Single Nucleotides Polymorphism analysis of *Salmonella typhi* isolated from Egypt

Soliman, Y. A.[1]; Karam, M.A.[2] and Seadawy, M.G.[3]

[1]Central Laboratory for Evaluation of Veterinary Biologics, Abassia, Cairo, Egypt
[2]Deparment of Botany Faculty of Science, Fayoum University, Egypt
[3]Main laboratories, chemical warfare, Egyptian army force, Egypt
dryousefadel@gmail.com, biologist202054@yahoo.com

Abstract: Meat and meat byproducts were taken from various areas in Egypt (Cairo, Sharqia, Mansoura, Ismailia, Fayoum and Suez). The samples (*n=200*) were subjected to bacteriological isolation of salmonella spp. Fifteen samples gave a suspected growth on the selective agar media. After biochemical isolation they were identified as salmonella spp the total 15 isolates were sampled from Cairo (4 positive samples), Mansoura (2 positive samples), Ismailia (2 positive samples), Sharqia (2 positive samples) Fayoum (2 positive samples) and finally Suez governorate (3 positive samples). Two of these samples were identified as *S. typhi* (isolated from Suez governorate) and one identified as ***S. paratyphi*** (isolated from Fayoum governorate). All the samples were further identified by serotyping and the results were all ***S. typhimurium*** except the two strains which were identified by biochemical reaction as ***S. typhi*** and ***S. paratyphi*** that gave the same results by serotyping. The isolated strains were further identified by traditional real time PCR or the portable identification system (R.A.P.I.D). All samples gave a positive Ct. Whole genome sequencing using ion torrent PGM The developed contigs were assembled and 4.1 mega bases have been developed when single nucleotide polymorphism analysis was conducted, about 9401 SNPS have been found.

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1. Introduction:

Salmonellosis is an infectious disease of human and animals caused ***Salmonella enterica*** and ***S. bongori***. Although primarily intestinal bacteria, ***Salmonella*** present in the environment and may commonly be found in farm effluents, human sewage and in any material subject to fecal contamination (***CDC, 2013***). Salmonellosis has been recognized in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs (***Winfield and Eduardo 2003***). Invasive disease due to serovar *Typhi* as well as Nontyphoidal ***salmonella***e (NTS) is common in children younger than 5 years old in developing countries, and multidrug resistance is an increasingly difficult problem to manage (***Graham, 2002***). ***Salmonella***are non-spore-forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella that are all around the cell body (***Fabrega and Vila, 2013***)

The genome of ***Salmonella revealed*** that, it consists of one the chromosome sequence of 4,809,037 bp (about 4.8M bp) in length (***Deng et al., 2003***) with a GC content of 52.09%. The chromosome was sequenced by shotgun method with 97,000 shotgun reads.

In order to in-depth understanding of the behavior of ***Salmonella*** spp in diseases and food intoxication, the current study aimed to analysis of the genetic material of the isolated ***Salmonella*** from different sources (Meat and meat byproducts) and different governorates and the whole genome sequencing was performed to study the single nucleotide polymorphism (SNPs).

2. Material and Methods:

Samples:

Meat and meat byproducts were (*n=200*) taken from various areas in Egypt (Cairo, Sharqia, Mansoura, Ismailia, Fayoum and Suez) and transferred on ice. The samples were collected during 2012-2013.

Bacterial isolation:

It was done according to ISO 6579 fourth edition 2002(E) and ***Davies et al., 2000***

The meet samples were sliced off with 0.1cm thickness. The meet byproducts were placed in a sterile stomacher bags containing 10 mL of buffered peptone water. The bags placed in the stomacher and subjected to homogenization for at least 10 min at high speed. The media was then transferred to a sterile 50 mL centrifuge tubes and incubated at 37°C/18 h ± 2 h then 0.1mL of the nonselective buffered peptone was transferred to 10mL of the RVS media and incubated at 41.5 °C ± 1 °C for 24 h ± 3 h. Other samples were platted directly onto the selective

agar media without the preselected enrichment. The incubated RVS media was platted out onto 15mmØ petri-dishes containing the XLD agar media and incubation was done at 37°C/24h. The suspected colonies were then plated out on XLD and Hektoen enteric agar for further selection and propagation of the isolated strain.

Identification of the isolated strains:

1. Biochemical identification:

It was done using Vitek II automated system with the manufacture instruction

2. Serotyping:

It was performed in the central laboratories of healthy ministry. Using serials of monoclonal antibodies against different salmonella spp

3. Identification by Real time qPCR:

Isolates grown onto Hekton enteric agar were collected in 1mL TE buffer pH8.0 and washed twice with cooled TE buffer. The bacterial pellets were subjected enzymatic digestion using 180 µl of enzymatic lysis buffer and incubation takes place at 37°C for 30 min. then the genomic DNA was extracted using QIAamp DNA Mini Kits (Qiagen cat # 51304) according to the manufacture instruction. The eluted DNA was electrophoresed on 1% agarose to check for its purity and then quantified using nanodrop (nanodrop8000, USA) stored at stored at -20°C till used. Two µL of the eluted DNA was mixed with 12.5 µL of Brilliant II QPCR master mix (Agilent cat # #600806) and 100nM of each of the sense primer (GCGTTCTGAACCTTTGGTAATAA) and antisense primer (CGTTCGGGCAATTCGTTA) and 200 nM of the FAM-TAMRA labeled probe (TGGCGGTGGGTTTTGTTGTCTTCT) these primers amplify a 150bp stretch of the *invA* gene of Salmonella spp. The primers were designed using CLC main workbench V6.7.1. the reaction was adjusted at 95°C/10 min for initial denature and 40 cycles of denature at 95°C/20 sec, annealing at 58°C/30 sec and extension at72°C/30 sec using Agilent MX3005P.

4. Identification with R.A.P.I.D:

A single colony plated onto Hekton agar media was picked off using the IT swab provided with the kit (R.A.P.I.D. Salmonella ID kit cat # 3826) and placed in the small bead tubes for bacterial cell wall disrupter using the mechanical agitation force of the vortex Genie with turbo Mix for 5 minutes. The released nucleic acid was mixed with 450 µl Buffer1 (Binding Buffer) and transferred to Small Spin column and spanned for 2 minutes at 7200Xg. the column was washed with 450 µl Buffer 2 (Wash Buffer) and Centrifuged/ 2 minutes at 7200 Xg. After brief centrifugation to remove the residual washing buffer, the nucleic acid was eluted using 200µl of elution buffer and centrifuged at 7200Xg/2min and stored at -20°C till used. A regent vial (contains all the master mix with primers and probes) was rehydrated with 20µl of 2X reconstitution buffer, and 20 µl of the eluted DNA was added. 19 µl of this mix was transferred to the capillary PCR tubes. The positive and negative controls provided with the kit were freshly rehydrated and 19µl were transferred to another 2 capillaries. The capillary tubes were briefly centrifuged to remove any air bubbles and placed in order in the RAPD PCR machine. The preinstalled program for salmonella was used to perform the reaction.

Whole genome sequencing:

It was done using Ion torrent PGM instrument according to the manufacture instruction. Briefly, the DNA was fragmented using The Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269) and then purified using Agencourt AMPureR XP Kit (Beckman Coulter A63880). The Preparation of template positive ion sphere particles and purification of the positive particles was performed using The Ion OneTouch™ Template Kit (Cat. no. 4468660) Dynabeads® MyOne™ Streptavidin Beads (Life Technologies cat # 650.01) respectively. The template positive ion sphere particles were subjected to sequencing using The Ion Sequencing Kit (life technologies cat #. 4468997) and Ion 314™ Chip Kit (Life Technologies cat # 4462923).

Analysis:

The Ion PGM sequencer was run automatically and the collected data through the server was transferred to DNASTAR V10 software to process the data. The genome assembly and Nucleotidesequence analyses and SNP were conducted with the LaserGenesequence analysis software package (LaserGene, version 10; DNAstar, Inc.).

3. Results:

Bacterial isolation:

Due to the presence of Salmonella in low concentration in the samples, the enrichment step is very essential; the enrichment process must not exceed 18 hours. The samples were then transferred to either Hektoen agar directly or to the RVS media and incubated at higher temperature as a method of selective enrichment of the Salmonella spp. Then the samples were plated out onto a ***Salmonella*** selective agar plates (either XLD or Hektoen agar media). Out of 200 meet and meet byproduct samples tested in the current study, fifteen samples were positive onto these selective media (7.5%) and were subjected to further analysis. One sample gave positive colonies when directly plated onto Hektoen agar media and were small black centered one. Another sample gave clear while colored colonies when plated directly onto XLD media these two samples were carefully sub cultivated

again onto XLD media. All samples were and subjected to the biochemical identification and Serotyping procedures.

4.2. Biochemical identification:

Vitek II analysis of the isolated strains revealed that all were identified as *Salmonella* spp. except two isolates which identified as *Salmonella enterica serovar typhi* that was isolated from Fayoum governorate and *S. enterica serovar paratyphi* that was isolated from Suez governorate. The total 15 isolates were sampled from Cairo (4 positive samples), Mansoura (2 positive samples), Ismailia (2 positive samples), Sharqia (2 positive samples) Fayoum (2 positive samples) and finally Suez governorate (3 positive samples)

4.3. Serological identification:

The Serotyping of the 15 isolates (table 1) revealed that those which identified as *S. entericaserovartyphi* and *S. enterica serovar paratyphi* were confirmed by serology where the others were typed *S. enterica serovar typhimurium*.

Real time qRT-PCR

The *inv*A gene was is present nearly in all *Salmonella* spp. and it was used as a target for the identification of the isolated strains. All the 15 isolates gave positive amplicon (Fig 1) confirming both the biochemical and serological identification of the strains.

R.A.P.I.D. PCR

RAPD PCR is a rapid identification system developed by Roch scientific. It utilizes a premade blind of primers and probe for identification of Salmonella spp. From purified colonies just in 30 min. on applying such technique with our isolates, all strains gave positive results which confirming that all the isolates are Salmonella spp (fig. 2).

Table (1) The serotyping of the *Salmonella* isolates

Isolates number	Governorate	Serotyping
1	Suez	*S. paraTyphi*
2	Suez	*S. entericaserovar Typhimuruim*
3.	Suez	*S. entericaserovar Typhimuruim*
4	Fayoum	*S. entericaserovar Typhi*
5	Fayoum	*S. entericaserovar Typhimuruim*
6	Sharqia	*S. entericaserovar Typhimuruim*
7	Sharqia	*S. entericaserovar Typhimuruim*
8	Ismailia	*S. entericaserovar Typhimuruim*
9	Ismailia	*S. entericaserovar Typhimuruim*
10	Mansoura	*S. entericaserovar Typhimuruim*
11	Mansoura	*S. entericaserovar Typhimuruim*
12	Cairo	*S. entericaserovar Typhimuruim*
13.	Cairo	*S. entericaserovar Typhimuruim*
14	Cairo	*S. entericaserovar Typhimuruim*
15	Cairo	*S. entericaserovar Typhimuruim*

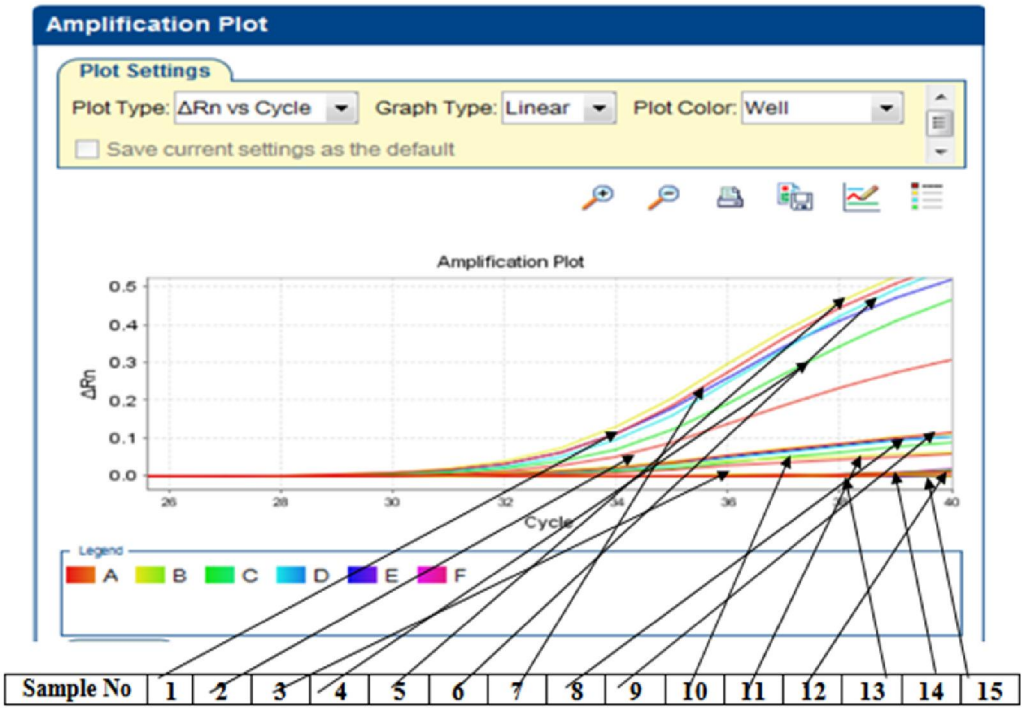

Fig (1) The amplification plot of the 15 *Salmonella*spp isolates

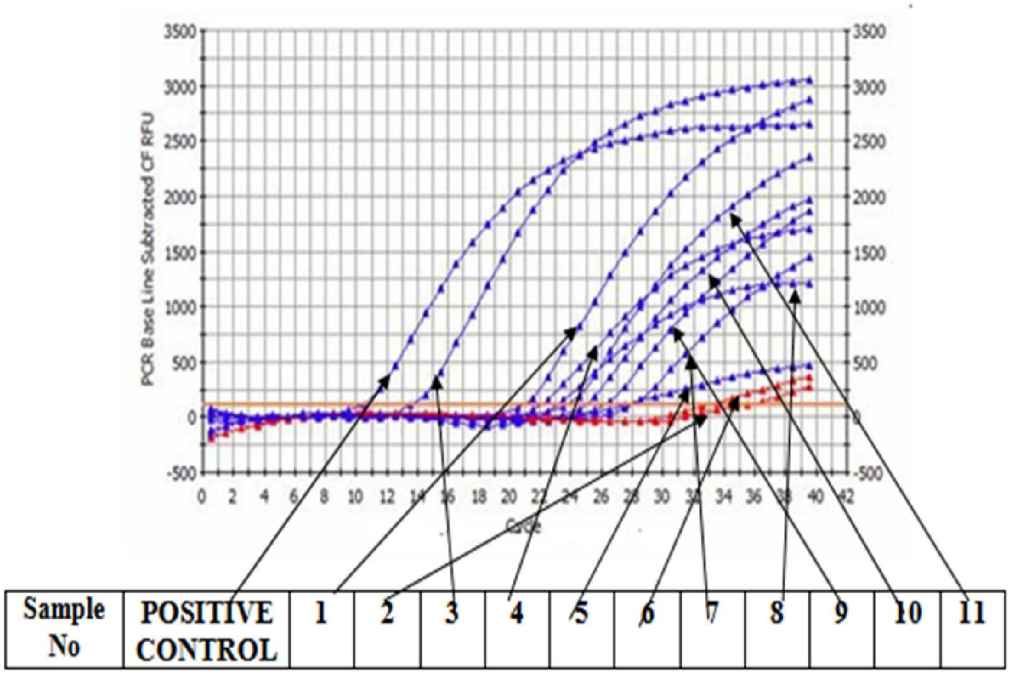

Fig (2) The amplification plot of the 15 Salmonella spp isolates using R.A.P.I.D technique.

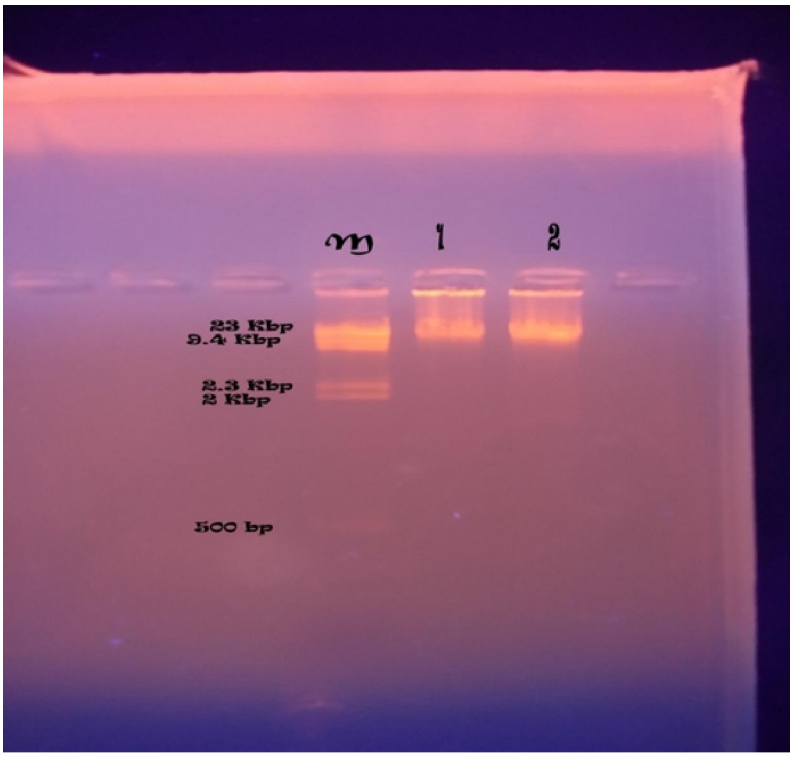

Fig (3) the purified genomic DNA from *S. typhi* (5µl and 10 µl lane 1and 2 respectively)

Table (2) example of *S. typhi YA strain* SNPs found on isolates in the current study

R_2013_04_23_10_40_26_ARMYCHEMICALWAR_SN1-3_yousef-adel_4 (single) (Reads) - locally realigned-1

Reference position	Consensus position	Consensus residue	Other residues
2170	330	T	Reference: C. Reads: T (1)
8751	562	T	Reference: C. Reads: T (1)
8759	570	T	Reference: C. Reads: T (1)
8804	615	T	Reference: C. Reads: - (1), T (1)
8807	618	T	Reference: G. Reads: T (2)
24843	810	T	Reference: C. Reads: T (1)
25569	863	T	Reference: C. Reads: T (1)
25579	873	T	Reference: G. Reads: T (1)
38235^38236	1280	T	Reference: -. Reads: T (1)
45025^45026	1618	T	Reference: -. Reads: T (1)
45052	1645	T	Reference: C. Reads: T (1)
45062^45063	1656	T	Reference: -. Reads: T (1)
50988	1873	T	Reference: C. Reads: T (1)
66848	2202	T	Reference: A. Reads: T (1)
70543^70544	2410	T	Reference: -. Reads: T (1)
77922	2749	T	Reference: C. Reads: T (1)
77928	2755	T	Reference: G. Reads: T (1)
77932	2758	T	Reference: A. Reads: T (1)
92878	3489	T	Reference: G. Reads: T (1)
114897	4204	T	Reference: C. Reads: T (1)
114909	4215	T	Reference: G. Reads: T (1)
129766	4782	T	Reference: G. Reads: T (1)
129771^129772	4788	T	Reference: -. Reads: T (1)
129782	4798	T	Reference: C. Reads: T (1)
129793^129794	4810	T	Reference: -. Reads: T (1)
129802^129803	4823	T	Reference: -. Reads: T (1)

Reference position	Consensus position	Consensus residue	Other residues
840465	32556	T	Reference: C. Reads: T (1)
840929	32625	T	Reference: A. Reads: T (1)
848454	32921	T	Reference: A. Reads: - (1), T (1)
851569^851570	32957	T	Reference: -. Reads: T (1)
852538	33058	T	Reference: C. Reads: T (1)
854540	33156	T	Reference: A. Reads: T (1)
854695	33158	T	Reference: G. Reads: T (1)
857429	33262	T	Reference: T. Reads: - (1), T (1)
871138	33793	T	Reference: A. Reads: T (1)
871276	33826	T	Reference: C. Reads: T (1)
871291	33841	T	Reference: C. Reads: T (1)
873102	33879	T	Reference: C. Reads: T (1)
873105	33882	T	Reference: C. Reads: T (1)
878493^878494	34073	T	Reference: -. Reads: T (1)
878493^878494	34074	T	Reference: -. Reads: T (1)
878493^878494	34075	T	Reference: -. Reads: T (1)
905330	34837	T	Reference: C. Reads: T (1)
905356	34863	T	Reference: G. Reads: T (1)
905387	34897	T	Reference: C. Reads: T (1)
905393	34903	T	Reference: C. Reads: T (1)
905423	34929	T	Reference: C. Reads: T (1)
905426	34932	T	Reference: T. Reads: - (1), T (1)
905464	34970	T	Reference: T. Reads: - (1), T (1)
917825	35356	T	Reference: C. Reads: T (1)
917840	35370	T	Reference: C. Reads: T (1)
918552	35400	T	Reference: A. Reads: T (1)
921874	35488	T	Reference: C. Reads: T (1)
925209	35563	T	Reference: G. Reads: T (1)
925243	35596	T	Reference: C. Reads: T (1)
928545	35692	T	Reference: C. Reads: T (1)
936674^936675	36093	T	Reference: -. Reads: T (1)

Reference position	Consensus position	Consensus residue	Other residues
731202^731203	27541	T	Reference: -. Reads: T (1)
731218	27557	T	Reference: C. Reads: T (1)
731223^731224	27563	T	Reference: -. Reads: T (1)
731228	27568	T	Reference: C. Reads: T (1)
731311	27594	T	Reference: C. Reads: T (1)
732441	27760	T	Reference: G. Reads: T (1)
733134	27869	T	Reference: C. Reads: T (1)
745577^745578	28412	T	Reference: -. Reads: T (1)
746904	28484	T	Reference: C. Reads: T (1)
753234^753235	28649	T	Reference: -. Reads: T (1)
759263^759264	29107	T	Reference: -. Reads: T (1)
759280	29122	T	Reference: C. Reads: T (1)
764916^764917	29404	T	Reference: -. Reads: T (1)
767692	29658	T	Reference: G. Reads: T (1)
773101	29844	T	Reference: C. Reads: T (1)
775347	29951	T	Reference: A. Reads: T (1)
775349	29953	T	Reference: G. Reads: T (1)
776540	30048	T	Reference: G. Reads: T (1)
778036	30092	T	Reference: C. Reads: T (1)
780077^780078	30258	T	Reference: -. Reads: T (1)
782139^782140	30371	T	Reference: -. Reads: T (1)
783312^783313	30432	T	Reference: -. Reads: T (1)
783353	30440	T	Reference: G. Reads: T (1)
794038	30778	T	Reference: C. Reads: T (1)
794119	30855	T	Reference: A. Reads: T (1)
798936	30980	T	Reference: C. Reads: T (1)
809120	31374	T	Reference: C. Reads: T (1)
813213	31480	T	Reference: C. Reads: T (1)
817259	31602	T	Reference: G. Reads: T (1)
826468	31842	T	Reference: A. Reads: T (1)
826534^826535	31908	T	Reference: -. Reads: T (1)

Reference position	Consensus position	Consensus residue	Other residues
319667^319668	12395	T	Reference: -. Reads: - (1), T (1)
319692	12421	T	Reference: A. Reads: T (2)
327601	12597	T	Reference: G. Reads: T (1)
341103^341104	12755	T	Reference: -. Reads: T (1)
345489	12857	T	Reference: C. Reads: T (1)
366483^366484	13349	T	Reference: -. Reads: T (1)
366536^366537	13365	T	Reference: -. Reads: T (1)
366536^366537	13366	T	Reference: -. Reads: T (1)
366537	13367	T	Reference: C. Reads: T (1)
369253	13619	T	Reference: C. Reads: T (1)
370294^370295	13690	T	Reference: -. Reads: T (1)
377791^377792	13861	T	Reference: -. Reads: T (1)
377812	13882	T	Reference: C. Reads: T (1)
379777	13923	T	Reference: G. Reads: T (1)
380871	13968	T	Reference: G. Reads: T (1)
384690	14219	T	Reference: C. Reads: T (1)
390086	14555	T	Reference: A. Reads: T (1)
391486^391487	14693	T	Reference: -. Reads: T (1)
391492	14699	T	Reference: C. Reads: T (1)
393674^393675	14805	T	Reference: -. Reads: T (1)
393718	14850	T	Reference: C. Reads: T (1)
393720	14852	T	Reference: A. Reads: T (1)
393730	14862	T	Reference: C. Reads: T (1)
393734^393735	14867	T	Reference: -. Reads: T (1)
393735	14868	T	Reference: C. Reads: T (1)
399259^399260	15259	T	Reference: -. Reads: T (1)
399268^399269	15269	T	Reference: -. Reads: T (1)
404070	15457	T	Reference: G. Reads: T (1)
404903	15496	T	Reference: C. Reads: T (1)
407125	15668	T	Reference: C. Reads: T (1)
407165	15708	T	Reference: C. Reads: T (1)

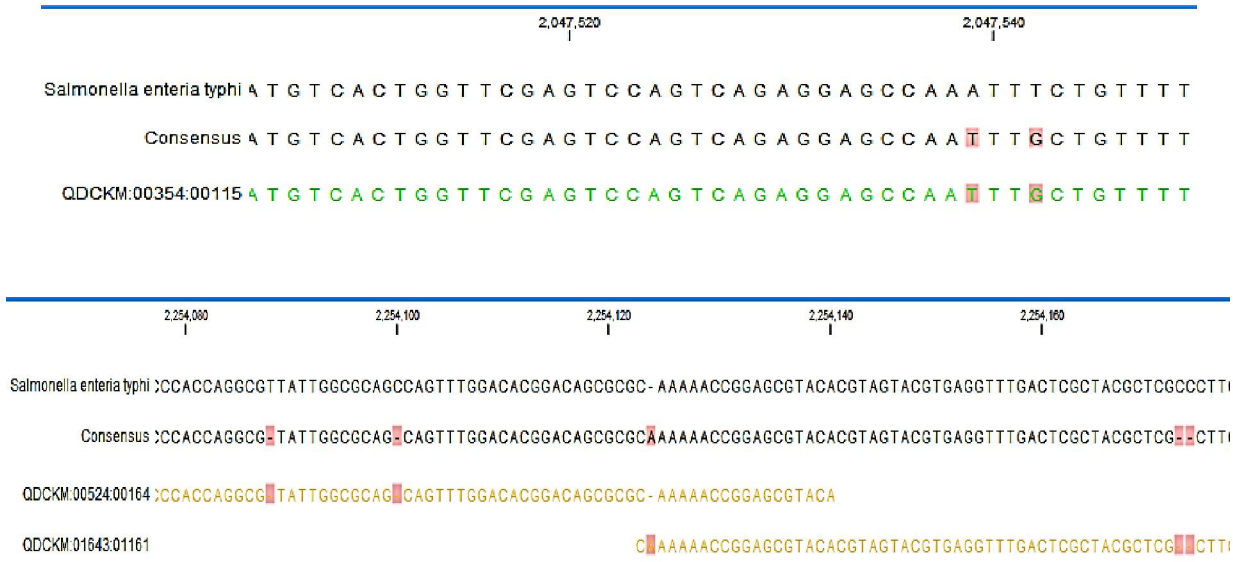

Fig (4) Example of the SNPs found on alignment of the *S. typhi* isolated in the current study with the published sequence in the gene bank accession number AE014613.1

Whole genome sequencing

The whole genome of the *S. entericserovar Typhi* (designed as YA isolate) was sequenced using ion torrent PGM platform. The sequence contigs were assembled in reference to the published sequence of *S. entericaserovar Typhi* in the gene bank data base accession number AE014613.1.

First the genomic DNA was purified and electrophoresed to chick the purity of the DNA and the absence of RNA contaminant. As in fig 3, a single band representing the genomic DNA is only seen. The genomic DNA was then fragmented and ligated to the adaptors then quantified using absolute quantification procedure. The relative quantity showed that on using 10 µl containing 100 Nano gram of gDNA, the library concentration was 0.96 while using 50 Nano grams of the gDNA yielded only 0.2.

The developed contigs were assembled and 4.1 mega bases have been developed when single nucleotide polymorphism analysis was conducted, about 9401 SNPS have been found. (Table 2 and fig 4 as examples).

4. Discussion:

Salmonellosis is one of the most common and widely distributed foodborne diseases and is caused by the bacteria ***Salmonella***. It is estimated that tens of millions of human cases occur worldwide every year and the disease results in more than hundred thousand deaths. For ***Salmonella*** species, over 2500 different strains (serovars) have been identified to date. ***Salmonella*** is a ubiquitous and hardy bacterium that can survive several weeks in a dry environment and several months in water.

First the samples were handled aseptically to prevent external contamination and processed as soon as possible (within 6 hours of samples). The bacteriological examination follows 3 steps for isolation and purification of the *salmonella* isolates according to the ISO-6579 standard manual.

To diminish the risk of obtaining false negative results, a non-selective pre-enrichment of the sample in buffered peptone water was first adopted then; a combination of selective enrichments (Rappaport Vassiliadis soy peptone (RVS) broth) and plating on two selective media XLD and Hektoen agar media was performed. Subsequent cultivation of the purified colonies was performed onto Hektoen agar media.

Out of 200 processed samples, 15 positive suspected *salmonella* spp was obtained with incidence of 7.5%. The colonies were small black centered colonies with green media beneath (due change in the pH to the alkaline side) on Hektoen, while on XLD the colonies were small black centered except on sample which gave a white colonies which was suspected as *S. Typhi*. This isolated was not selectively pre-enriched as it was plated directly from the buffered peptone media.

Biochemical identification was performed using Vitec II instrument utilizing the gram negative detection cards. All the isolated strains confirmed to be *salmonella* spp. Without identification to the genus level except two strains which were originally grown when cultivated directly onto the selective agar media

without the selective pre-enrichment on RVS. One isolate confirmed to be *S. Typhi* and the other S. para*typhi*. The strain *S. paratyphi* was originally isolated from a meet byproduct from Suez governorate, although this strain is not common food intoxication *salmonella*e **(*Anne 2014)***, yet contamination of the meet with underground water that is near the water sewages could explain how this strain could get in contact with the sample. Another explanation is the food contamination by the personal that might be recovers from an infection with paratyphoid fever **(*Jeremy 2012*)**. For the ***S. Typhi***, it was isolated from meet byproduct sample from Fayoum governorate. The source of the contamination might be the same as in case of ***S. paratyphi*** especially hospitals receive sporadic cases of typhoid fever every summer (personal communication).

The serotyping of the purified isolates was used for further characterization. A panel of monoclonal antibodies was used to identify the isolates to the genus level, a part from the ***Typhi*** and ***Paratyphi*** (which also confirmed by serotyping) all the other isolates confirmed to be ***S. Typhimurium***, a commonly ***salmonella*** spp that is found in the cattle (the main source of the meat and meat byproduct in Egypt). The infection in cattle can be largely symptomatic or mild symptoms such as diarrhea with slight fever and usually the cases are sporadic and treated largely with antibiotic. In such case some infected cattle that might not respond to the treatment or when inadequate regimen was adopted yielded colonization of the ***salmonella*** in the liver or enteric part of the animal and might be the cause of the contamination of the meat and its byproducts (***Peterson & Coon 1967, Bairey 1978,* and *Robertsson et al., 1983).***

The isolated strains were further identified on the genomic by polymerase chain reaction. Two techniques were adopted in the current study. The first technique is the R.A.P.I.D. which is fast and portable system for rapid identification of bacteria developed by Roch. The system composed of ready to use extraction system from a swap of the isolate from the agar media and the extracted DNA subjected to real time amplification using ***salmonella*** specific primer and probe in comparison of control positive sample. All the tested isolates where positive and gave amplification plot as early as Ct_{13}. Second, the isolates were identified by traditional real time PCR using primers and probe targeting the *invA* gene., *invA* gene has been characterized that allow along with other genes (*invB, C, D*) ***Salmonella*** to enter cultured epithelial cells ***(Rahn et al., 1992)***. The use of these genes as a potential target for ***Salmonella*** identification has recently been suggested since these genes were shown to be present in a number of *Salmonella* strains. All samples also gave positive results, although there was variability in the concentration of the amplicons (as measured by Ct) yet all were positive.

The *Salmonella typhi* was then subjected to whole genome sequencing using ion torrent PGM platform. Sequencing analysis revealed the presence of 4.1mega base when assembled against reference sequence (gene bank accession number AE014613.1). The genome sequencing of ***S. Typhi*** allows study the genomes of individual species, subspecies, serovars, and even different isolates within the same serovar (***Parkhill et al., 2001and Deng et al., 2003***) Thus, one can examine the genetic blueprint of such bacteria and make simple comparisons with the genomes of bacteria that share certain phenotypic characteristics (such as host restriction or biotype). It might also be possible to compare the genome of ***S. Typhi*** with the genomes of other bacteria that are limited in their pathogenic potential for particular hosts (i.e., host promiscuity or restriction). In the current study, only the SNPs have been characterized, it was found that 9401 SPNs ranges from *indel* or base substitutions. These SNPs was found to be distributed all over the genome.

The outcome of such investigation will enables the scientists in Egypt to understand and develop a genetic marker for molecular epidemiological surveillance and to direct the attentions towards the different kinds of toxins produced by such strain.

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