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A Microbiological Study of Ileal Perforation due to *Salmonella enterica* sub specie *enterica* serovar Typhi Associated with Typhoid Fever in the City of Kinshasa, D.R. Congo

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Abstract: - Typhoid fever leads to a major consequence, the intestinal perforation, which occurs to the very infected patients. The intestinal perforation remains one of the most complication of this disease in developing countries. To share responsibility with the surgeon who declares this perforation and repairs the damage, this study is performed, not only to isolate the *Salmonella enterica* subspecie, *enterica* serovar Typhi strains, but also to detect their DNA fragment in peritoneal fluid, collected from peritoneal cavity of the surgically operated patients. To attain this goal, the peritoneal fluid collected from some hospital of the City of Kinshasa, the Democratic Republic of the Congo (R.D.C.) has been carried to the National Institute for Biomedical Research (INRB) in Kinshasa and at Kenya Medical Research Institute (KEMRI) in Nairobi, respectively, to perform bacterial culture for the *Salmonella* Typhi isolation and to detect its DNA. Of 28 patients who have been operated with ileal perforation, associated to typhoid fever, 71.4% were male with a median age of 20.5 years. This prospective study, performed by culture of peritoneal fluid in MacConkey agar and by polymerase chain reaction in the same specimen did not, neither isolate the *Salmonella* Typhi nor detect its DNA.

Keywords:- Intestinal perforation, peritoneal fluid, Isolation, *Salmonella* Typhi, INRB, Kinshasa, D.R. C, Detection, DNA, KEMRI.

I. INTRODUCTION

This enterobacterium of the genus of *Salmonella* has two species which are *enterica* and *bongori*. *Salmonella enterica,* subsp *enterica* has more than 2,500 different serovars of which Typhi (1, 2).

Typhoid fever is estimated to 33 million cases per year worldwide with 600,000 to 700,000 deaths occurring in the developing countries of Southeast Asia and Sub-Saharan Africa. These mortality rates vary region to region. About 15% to 30% are reported from India, Cambodia, Indonesia, Nigeria (3, 4)

With appropriate and early antimicrobial therapy, some patients recover their health with minor complications. On the contrary, the illness major consequence, which is the ileal perforation, occurs to the penniless patients, who are in weak income region (3).

In urban and popular area of developing countries, including the DRC, the intestinal perforation remains an absolute and first cause of the urgent laparotomy among surgical emergencies.

The typhic perforation occurs in the typhoid epidemic outbreak with the highest rates of deaths (5, 6, 7). It is a health major matter in communities.

After ingestion, the rods of Eberth get over the barrier of the intestinal mucosa that they leave intact, without injure it and reach the lymphatic stage in the mesenteric nodes where they multiply in under mucosa layer. After this, some of the *Salmonella* Typhi cells earn the circulation of the blood through thoracic canal to cause the septicemia (1, 2, 8 and 9).

In the same human immune system, in the mesenteric nodes, the *Salmonella* Typhi cells are destroyed by the macrophages releasing the endotoxin in under-mucosa of the ileal wall.

This endotoxin, of lipopolysaccharidic nature, in the bacterium cell wall, is a real poison that remains the major cause of the ileal perforation (10).

The Peyer patches and the under mucosa layer get irritated and bleed until the complete intestinal perforation. The opening is established between intestinal mucosa and the peritoneal envelope where the fluid that flows from the injured layers and bowels is collected (5, 11).

To diagnose a typhic infection, one performs in routine, classical bacteriological analyses in blood, stool and urine culture with appropriate techniques (12).

These performed tests lead to the isolation of the Salmonella Typhi as bacterium cell in different appropriate specimens (13, 14).

The Widal and Felix serological test is also performed to detect the *Salmonella* Typhi infection from anti bodies (9, 12).

However, in peritonitis with the intestinal perforation pathology, one cannot successfully perform the above tests in the peritoneal fluid to objectively get the expected results, the *Salmonella* Typhi, bacteria cells. Normally, these bacteria cells which have created the opening by their endotoxin from intestinal mucosa until the peritoneum have been destroyed.

That precedes has to keep our attention to assure an accurate diagnostic, in determining the etiological agent in spite of the surgeon who, in his laparotomy, asserts an acute typhic perforation that he observes. That is not enough to do so without the detection of an etiological agent to establish the cause and the effect relation.

The peritoneal fluid collected as an appropriate specimen cannot, normally, contain the *Salmonella* Typhi as bacterium cell or allow to its isolation, except only the possible cellular strains drained from bowels to peritoneal cavity through the perforation sites. The *Salmonella* Typhi DNA fragments are though expected to be detected by Polymerase Chain Reaction test (PCR).

This fundamental experiment relies on solid bases as followed:

- Peritoneal fluid to diagnose the *Salmonella* Typhi infection at its last and critical stage of the pathology ;
- Important means leading to diagnose the *Salmonella* Typhi infection even there is no alive bacterium cell that should grow in the classical bacteriological culture media (blood, bone marrow and stool) ;
- It is an epidemiological value aiming at the improvement of the diagnosis quality ;
- May perform the peritoneal fluid culture to isolate the *Salmonella* Typhi cells and other bacteria cells drained from bowels;
- The peritoneal fluid is rare to be used as a classical sample to diagnose the *Salmonella* Typhi infection ;

The study, mainly, consists to assess the *Salmonella* Typhi infection by its evidence in the peritoneal fluid and specifically,

- To isolate the *Salmonella* Typhi strains by bacteriological culture ;
- To detect the *Salmonella* Typhi DNA fragment by polymerase chain reaction in peritoneal fluid.

II. MATERIALS AND METHODS

This study has been approved by both of scientific and ethical committee of the School of Public Health of the University of Kinshasa, D. R.C. with approval n° ESP/CE/ 060/2014.

This prospective study has been extended from February 20th, 2015 to February 24th, 2016 for 28 surgically operated patients with intestinal perforation at the Masina Roi Baudouin Hospitable Center (CHRB-M), in the East of Kinshasa and at the Makala Reference General Hospital (HGR-M), in the Middle West of the City. Of 28 patients who have been operated with ileal perforation, associated to typhoid fever, 71.4% were male with a median age of 20.5 years with the extremes, 5 and 59 years old and the sex ratio M/F was 5:2.

The stercoral fluid drained from the ileal mucosa with the necrotic secretion from the different intestinal layers due to the *Salmonella* Typhi endotoxin and collected from the peritoneal cavity in two different labelled containers was carried at the bacteriological laboratory of the Unit of the Potential Epidemic Diseases of INRB.

After each sample collection, the first specimen dispensed in the Selenite F broth was cultured on MacConkey agar (Oxoid) and placed in the incubator (Memmert D model 600) for 24 hours at 37°C.

The day after, all suspicious colonies (negative lactose) were sub cultured on the identification media for the biochemical tests, according to the classical principles of analytical bacteriology. The second aliquot dispensed in the dry tube was frozen, at minus 20° C, and has been sent at KEMRI in Nairobi for the *Salmonella* Typhi DNA detection.

At Centre for Microbiology Research (CMR)-KEMRI, the specimen were performed according to the PCR protocol committed to the isolation of pathogen bacteria from stool by Qiamp technique after repeating the peritoneal fluid culture of on the MacConkey agar as previously performed at INRB.

In molecular lab, the detection kit of multiple diarrhoegenic pathogens DNA, as a selective test using the diarrhea- B1 ACE Detection as followed has been used:

- Internal control……………….1000 base-pair;
- *Vibrio* spp……………………..651 bp;
- *Clostridium difficile* toxin B….475 bp;
- *Salmonella* spp……………….395 bp;
- *Shigella* spp…………………..330 bp;
- *Campylobacter* spp…………...227bp.

This analytical PCR kit has been used to detect the *Salmonella* Typhi DNA fragment in the peritoneal fluid. The test was performed with a Salmonella DNA positive control in Thermal cycler DYAD MJ Research, PTC DNA Engine, serial N° AL039259 according to the appropriate procedure.

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The filter tips and tight gloves have been used for specimen and control preparation to prevent splashing and potential cross contamination. The extreme care also has been used to ensure specific amplification. The DNA has removed from -20°c and placed in ice or in a fridge (4°c) to allow thawing.

The components of the PCR kit have similarly been thawed. The PCR reaction tubes have been labeled with numbers corresponding to those of isolates to be analyzed.

The reagent tubes have been briefly centrifuged to remove drops from the inside of the cap. Once the DNA was half-way thawing, the following master mix have been made for detection of possible *Salmonella* Typhi DNA.

The amount of each reagent needed based on total number of reactions(samples + controls) has been calculated and mixed by quick vortexing and centrifuged briefly.17µl of the reaction mix have been dispensed into 0.2 ml PCR tubes and 3µl of each of 28 peritoneal fluid samples have been added into the mix. In each 17µl of the reaction mix of the negative and positive PCR control have been added 3µl, respectively, PCR distilled water and *Salmonella* Typhi DNA. After briefly vortexing, the test and controls tubes were placed in a preheated (94° C) of thermal cycler for running the PCR reactions about 3 hours for 35 cycles.

The electrophoretic migration of the nucleotide sequences was carried out at the end of the amplification. To 1% (w / v) of agarose gel prepared and homogenized at the boiling point in the microwave, for about 3 minutes were added, with careful mixing, 2 drops of ethidium bromide once the solution has reached lukewarm temperature.

This gel still liquid was poured, without delay, into a tank with a suitable comb to form wells at its removal from the gel solidified (cooled). Already solid, the gel was transferred to the bottom of the electrophoretic migration vessel, submerged TAE buffer.

To each well of the support, were deposited, in the order of alignment, 12 µl of each of the following products: the molecular weight marker (Ladder) DNA (whose size of each band is 200 bp), the positive control, the negative control and the supposed amplicons of the study which were all mixed with 3 µl of bromophenol blue colored loading buffer.

An alternating electric current of 220 to 240 volts, converted to 120 volts, was applied to the electrophoresis tank for a migration time of 60 minutes. At the end of this time interval, the anionic amplicons migrated in bands towards the anode of the tank. The solid gel, carrying the migration of the DNA bands, was transferred from the migration vessel to the developer support (GelMaxImager) connected to the computer (*hp*).

The anionic bands of DNA molecular weight marker, positive control, negative control and amplicons of the study were revealed and visualized, under ultraviolet rays, to the fluorescence of ethidium bromide. Based on knowledge of the known size of each DNA marker fragment, the base pair size of positive control band was determined. These results of electrophoretic migration collected in bands were recovered in electronic format.

III. RESULTS

Of 30 surgical operated patient for peritonitis, it has been registered 28 cases due to the typhoid ileal perforation and 2 cases of appendicular perforation.

Age group of operated patient and bacteriological test results. Twenty-eight operated patients (20 male and 8 female) with clinical typhoid perforation were enrolled into the study. Patient details, age group and bacteriological results are shown in tables 1 and 2.

Table 1.Patients operated with typhoid intestinal perforation distributed according to the age group and by sex.

Age group (y)	Male (%)	Female (%)	Total (%)
5 – 9	6 (21.4)	2 (7.1)	8 (28.6)
10 – 14	2 (7.1)	2 (7.1)	4 (14.3)
15 – 19	3 (10.7)	1 (3.3)	4 (14.3)
20 – 24	2 (7.1)	2 (7.1)	4 (14.3)
25 – 29	1 (3.6)	0	1 (3.6)
30 – 34	1 (3.6)	1 (3.3)	2 (7.1)
35 – 39	1 (3.6)0	0	1 (3.6)
40 – 44	1 (3.6)0	0	1 (3.6)
45 – 49	1 (3.6)0	0	1 (3.6)
50 – 54	1 (3.6)0	0	1 (3.6)
55 – 59	1 (3.6)0	0	1 (3.6)
Total	20 (71.4)	8 (28.6)	28 (100)

Table 2.Result of the bacteriological analyses performed at INRB according to the age group (year).

Bacterial culture and Biochemical tests results

Age group (y)	Eff.	*S*.T (%)	*E c* (%)	*Cit* sp (%)	*K* sp (%)	*P mi* (%)	*P vu* (%)	*P. aer* (%)	Tot (%)
5 – 9	8	0	2 (7.1)	1(3.6)	2 (10.0)	0	3 (10.7)	0	8(28.6)
10 – 14	4	0	3 (10.7)	0	1 (3.6)	0	1 (3.6)	0	5 (17.8)
15 – 19	4	0	4 (14.3)	0	0	1(3.6)	0	1 (3.6)	6 (21.4)
20 – 24	4	0	1 (3.6)	0	1 (3.6)	1 (3.6)	1 (3.6)	0	4 (14.3)
25 – 29	1	0	0	0	0	0	0	0	0
30 – 34	2	0	0	0	0	0	0	0	0
35 – 39	1	0	1 (3.6)	0	0	0	0	0	1 (3.6)
40 – 44	1	0	1 (3.6)	0	0	0	0	0	1 (3.6)
45 – 49	1	0	0	1 (3.6)	0	0	0	0	1 (3.6)
50 – 54	1	0	0	1 (3.6)	0	0	0	0	1 (3.6)
55 – 59	1	0	0	0	1(3.6)	0	0	0	1 (3.6)
Total	28	0	12 (42.8)	3(10.7)	5(17.8)	2(10.0)	4(14.3)	1(3.6)	28(100)

Legend: *S*.T= *Salmonella* Typhi, *E. c*.= *Escherichia coli*, *Cit*. sp = *Citrobacter* sp, *K*. sp = *Klebsiella* sp, *P. mi*.= *Proteus mirabilis*, *P. vu*.= *Proteus vulgaris*, *P. aer*. = *Pseudomonas aeruginosa.*

With the *Salmonella* Typhi DNA positive control, demonstrated by 395bp, no *Salmonella* Typhi DNA fragment from any peritoneal fluid samples was neither detected while PCR process nor revealed by electrophoretic migration on agarose gel.

IV. DISCUSSION

71.4% of the patients admitted for ileal perforation of typhic origin were male and the first 4 age groups were the most affected, with 71.5%, of which more than half were male, i.e. 46.3% (Table 1).

A study has demonstrated and attributed high rates of the typhic infection more to males than to females because of their behavior of getting diner outside of the own house. The young boys also, spend a lot time outside more than girls for leisure activities, where they do not miss to be drinking or eating what they do find (1).

In their study led on *"Salmonella* Typhi in the Democratic Republic of the Congo: Fluoroquinolone Decreased Susceptibility on the Rise" Lunguya *et al*, have reported the prevalence of the *Salmonella* Typhi infection among the teenage and young adults. On top of that, the same author, has found the rate of 32.8% of children under 10 years old who were infected by *Salmonella* Typhi (13).

Salmonella Typhi infection appears to prevail among school-aged children and young adults. (14).

Bacterial cultures, performed on 28 peritoneal fluid samples at INRB and repeated at CMR-KEMRI laboratories, gave the similar results, no *Salmonella* strain was isolated as bacterial cell apart from some enterobacteria mentioned (table 2).

In Vietnam, from April 1997 to February 1998, Chanh*et al,* isolated 3 strains of *Salmonella* Typhi from 27 peritoneal fluid samples from admitted and operated patients following suspected intestinal typhoid perforation, at the Dong Thap Provincial Hospital. The bacteriological culture performed in the secretions collected at the sites of perforation has also allowed the isolation of 3 other strains of *Salmonella* Typhi in these patients (15).

In this study, although many peritoneal fluid samples were taken from Selenite F broth, they were not delivered in time to the laboratory. Not only all the hospitals that performed laparotomies with intestinal perforation indications and where our samples came from were far from the INRB, but most of these surgeries were done late at night.

Two reasons are advanced to explain the late treatment of these patients. Firstly, it is a technical reason, laparotomy is a septic surgery that is often performed, last after all interventions, essentially, aseptic. Secondly, it is in general, a disease of the poorest who have no means to pay their bills care in time to be scheduled for surgery. Thus, they arrive to be operated, by duty of conscience, only when, sometimes their general condition becomes more and more worrying (5, 6 and 16).

After running PCR and electrophoretic migration on agarose gel, no *Salmonella* DNA fragment was neither detected after PCR running nor revealed by electrophoretic migration on agarose gel.

In their study, Chanh *et al*, have detected, on the other hand, the *Salmonella* Typhi DNA fragment by PCR on all the 25 biopsy samples (100%) of the suspected patients with the intestinal perforation due to typhoid fever.

The *Salmonella* Typhi DNA fragment detection is more sensitive in the biopsy of the perforation sites according to the Chanh's study than in the peritoneal fluid of this current experiment (15).

Some hypotheses which may justify the result of this current experiment:

- The peritoneal fluid may have diluted the *Salmonella* Typhi strains and decrease their isolation probability.
- The concentration of the *Salmonella* Typhi DNA molecule, would be very weak and highly diluted in peritoneal fluid, so the detection was difficult.

V. CONCLUSION

This study has shown, like many others that young boys and men are much more affected by typhoid fever and especially, its greater consequence is peritonitis on intestinal perforation.

The irregularities experienced, even during the surgical treatment of the patients in relation to this pathology, affected the quality of the samples of the study and to this was added the late delivery of the latter to the laboratory. At the end of the analyzes of the bacteriological cultures and molecular tests by PCR of the peritoneal fluid, no strain of *Salmonella* Typhi was not isolated there nor its DNA fragment either, was not detected there.
In our context, in order to bring surgical considerations closer to these intestinal lesions and biological approaches in establishing formal evidence with the isolation of *Salmonella* Typhi, further studies are envisaged while reducing the irregularities inherent in sampling conditions.
Other experiments are recommended to increase the chance of the detection of the *Salmonella* Typhi DNA fragment by PCR technique in the biopsy of the perforation sites tissue. Typhoid isolates from the lesions associated with intestinal perforations are of great interest for the monitoring of microbial resistance.

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