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A Single Nucleotide Polymorphism on *rpoB* Gene Allows Specific Identification of *Salmonella enterica* Serotype Typhimurium

K. Hernández Guijarro, S.E. Feingold and H.R. Terzolo
Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agronómica Balcarce, Ruta Nac. 226, km 73,5. Balcarce, Buenos Aires, Argentina

Corresponding Author: Keren Hernández Guijarro, Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Balcarce, CC 276, Balcarce, Buenos Aires, Argentina Tel: +54 2266 439100 Fax: +54 2266 439101

ABSTRACT
The aim of the study was to develop a specific test for the detection of *Salmonella enterica* serotype Typhimurium based on *rpo*B gene sequence. A 622 bp *rpo*B gene fragment was amplified and sequenced from 105 bacterial strains, comprising 26 different *Salmonella* serovars and other Enterobacteriaceae members isolated from diverse sources. Comparison of in house generated sequences with other public bacterial *rpo*B sequences allowed the identification of a specific Single Nucleotide Polymorphism (SNP) for *Salmonella* Typhimurium. A molecular test based on this SNP using *Taq*Man® real-time PCR was designed and validated. This test showed 100% specificity and all *Salmonella* Typhimurium strains assayed were identified correctly. The designed test specifically identifies *Salmonella* Typhimurium serovar based on a SNP on *rpo*B gene. This test is rapid, sensitive and reliable and it is a useful additional tool for veterinarian and heath care professionals in general.

Key words: *Salmonella* Typhimurium, *rpo*B gene, single nucleotide polymorphism, strain identification, food poisoning

INTRODUCTION
Salmonella enterica subspecies *enterica* serotype Typhimurium is a ubiquitous salmonella that causes gastroenteritis and sometimes septicemia in a broad range of unrelated animal species. This non-typhoid *Salmonella* serovar also causes disease in humans, generally related to food poisoning. In farm animals, it can reduce productivity and can also lead to mortality. Furthermore, asymptomatic carrier animals constitute a serious epidemiological risk due to contamination during processing of food products, in particular the ones derived from meat and eggs (Humphrey, 2006).

The identification of *Salmonella* Typhimurium, as well as other serotypes of the genus *Salmonella*, is a very complex task. Conventional methods often require the use of selective enriched culture media and subcultures on agar plates followed by biochemical and serological tests. Species and subspecies are phenotypically classified by using different growth media and biochemical tests, while serotyping using the Kauffmann-White scheme (Kauffmann, 1972) remains the standard for serotype determination.

Several molecular methods have been proposed as alternatives to improve both specificity and rapidness of results. Conventional and real-time PCR based methods, mono or multiplex, have been reported for the identification *Salmonella* serovars. Different genetic regions encoding for

pathogenicity, invasiveness and antigenic properties have been assayed to detect *Salmonella* Typhimurium by means of molecular techniques (Bennasar *et al*., 2000; Lim *et al*., 2003). On the other hand, the increasing availability of sequenced bacterial genomes allows the identification of specific regions of *Salmonella* serovars based on comparative genomics. Kim *et al*. (2006b) used this methodology for identifying *Salmonella* Typhimurium using the genome of *Salmonella* Typhimurium LT2 in comparative genomic with the available genomes of other *Salmonella* serovars published at that date. Concordantly, some other studies have reported different genetic regions for detection both of *Salmonella* Typhimurium and other serotypes, as well as specific genetic regions of species, serogroups and serotypes of *Salmonella* (Kim *et al*., 2006a, c; Mccarthy *et al*., 2009; Chen *et al*., 2010; Liu *et al*., 2011).

In this study, a novel target sequence located at *rpo*B gene, is proposed for detecting *Salmonella* Typhimurium serotype based on a Single Nucleotide Polymorphism (SNP). *rpo*B is a single-copy chromosomal gene encoding the RNA polymerase β-subunit. This gene has been previously used in phylogenetic analysis for bacteria species and genus delineation (Mollet *et al*., 1997), since it is highly conserved across organisms. Based on this, and since SNPs are the most prevalent form of genetic variation, the discovered SNP could constitute a useful tool for universal bacterial identification (Hajibabaei *et al*., 2007).

In this study, the specificity, selectivity and sensitivity of the rpoB SNP-based assay was evaluated by testing Salmonella serovars isolated from different animal and food sources.

MATERIALS AND METHODS

Bacterial strains and Genomic DNA extraction: A total of 105 strains comprising 26 different *Salmonella enterica* serotypes and strains from *Escherichia*, *Shigella*, *Proteus*, *Klebsiella*, *Enterobacter* and *Citrobacter* genera were studied (Table 1). *Salmonella* Typhimurium strains included the ATCC 6994 strain and other 21 field strains isolated in Argentina from 1974 to 2010, deposited in the Bacterial Collection of INTA EEA Balcarce. Serotypes *Salmonella* Aberdeen, *Salmonella* California *Salmonella* Derby, *S*. Kentucky, *Salmonella* Paratyphi C and *Salmonella* Virchow, were kindly provided by Dr. Maria Ines Caffer from The Enterobacteriaceae Service, National Institute of Infectious Diseases (ANLIS- INEI) “Dr. Carlos G. Malbran”.

Salmonella strains were serotyped according to the Kauffmann-White typing scheme (Kauffmann, 1972). DNA isolation was performed from cultures grown overnight at 37°C in Luria Bertani broth (LB). DNA was extracted using Wizard Genomic DNA Purification kit (Promega, USA) according to the manufacturer’s instructions. The DNA concentration and A260/A280 ratios were determined spectrophotometrically.

Construction of rpoB primers: All strains of *Salmonella* Typhimurium (32), *S*. Enteritidis (13) and *Salmonella* Gallinarum (17) mentioned above and one strain from the rest of *Salmonella* serotypes and Enterobacteriaceae members, listed in Table 1, were amplified by PCR and sequenced for a 622 bp region of *rpo*B gene, using the following forward (5’-GTCAGATCCGTGGCGTGACC; corresponding to *Salmonella* Typhimurium position 254) and reverse (5’-TGTACTCAACCGGA ACTTCG; corresponding to *Salmonella* Typhimurium position 855) primers. Primers were designed by using Fast PCR program (Kalendar *et al*., 2009). Standard PCR reactions were performed with a touchdown profile with annealing temperature from 63-57°C with 0.5°C decrease per cycle.

Table 1: Bacterial strains and values of delta Rn obtained from *Taq*Man® genotyping analysis

			Real Time-PCR	
Serotypes	No. of strains	Source	ΔRn STspc probe	ΔRn STnonspc probe
Salmonella Typhimurium ATCC 6994		Chicken	3.687	0.845
Salmonella Typhimurium	25	Chicken	3,486*	0,821*
	2	Bovine	3,600*	0,896*
	1	Sheep	3.349	0.965
	1	Otter	3.416	0.831
	1	Pork	3.083	0.790
	1	Pork sausages	3.277	0.954
	1	Hen	3.192	0.797
Salmonella Enteritidis PT1	1	Chicken	0.763	1.371
Salmonella Enteritidis PT4	1	Chicken	0.641	1.587
Salmonella Enteritidis PT34	1	Chicken	0.854	1.354
Salmonella Enteritidis	1	Chicken	0,767	1,591
	5	Pork	0,769*	1,665*
	2	Animal food	0,794*	1,557*
	1	Pork sausages	0.733	1.738
	1	Soybean meal	0.794	1.493
Salmonella Gallinarum	17	Chicken	0.856*	1.836*
Salmonella Senftenberg	1	Chicken	0.759	1.543
Salmonella Muenchen	1	Chicken	0.873	1.871
Salmonella Dublin	3	Bovine	0.854*	1.673*
Salmonella Panama	1	Pork	0.863	1.700
Salmonella Livingstone	1	Chicken	2.447	0.874
Salmonella Montevideo	1	Chicken	0.786	1.406
Salmonella Virchow	1	Chicken	2.504	0.865
Salmonella Aberdeen	1	Bovine	2.766	1.001
Salmonella California	1	Animal feed	0.894	1.734
Salmonella Kentucky	1	Chicken	0.904	1.597
Salmonella Paratyphi	1	Human	0.865	1.648
Salmonella Havana	1	Soybean meal	0.853	1.477
Salmonella San Diego	1	Human	0.809	1.445
Salmonella Bredeney	1	Chicken	0.793	1.600
Salmonella Mbandaka	1	Bovine	0.816	1.552
Salmonella Agona	4	Pork	0.805*	1.601*
Salmonella Javiana	1	Chicken	0.865	1.830
Salmonella Derby	1	Sheep	0.867	1.599
Salmonella Newport	3	Chickens/ bovine	0.855*	1.699*
Salmonella Infantis	1	Broiler	2.688	0.861
Salmonella pullorum	2	Chicken	0.841*	1.819*
Salmonella Schwarzengrund	1	Pork sausages	0.845	1.687
Salmonella Hadar	1	Chicken	0.901	1.775
Escherichia coli		Chicken	0.733	1.149
Shigella flexneri 1755/05		Human feces	0.860	0.961
Shigella boydii		Human feces	1.147	1.089
Shigella sonei 1547/05		Human feces	1.140	0.944
Proteus vulgaris		Chicken	1.733	1.168

Table 1: Continue

			Real Time-PCR	
Serotypes	No. of strains	Source	ΔRn STspc probe	ΔRn STnonspc probe
Klebsiella pneumoniae (2 strains and ATCC 700603)		Milk, bovine and human	0.706*	0.992*
Enterobacter cloacae (2 strains)		Chicken and human	0.566*	0.967*
Citrobacter freundii 709/05		Chicken	1.172	0.978

*Average values from all strains tested

The PCR products were purified by using Polyethylene glycol (PEG) 6000 and cycle sequenced from both strands at a MegaBace 750 Sequencer (GE, Sweden). In house generated sequences and all homologous sequences from bacteria available in public data bases (NCBI http://www.ncbi.nlm.nih.gov and *Salmonella* sp. comparative sequencing Project http://www.sanger.ac.uk/Projects/Salmonella) were compared. Alignment of all sequences was achieved by using BioEdit software (http://www.mbio.ncsu.edu).

Design and validation of the specific test: A *Taq*Man® real-time PCR assay was designed for the specific identification of *Salmonella* Typhimurium serotype. Two primers (forprimerST 5'-GGTACTGAGCGTGTTATCGTTTCTC ; revprimerST 5' -GGTTTTACCTTTGTCGGAGTCAA) were designed to amplify a 75 bp fragment encompassing the *Salmonella* Typhimurium specific SNP. Twofluorogenic, Minor Groove Binding (MGB) probes targeted to the specific SNP were designed and synthesized with two different fluorescent dyes. The probe labeled with VIC (targeted to *Salmonella* Typhimurium strains; STspc 5'-VIC- TGCACCGTAGCCCT-MGBNFQ), presented a Cytosine at the SNP position, while the probe labeled with FAM (targeted to non *Salmonella* Typhimurium strains; STnonspc 5'-FAM-CACCGTAGTCCGGG-MGBNFQ) presented a Thymine at the SNP position. The probe region also included an additional SNP present in *Salmonella* Typhimurium but also present in *Salmonella* Aberdeen, *Salmonella* Infantis, *Salmonella* Livingstone and *Salmonella* Virchow serotypes (underlined bases) which inclusion was unavoidable. Primers and probes were obtained from Applied Biosystems.

Real-time PCR reactions were carried out in a 15 μL reaction volume that included 7.5 μL of *Taq*Man universal PCR master mix kit (Applied Biosystems), 800 nmol L^{-1} concentration of each primer, 300 nmol L^{-1} concentration of each probe and 5 μL of sample DNA. Thermocycling was performed on an ABI 7500 (Applied Biosystems) and the program consisted of 2 min at 50°C, a Ampli*Taq* Gold DNA Polymerase activation for 10 min at 95°C and 40 cycles of 15 s at 92°C and 1 min at 60°C. Each sample was assayed in duplicate and a negative control without template (NTC) was included.

The results were analysed by using SDS software v 1.3.1(Applied Biosystem).

Sensitivity and detection-limit of the real-time PCR test: The DNAs from *Salmonella* Typhimurium ATCC 6994 and *S*. enteritidis PT 4 were serially diluted until obtaining concentrations of *circa* 10^7 to 10^0 copies of DNA per reaction. Dilutions of both strains were amplified as described above and standard curves were prepared for each one. The resulting Ct values (the fractional cycle number at which the fluorescence reaches 20 standard deviation above background emissions) were plotted as a function of the log DNA copy number. Three replicas of

each strain were simultaneously performed. Samples were considered positive if they reach Ct values greater than 30 cycles. PCR efficiencies were calculated from the standard curves by using the equation (Rasmussen, 2001):

$$\text{PCR efficiency} = [10^{(-1/M)}]-1\times 100$$

where, M is the slope of the standard curve.

RESULTS

The analysis of *rpoB* gene fragments sequenced in house revealed a SNP specific of *Salmonella* Typhimurium serotype at position 456 of the coding sequence. This polymorphism involves a silent T-to-C transition, which is present in all *Salmonella* Typhimurium strains analyzed, including all the ones available in public genetic databases, at time of writing. This specific SNP was not present in the other *Salmonella* serovars and was found in some Enterobacteria strains of *Enterobacter sakazakii, Citrobacter koseri, Klebsiella pneumoniae, Vibrio cholerae, V. fischeri* and *V. vulnificus* reported at NCBI. The divergence of sequences neighboring the candidate SNP between *Salmonella* and non-*Salmonella* strains permits the design of a specific test. This SNP was chosen for the development of a *Taq*Man real-time PCR genotyping assay. The *Taq*Man assay showed 100% specificity, successfully genotyping all 32 *Salmonella* Typhimurium strains correctly. Figure 1 shows a representative genotype discrimination plot derived from a *Taq*Man real-time PCR assay. Two groups of data points were easily separated in opposite quadrants of the graph, discriminating *Salmonella* Typhimurium (dots) and non-*Salmonella* Typhimurium (diamonds) strains. The Cycle Threshold (Ct) required for detecting *Salmonella* Typhimurium strains ranged from 11-15 cycles. Based on the relative fluorescence of specific labeled probes (ΔRn), *Salmonella* Typhimurium strains showed values greater than three for STspc probe while the other *Salmonella* serotypes generally showed Δ Rn values minor than one for this probe (Table 1). Some other strains represented by *Salmonella* Aberdeen, *Salmonella* Infantis, *Salmonella* Livingstone and *Salmonella* Virchow serotypes presented ΔRn values associated to STspc probe ranging between 2.4 and 2.7, due to latter amplifications (Ct>20) induced by a non specific SNP located at the probe sequence which could not be avoidable in the design of the test. These serotypes have a cytosine at

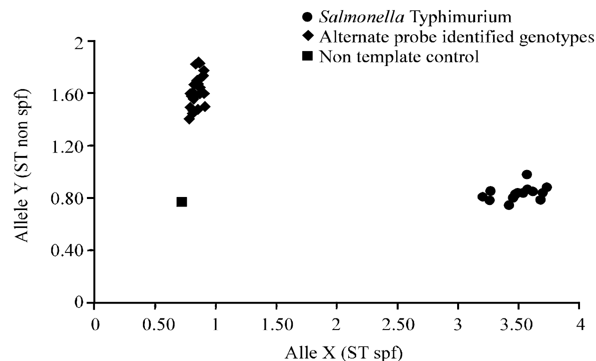

Fig. 1: Representative genotype discrimination plot of *Salmonella* Typhimurium (dots) vs. other *Salmonella serotypes* (diamonds). Non template control (NTC) is depicted by the square

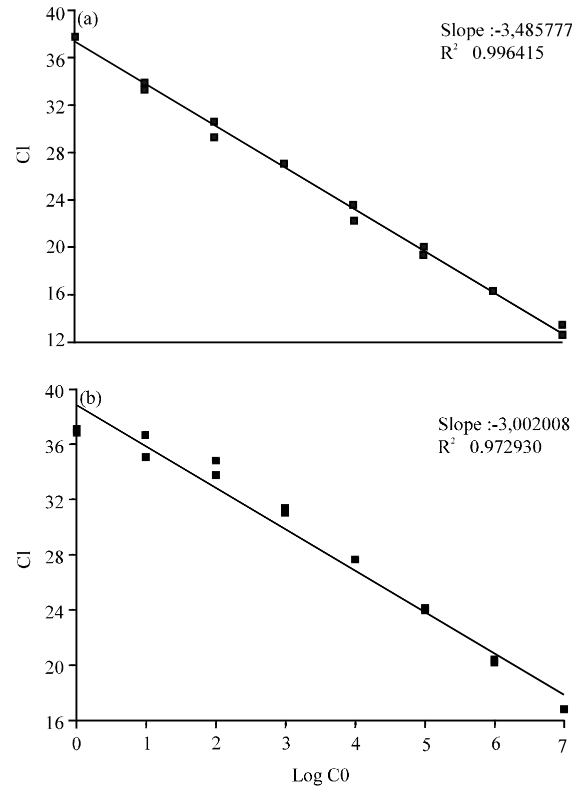

Fig. 2(a-b): Real time PCR standard curve for (a) *Salmonella* Typhimurium ATCC 6994 and (b) *Salmonella* Enteritidis PT4, The resulting Ct values were plotted as a function of the log of DNA copy number

456 position and not a thymine specific of *Salmonella* Typhimurium serotype and they are distinguished from *Salmonella* Typhimurium by higher ΔRn and Ct values.

All Enterobacteria tested were grouped close to control samples containing no template and their ΔRn values for both probes are shown in Table 1. *In silico*-PCR was performed with the sequences of these strains presenting the SNP specific of *Salmonella* Typhimurium serotype reported at NCBI and no amplification product was obtained.

The sensitivity of the *Taq*Man assay was tested by means of serial dilutions of *Salmonella* Typhimurium ATCC 6994 and *S.* enteritidis PT 4 genomic DNAs. The linear range of detection was determined to be 10^7-10^2 for *Salmonella* Typhimurium and 10^7-10^3 for the other *Salmonella* serotypes (Fig. 2a, b, respectively). The amplification efficiency was 93.5% and correlation coefficient for *Salmonella* Typhimurium detection was 0.996 (Fig. 2a).

DISCUSSION

Many molecular PCR based methods (single or multiplex) have been developed for detecting *Salmonella* Typhimurium targeting antigenic protein genes and/or virulence factors. Khan *et al.* (2000) reported the detection of the multidrug-resistant *Salmonella* Typhimurium DT104 by multiplex PCR simultaneously amplifying four genes: florfenicol (flo(st)), virulence (spvC), in vasion (invA) and integron (int). Soumet *et al.* (1999) proposed the identification of *Salmonella* Typhimurium by targeting a flagellin gene (*fli*C). However, both studies have a

limitation on specificity, since the selected sequences are not exclusive of *Salmonella* Typhimurium. Lim *et al*. (2003) developed a multiplex PCR test based on somatic and flagellar antigen genes (rfbJ, fliC and fljB) putatively specific to *Salmonella* Typhimurium. Due to the few numbers of other serotypes evaluated the exclusive occurrence of these sequences in *Salmonella* Typhimurium remains uncertain. Moreover, multiplex PCR is cumbersome and sometimes lacks reproducibility between laboratories because the specific conditions needed for simultaneous amplification of several regions.

The increased availability of bacterial genomic sequences has allowed a comparative analysis targeted to find *Salmonella* regions that are specific for serotypes. On this respect, Kim *et al*. (2006b) found a region (named STM 4497) coding a putative cytoplasmatic-protein that specifically identifies *Salmonella* Typhimurium. The same region was included in multiplex tests, aiming at the simultaneous identification of several *Salmonella* serotypes (Park *et al*., 2009; Shanmugasundaram *et al*., 2009). These tests have been reported to be highly selective. However, since STM 4497 tests are based on presence vs. absence of PCR amplified bands, failed reactions can be misleading.

This study presents a new molecular test designed for the specific differentiation of *Salmonella* Typhimurium, which complements existing detection methods. The novel targeted region corresponds to *rpo*B gene where a specific SNP to *Salmonella* Typhimurium serotype was found. *rpo*B is a highly conserved single-copy gene, that codifies for RNA polymerase **β** subunit, a ubiquitous enzyme vital for cell survival. Among the core bacterial genes, *rpo*B has emerged as one of the few potential candidates for bacterial phylogenetic analyzes and identification (Adekambi *et al*., 2008a) This gene has been recommended previously for species and genus delineation (Adekambi *et al*., 2008b) and it has been used to study the Enterobacteriaceae family (Mollet *et al*., 1997) and some other related and unrelated genera (Peixoto *et al*., 2002; Khamis *et al*., 2004). The phylogenetic resolution of *rpo*B is comparable with the given by 16S rRNA gene at taxonomic level, with a better performance for closely related organisms within species and subspecies (Case *et al*., 2007). The silent or synonymous SNPs are used as a simple and rapid way to compare genomes in many bacterial strains. They provide useful targets for large-scale population genetic molecular studies and to assess relationships among bacterial strains. In this respect, the discovered SNP can be used as a complementary datapoint in such studies where salmonellas are included.

The test relies on a *Taq*Man real-time PCR strategy, a powerful tool in microbiological diagnosis due to its high specificity, sensitivity and rapidness, without the need of post-amplification steps. The test showed consistent detection and genetic discrimination of *Salmonella* Typhimurium from other *Salmonella* serotypes at DNA concentrations as low as 10^2 estimated genome copies. The use of a single target region eliminates the drawbacks of multiplex systems and the use of specific probes differentially labeled increase test performance and eliminates the occurrence of false negatives if PCR reaction fails.

In our hands, *rpo*B region has proved to be 100% accurate for genotyping *Salmonella* Typhimurium among other *Salmonella* serotypes that were examined. Due to its high conservation among genera, the *rpo*B SNP may be also be used in genotyping chips or in any other device for universal detection and classification of bacteria.

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REFERENCES

Adekambi, T., M. Drancourt and D. Raoult, 2008a. The rpoB gene as a tool for clinical microbiologists. Trends Microbiol., 17: 37-45.

Adekambi, T., T.M. Shinnick, D. Raoult and M. Drancourt, 2008b. Complete rpoB gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. Int. J. Syst. Evol. Microbiol., 58: 1807-1814.

Bennasar, A., G. de Luna, B. Caber and J. Lalucat, 2000. Rapid identification of *Salmonella* Typhimurium, *S. enteritidis* and *S. virchow* isolates by polymerase chain reaction based fingerprinting methods. Int. Microbiol., 3: 31-38.

Case, R.J., Y. Boucher, I. Dahllof, C. Holmstrom, W.F. Doolittle and S. Kjelleberg, 2007. Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. App. Environ. Microbiol., 73: 278-288.

Chen, J., L. Zhang, G.C. Paoli, C. Shi, S.I. Tu and X. Shi, 2010. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. Int. J. Food Microbiol., 137: 168-174.

Hajibabaei, M., G.A. Singer, P.D. Hebert and D.A. Hickey, 2007. DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet., 23: 167-172.

Humphrey, T., 2006. Public Health Aspects of *Salmonella enterica* in Food Production. In: Salmonella Infections: Clinical, Immunological and Molecular Aspects, Mastroeni, P. and D. Maskell, (Eds.). AMCM Series: Advances in Molecular and Cellular Microbiology, Cambridge University Press, Cambridge, pp: 89-116.

Kalendar, R., D. Lee and A.H. Schulman, 2009. Fast PCR Software for PCR Primer and Probe Design and Repeat Search. In: Focus on Bioinformatics. Genes, Genomes and Genomics 3, Mansour, A. (Ed.). Zagazig University, Egypt, pp: 1-14.

Kauffmann, F., 1972. Serological Diagnosis of *Salmonella* Species. Kauffmann White Scheme Minkagaard, Copenhagen, Denmark.

Khamis, A., D. Raoult and B. La Scola, 2004. rpoB gene sequencing for identification of *Corynebacterium* species. J. Clin. Microbiol., 42: 3925-3931.

Khan, A.A., M.S. Nawaz, S.A. Khan and C.E. Cerniglia, 2000. Detection of multidrug-resistant *Salmonella* Typhimurium DT104 by multiplex polymerase chain reaction. FEMS Microbiol. Lett., 182: 355-360.

Kim, H.J., S.H. Park and H.Y. Kim, 2006a. Comparison of *Salmonella enterica* serovar Typhimurium LT2 and non-LT2 *Salmonella* genomic sequences and genotyping of *Salmonellae* by using PCR. Appl. Environ. Microbiol., 72: 6142-6151.

Kim, H.J., S.H. Park, T.H. Lee, B.H. Nahm, Y.H. Chung, K.H. Seo and H.Y. Kim, 2006b. Identification of *Salmonella enterica* serovar Typhimurium using specific PCR primers obtained by comparative genomics in *Salmonella* serovars. J. Food Prot., 69: 1653-1661.

Kim, S., J.G. Frye, J. Hu, P.J. Fedorka-Cray, R. Gautom and D.S. Boyle, 2006c. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. J. Clin. Microbiol., 44: 3608-3615.

Lim, Y.H., K. Hirose, H. Izumiya, E. Arakawa and H. Takahashi *et al*., 2003. Multiplex polymerase chain reaction assay for selective detection of *Salmonella* enterica serovar typhimurium. Jpn. J. Infect. Dis., 56: 151-155.

Liu, B., L. Zhang, X. Zhu, C. Shi and J. Chen *et al*., 2011. PCR identification of *Salmonella* serogroups based on specific targets obtained by comparative genomics. Int. J. Food Microbiol., 144: 511-518.

Mccarthy, N., F.J. Reen, J.F. Buckley, J.G. Frye, E.F. Boyd and D. Gilroy, 2009. Sensitive and rapid molecular detection assays for *Salmonella enterica* serovars typhimurium and heidelberg. J. Food Prot., 72: 2350-2357.

Mollet, C., M. Drancourt and D. Raoult, 1997. *RpoB* sequence analysis as a novel basis for bacterial identification. Mol. Microbiol., 26: 1005-1011.

Park, S.H., H.J. Kim, W.H. Cho, J.H. Kim and M.H. Oh *et al*., 2009. Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars typhimurium, enteritidis and typhi using multiplex PCR. FEMS Microbiol. Lett., 301: 137-146.

Peixoto, R.S. H.L.D. Coutinho, N.G. Rumjanek, A. Macrae and A.S. Rosado, 2002. Use of *rpo*B and 16S rRNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE. Lett. Appl. Microbiol., 35: 316-320.

Rasmussen, R.P., 2001. Quantification on the Lightcycler. In: Rapid Cycler Real-Time PCR, Methods and Applications, Meuer, S., C. Wittwer and K. Nakagawara, (Eds.), Singer-Verlag, Heidelberg, pp: 21-34.

Shanmugasundaram, M., M. Radhika, H.S. Murali and H.V. Batra, 2009. Detection of *Salmonella enterica* serovar Typhimurium by selective amplification of *fli*C, *flj*B, *iro*B, *inv*A, *rfb*J, STM2755, STM4497 genes by polymerase chain reaction in a monoplex and multiplex format. World J. Microbiol. Biotechnol., 25: 1385-1394.

Soumet, C., G. Ermel, N. Rose, V. Rose, P. Drouin, G. Salvat and P. Colin, 1999. Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella enteritidis* strains from environmental swabs of poultry houses. Lett. Applied Microbiol., 29: 1-6.