threat is becoming increasingly worrying in both developing and industrialized countries. In Europe, the results of a recent study on antimicrobial resistance mortality reported 25,000 people (Soumois F., 2016). Some American dailies report that death rates attributable to some multidrug-resistant strains exceed those caused by HIV-AIDS infection (Tortora G.J. *et al*, 2003). The main objective of the study was to evaluate antimicrobial resistance profiles of *Salmonella* Typhi isolates associated with typhoid fever epidemics in the DRC, from 2002 to 2014. Specifically, to determine multidrug resistant of the *Salmonella* Typhi isolates to common antimicrobials in the DRC.

II. MATERIALS AND METHODS

All available strains of *Salmonella* Typhi isolated from blood or stool were from typhoid fever outbreaks in the DRC endemic areas and clinical cases recorded from 2002 to 2014. Stored in nutrient agar, these isolates were sent at National Institute for Biomedical Research (INRB) for further bacteriological analyzes. From DRC, *Salmonella* Typhi isolates were shipped out to the Center for Microbiology Research at Kenya Medical Research Institute (CMR-KEMRI) in Nairobi for this current study. The compliant and sorted isolates from the consignment were cultured on MacConkey agar (Oxoid, UK) and placed in the incubator (Memmert D model 600) for 24 hours at 37°C. The recovered isolates (negative lactose colonies) were purified by streaking them on Mueller-Hinton agar (Oxoid) and incubated as previously demonstrated. The confirmation of these isolates was performed by serotyping using the slide agglutination method.

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During this test, two drops of a suspension of the mixture of two identical colonies, pure and fresh, presumed typhoid isolates were separately emulsified on a slide, the first with a drop of physiological solution and the second with a drop of antiserum anti *Salmonella* Typhi (Remel, Dartford, UK), anti O, then with anti H.
The agglutination was obtained within 60 seconds after mixing each solution by turning the preparation and reading was done in good lighting.

The isolates were confirmed *Salmonella* Typhi by the presence of agglutination, which reflected the positive test. Otherwise, the test was declared negative. This test made it possible to close the selection process of working isolates, of which 95 were selected. In addition to the American Type Culture Collection (ATCC) 25922 *Escherichia coli* quality control sample, these 95 confirmed and selected typhoid isolates were tested for antimicrobial susceptibility. This test was performed using the Kirby-Bauer diffusion method on Mueller-Hinton agar with 14 usual antimicrobials (Oxoid) for a control sample and typhoid isolate from the study for each batch tested. Mueller-Hinton agar was cast in 4 mm thickness in each Petri dish (Oxoid, UK), sterile, plastic 10 cm in diameter. By test, for the quality control sample as well as for the working isolate, 2 Petri dishes were used. The transparency of the suspension of the inoculum obtained after dilution of 4 to 5 colonies of the control strain and those of each *Salmonella* Typhi isolate in 5 ml of 0.9 g / L NaCl saline was compared and held equal to that of the MacFarland standard 0.5. This inoculum, freshly prepared, was taken using a sterile cotton swab (MW-108). Once the swab cleared the excess liquid from the inoculum, by squeezing the inner wall of the containment tube, bacterial cell streaking was uniformly performed over the entire surface of the agar plate after several passages. On the bacterial mat of the Mueller-Hinton agar of the first Petri dish, devoid of wet reflection, 6 disks of β-lactamine antibiotics were deposited using the automatic dispenser (Disp-FG) forming a circle.

These are: Ampicillin (AMP) 10 µg, Ceftriaxone (CRO) 30 µg, Cefotaxime (CTX) 30 µg, Ceftazidime (CAZ) 30 µg, Cefepime (FEP) 30 µg and Cefoxitin (FOX) 30 µg, with at center, Amoxicillin-clavulanate of potassium (AMC) 20/10 µg manually deposited as a 7th disc. On the bacterial mat of the agar of the second Petri dish, the other 6 antibiotics, not β-lactamines, have also been automatically deposited in a circle with the 7th in the middle, deposited manually. These are: Nalidixic acid (NA) 30 µg, Ciprofloxacin (CIP) 5 µg, Gentamicin (CN) 30 µg, Chloramphenicol (C) 30 µg, Trimethoprim (W) 5 µg, Sulfamethoxazole (RL) 25 µg, and Tetracycline (TE) 30 µg in the center of the others forming a circle. All petri dishes loaded with the antimicrobial disks, the control strain and the typhoid isolates of the study were incubated as previously described. After incubation, the diameters of the zones of bacterial inhibition obtained were determined using the slats millimeter. Codified in millimeters, the numerical values of diameters were interpreted on the basis of the interpretative instructions manufacturer (Oxoid), by the three traditional categories known: Resistant = R, Intermediate susceptibility = I, Susceptible = S. For analyzes performed, all data was initially recorded on Excel 2010 (Microsoft Corporation) and SPSS (IBM SPSS, Statistics, version 20). Approval of the Ethics Committee: On the basis of the study protocol developed to carry out this one, with the prior consent of the participants, the Ethics Committee of the School of Health of the University of Kinshasa, DRC approved its implementation by N ° Approval Committee Ethics: ESP / CE / 060B / 2016, of January 20, 2016, UNIKIN, DRC.

III. RESULTS

On the basis of the analyzes carried out in this phenotypic resistance-based study, we obtained the following result : RL 87% of *Salmonella* Typhi isolates resistant (R), 6% susceptible (S) and 6% of intermediate susceptibility (SI) ; C: 48% R, 43% S and 8% SI; W: 46% R, 41% S and 13% SI; AMP: 42% R, 35% S 23% and SI; TE: 22% R, 73% S and 5% SI; NA: 8% R, 87% S and 4% SI; AMC: 8% R, 80% S and 12% SI; FOX: 4% R and 96% S; CN: 2% R, 92% S and 6% SI; CIP: 1% R and 99% S; CTX: 1% R and 99% S; CAZ: 0% R and 100% S; CRO: 0% R and 100% S; FEP: 0% R and 100% S.

Table 1: Susceptibility of *Salmonella* Typhi isolates to all antimicrobials tested

Resistant	Susceptible	Intermediate
19%	75%	6%

Table 2: Susceptibility of *Salmonella* Typhi isolates to the former first-line tested antimicrobials

Resistant	Susceptible	Intermediate
49%	40%	11%

Table 3. Resistance profiles of *Salmonella* Typhi isolates to the former first-line antimicrobials including TE

	n=95					
Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
RL	83	**87**	6	6	6	6
C	46	**48**	41	43	8	8
W	44	**46**	39	11	12	13
AMP	40	**42**	33	35	22	23
TE	21	22	69	73	5	5

Table 4. Resistance profiles of *Salmonella* Typhi isolates to antimicrobials other than the former first line antimicrobials

	n=95					
Antimicrobial	Resistant		Susceptible		Intermediate	
	n	%	n	%	n	%
Nalidixic acid (NA)	8	8	83	87	4	4
Amoxicillin + Clavulanic acid (AMC)	8	8	76	80	11	12
Cefoxitin (Fox)	4	4	91	96	0	0
Gentamicin (CN)	1	1	87	92	6	6
Ciprofloxacin (CIP)	1	1	94	99	0	0
Cefotaxime (CTX)	1	1	94	99	0	0
Ceftazidime (CAZ)	0	0	95	100	0	0
Ceftriaxone (CRO)	0	0	95	100	0	0
Cefepime (FEP)	0	0	95	100	0	0

The most important peak of the *Salmonella* Typhi isolates multidrug-resistant was the former first-line antimicrobials, still used in the treatment of typhoid fever. On the other hand, fluoroquinolones including Ciprofloxacin and third-generation cephalosporins (alternating on the first-line) and Cefepime, which is in the 4th generation Cephalosporin, showed good activity on these studied isolates. In addition to these antimicrobials of good activity, other molecules tested together, also showed their good activity against these same typhic isolates as illustrated on curve.

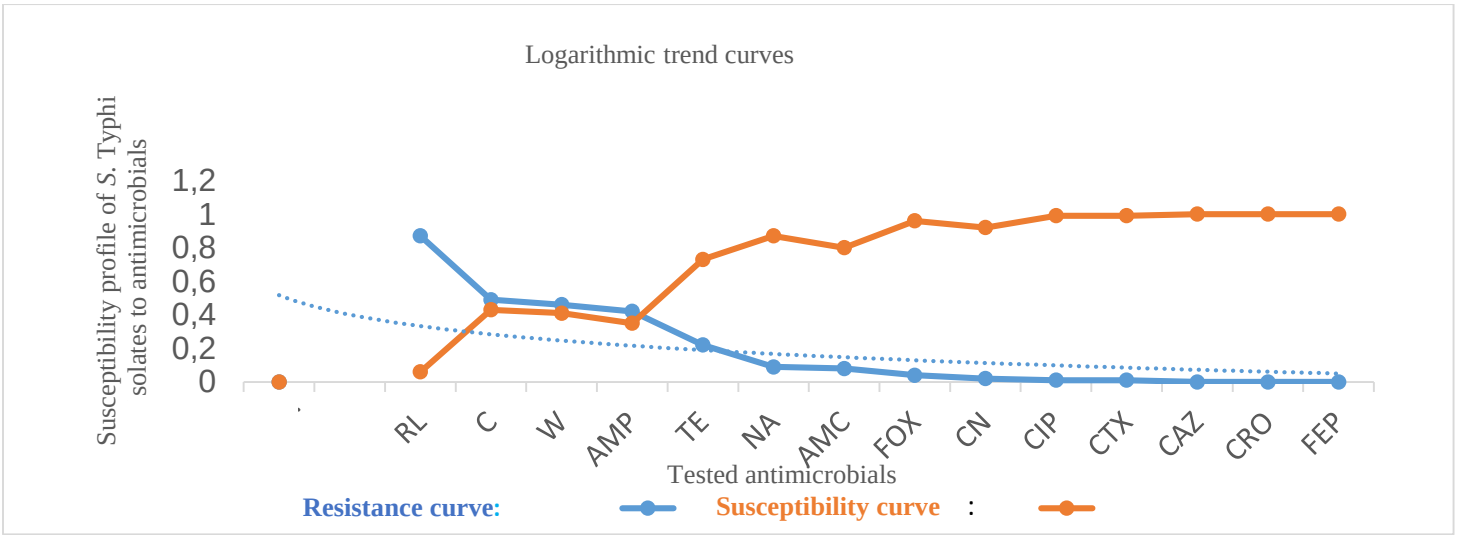

Figure: Panoramic profile of *Salmonella* Typhi isolates tested to antimicrobials recommended by WHO.

IV. DISCUSSION

Experimental work conducted on the determination of the antimicrobial phenotypic resistance profile of 95 isolates of *Salmonella* Typhi, of the typhoid fever epidemics in the DRC, has resulted from three categories of results: resistant, susceptible and intermediate susceptibility. The average resistance rate of *Salmonella* Typhi isolates to all 14 antimicrobials used was 19% versus 49% of multidrug-resistant isolates to the former first-line antimicrobials (RL 87%, C 48%, W 46%, and AMP 42% in addition to TE 22%) (Tables 1 and 2). The decrease in antimicrobial activity of these molecules has been proven in the typhic isolates tested in this study.

However, the susceptibility of these typhic isolates has been encouraging to antimicrobials other than the former antimicrobials of the first line namely:

- Cephalosporins: FEP 100% Susceptible (S) and 0% resistant (R) (4th generation); CAZ 100% S and 0% R; CRO 100% S and 0% R; CTX 99% S and 1% R (3rd generation) and FOX 96% S and 4% R (2nd generation);
- Quinolones: CIP (fluoroquinolone) 99% S and 1% R (2nd generation); NA 87% sensitive (S), 8% resistant (R) and 4% intermediate susceptibility (IS) (1st generation);
- Aminoglycoside: CN 92% S, 6% IS and 2% R;
- Penicillins: AMC 80% S, 12% IS and 8% R.

In the DRC, from October 2004 to January 2005, out of 144 cases of *Salmonella* Typhi infection in Kinshasa, during an outbreak, 63% of operated patients with typhic perforation were previously treated with C, AMP and to RL-W (TMP-SXT), except TE. Of 11 strains of this typhic bacillus isolated by the routine blood culture test, all were 100% resistant to these molecules, including TE. These same isolates were, on the other hand, susceptible to the third-generation cephalosporins and fluoroquinolones (Muyembe J.J. *et al*, 2009). Compared with the result of our study, 49% of multidrug-resistance rate of *Salmonella* Typhi to the former first-line antimicrobials the result of Muyembe J.J. *et al*, would testify strong pressure of selection of typhic strains. In observing after, the susceptibility result of the typhic isolates with fluoroquinolones and 3rd generation Cephalosporins, our results are consistent with those of Muyembe J.J. *et al*. In the Republic of Kenya, during the typhoid fever epidemics occurred between 1988 and 2008, 323 isolates of *Salmonella* Typhi tested by AMP, C, TE and W-RL (TMP-SXT), 60.4% were found to be resistant compared to 16.7% susceptible to these former first-line antimicrobials (Kariuki S. et al, 2008). During the period from 2001 to 2008, another study reported the rate of 64% multidrug-resistant S. Typhi isolates in Kenya (Kariuki S. op. cit.). The result of this work is joining the previous one. However, taken separately, each result of those two Kenyan studies remain higher to ours. In contrast to the significant reduction in susceptibility of Tetracycline 30 µg, as reported by Muyembe J.J. et al, in Kinshasa and Kariuki S. *et al*, in Kenya, our typhic isolates have been susceptible up to 73% versus 22% resistant, followed by intermediate susceptibility at 5%.

In Kenya, the rates of multidrug-resistance of some other studies carried out thereafter have been greater than ours (49%), particularly: 70% (2004 - 2006), 79% (2010), 78% (2012), and 85.7% (2013). In Tanzania, 89% (2010) (Al-Emran H. M. et al, 2016). These data show the trend of increasing the prevalence of multidrug-resistance, particularly in this eastern part of the continent and yet in Burkina Faso, Guinea Bissau, Senegal and Madagascar, countries concerned by the same study, to our knowledge, no multidrug resistance has been reported for all their isolates.

However, in Senegal, a previous study that was carried out in 2002 had identified 0.4% of multidrug-resistant S. Typhi isolates (Hassan M. A. et al, 2016). Multidrug-resistant prevalence of former first-line antimicrobials in West African countries is low (Parry C. et al., 2011). From this study, 9% of resistance rate of Salmonella Typhi versus 87% of susceptibility, followed by 4% intermediate susceptibility to Nalidixic acid (NA) 30 µg have been obtained (Figure). Nigeria, however, constitutes, as far as Kenya, African countries where the resistance of Salmonella Typhi to Nalidixic acid (NA) extends the list of molecules, formerly defined in multidrug resistance in relation to previous antimicrobial drugs of the first line (Parry CM et al, 2003, Parry C. op.cit.). In Kenya, the rate of resistance of typhic bacillus to Nalidixic acid, raised to 5.6% after work on isolates collected during the period from 1994 to 2000, has reached 18.4% on also collected isolates carried out from 2001 to 2008 (Kariuki S. et al, 2008).This rate of resistance to Nalidixic acid, which is double of ours, is practically three times of the kenyan study, in the space of about eight years. This generalization of resistance of *Salmonella* Typhi to Nalidixic acid was well known before, in India, Saudi Arabia and Laos, in the Asian continent (Parry C.M. et al, 2000).

Reported from broilers, high to very high resistance has been observed in Europe with Tetracycline and Nalidixic Acid. In humans and poultry, resistance levels of *Salmonella enterica* to the following antimicrobial molecules were noted: TE 30%, AMP 28.2% and RL 28.2% (Soumois F., 2016). With regard to the behavior of the isolates of this typhic bacillus with Ciprofloxacin, the results of several works were noted in margin of ours which was of 1% of resistance (Table IV) as much as of Muyembe J.J. *et al*, which has obtained no case of resistance (Muyembe JJ et al, 2009). In Kenya, between 2004 and 2006, the 13% reduction in susceptibility of *Salmonella* Typhi isolates to Ciprofloxacin was reported (Al-Emran H.M. *et al*, 2016). In their study which used the susceptibility test on *Salmonella* Typhi isolates with fluoroquinolones in the DRC, Lunguya O. *et al*, found 15% of the reduction rate of this susceptibility (Lunguya O. et al, 2012). In Kenya, thereafter, in 2012 and 2013, the susceptibility reduction rates of *Salmonella* Typhi strains to fluoroquinolones were 19.5 and 42.9%, respectively. This reduction has also been reported in some countries such as Egypt (36%), Iraq (81%) and Cambodia (90%) (Vlieghe ER, *et al*, 2012, Rahman BA, *et al*, 2014).

These results, not only raise concern, but even call into question the WHO recommendation that in 2003, Ciprofloxacin or Cefixime (C3G) were considered as anti-*Salmonella* Typhi molecules in first-line treatment of typhoid fever (World Health Organization, 2003).

The resistance rate of *Salmonella* Typhi isolates to the combination to Amoxicillin and Potassium clavulanate was 8% compared to 80% of the susceptibility rate followed by 12% intermediate susceptibility (Table IV). These 80% susceptibility of these isolates to this duo is attributable to the activity of Amoxicillin in its combined state with potassium clavulanate. Potassium clavulanate inhibited the so-called β-lactamase bacterial proteins which are susceptible to it so that only the β-lactamine antibiotics act on bacterial isolates. *Salmonella* Typhi isolates have been resistant to Cefoxitin at 4% versus 96% susceptible (Table IV). This second-generation Cephalosporin still has a considerable susceptibility rate. As observed in this study, the susceptibility of some typhic isolates to certain common antimicrobials has been encouraging. It has been estimated that, there is the typhic strains which may be still keeping a wild character in many parts of the DRC. Those typhic strains maybe probably come from poor areas with low antimicrobial use and consequently, there is an absence of selection pressure for typhic strains.

V. CONCLUSION

The average multidrug resistance rate for the former first line antimicrobials was 49% as a resistance profile in the DRC (Sulfamethoxazole 87%, Chloramphenicol 48%, Trimethoprim 46%, and Ampicillin 42% in addition to Tetracycline 22%). In comparison with these former first line antimicrobials tested, the other category of antimicrobials used in the same study has demonstrated a good profile and satisfactory susceptibility. It is about Ciprofloxacin and the third-generation Cephalosporins, recommended in 2003 by the WHO as a first-line molecule to the treatment of typhoid fever despite their high cost among the most impoverished communities. While the degradation of Ciprofloxacin is increasingly being shown by the results of studies conducted in South East Asia and in Eastern and sub-Saharan Africa, third-generation Cephalosporins are useful and still hopeful in the typhoid fever antibiotherapy. Like the epidemiological surveillance of typhoid fever in the DRC, biological confirmations are recommended at the same time as monitoring for antimicrobial resistance.

- *Perspective*

Encourage sectorial studies of antimicrobial resistance profiles in the DRC, depending on whether it is high access and low antimicrobial access, respectively, of high and low consumption. These low consumption antimicrobial areas can still use active molecules on their bacterial isolates that have already lost their potency in communities where the same typhoid serovar has undergone significant selective pressures.

- *Weakness of the study*

The *Salmonella* Typhi isolates investigated did not all have complete identification of their geographic isolation areas. This did not allow us to establish the association between the isolates of the less equipped communities and those of the urban centers.

- *Strength of the study*

This is an update of the resistance profiles of *Salmonella* Typhi isolates to antimicrobials, in light of the findings of others for the assessment of the emergence status of this antimicrobial resistance in the DRC. On the fringes of these determined resistance profiles, this study, supported by other results is revealing the trend of this phenomenon in Africa.

From the East, with higher prevalences, through the Center, the DRC, with considerable values increasing, but below the previous ones, the antimicrobial resistance trajectory ends in West with low rates.

- *Conflicts of interest:* None declared.

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