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Development of a Duplex Real-time PCR for Differentiation of *Salmonella* Typhimurium and Monophasic Serovars

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ABSTRACT

Salmonella Typhimurium is the most *Salmonella* serovar causing acute gastroenteritis and diarrhea. Serovar 1, 4, [5], 12: i:- is considered a monophasic variant of *S.* Typhimurium that threaten public health. Fifty-eight serologically confirmed *Salmonella* strains were investigated by PCR using *16S rRNA* and *fliC* genes. All 58 strains harbored *16S rRNA* while 21 strains harbored *fliC* gene that included *S.* Typhimurium (12), *S.* Kentucky (6), *Salmonella* variant strain serotype 1, 4, [5],12:i:- (1), *S.* Lagos (1), and *S.* Kedougou (1). A duplex TaqMan real-time PCR was performed for differentiating between biphasic *S.* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *fljB1, 2* and *fliB/IS200* in the *fliA-fliB* intergenic region. Ten out of twelve *S.* Typhimurium harbored *fljB 1, 2*, while *Salmonella* variant strain serotype 1, 4, [5], 12:i:- lacked this gene. Thirteen strains (12 *S.* Typhimurium and the variant strain serotype 1, 4, [5], 12:i:-) were positive for *fliB/IS200* that is a specific gene for *S.* Typhimurium (biphasic and monophasic). The result of duplex TaqMan real-time PCR indicated that 10 *S.* Typhimurium strains were biphasic while two *S.* Typhimurium strains and the variant strain serotype 1, 4, [5], 12:i:- lack *fljB1,2* and had *fliB/IS200* were monophasic *S.* Typhimurium. It is noticed that prolonged subculture and repeat phase inversion method leads to the formation of flakes that in turn cause wrongly serotype identification, therefore, real-time PCR is rapid and can be used for identifying and differentiating between biphasic and monophasic *S.* Typhimurium.

Key words: Biphasic and monophasic *S.* Typhimurium, *flj* gene, Real-time PCR, *Salmonella*.

INTRODUCTION

Salmonella enterica is zoonotic bacteria transmitted through the food chain and is an important cause of disease in humans (Osman et al., 2014a; Shaw et al., 2018). It is the second leading cause of bacterial foodborne illness (Foley et al., 2008; Persad and LeJeune, 2018). The genus *Salmonella* has a large number of serotypes that differ in pathogenicity and host specificity. Despite the widespread use of serotyping, it has deficiencies that limit its utility, including that it often takes three or more days to give a result and approximately 5-8% of isolates are partially typed.

In addition, prolonged subculture can affect the antigenic properties of strains. Highly trained laboratories are required to type strains accurately, also high costs of producing and validity specific antisera to rare antigens are problematic (Kim et al., 2006). Delay caused by identification can hinder the response to a disease outbreak and/ or epidemiological surveillance. Therefore, various studies have been explored alternative assays to differentiate *Salmonella* isolates, such as the search for genes that can be used as potential molecular substitutes for serotyping. However, the genes tested so far have often yielded inconsistent results (Osman et al., 2014b; Hua Zou et al., 2016). Real-time PCR for detection of *Salmonella* has been brought to inter-laboratory trial, the results of which support their use as international standard methods (Malorny et al., 2007).

Two genomic sites, *16S rRNA* and *fli*C gene have been reported as candidates suitable for common and specific detection of Genus *Salmonella*, and *S.* Typhimurium, respectively by real-time PCR (Imre et al., 2005). The *16SrRNA* can be used for the rapid and multiple detections of the 16 pathogenic bacteria frequently isolated from contaminated foods that are important for food safety (Shin et al., 2016). The 16S ribosomal RNA (rRNA), approximately 1500 nucleotides in length of the prokaryotic ribosome, provides sufficient highly-conserved sequences to design the probes for developing microbial detection (Woo et al., 2003). The

fliC gene codes for the Hi antigen of *Salmonella* targeting the *fliC-i* allele greatly increases the specificity for *S.* Typhimurium identification (Pathmanathan et al., 2003).

S. Typhimurium, according to the White–Kaufmann–Le Minor serotyping scheme (Grimont and Weill, 2007), exhibits the antigenic formula 1, 4, [5], 12: i:1, 2, where "i" and "1,2" are the first and second flagellar antigens expressed by the bacterium at different times, hence the serotype description as biphasic (Soyer et al., 2009). Antigenic variants that lack either the first or second H antigens or both have been described. In recent years isolates with antigenic formula 1, 4, [5], 12:i:– have become increasingly important as a public health risk and more frequently recovered from humans and food-producing animals (Hopkins et al., 2010). The European Food Safety Authority (EFSA, 2010) recently recommended the confirmation of the serological identification of monophasic *S.* 1, 4, [5], 12:i:- strains using a polymerase chain reaction (PCR) protocol based on the detection of *fljB* gene and the *fliA-B* intergenic region. The *fljB*1,2 gene codes for second phase flagellar antigen present in *S.* Typhimurium. Indeed, all serovar Typhimurium strains and its monophasic/ nonmotile variants have an IS200 fragment of 1 kb in the *fliA-B* intergenic region, which is not detected in the other serovars. Within the flagellin gene cluster of *Salmonella* Typhimurium carries a conserved IS200 insertion sequence located downstream of the flagellin N-methylase gene (*fliB*) and upstream of the flagellar biosynthesis sigma factor gene (fliA), this element found in *Salmonella* Typhimurium and its variant (Burnens et al., 1997). Several studies have reported DNA sequences for *Salmonella* flagellin genes. As of June 2003, 74 complete or partial *Salmonella fliC* alleles and 25 complete or partial *Salmonella fljB* allele sequences had been documented in GenBank release no. 132, excluding complete genome sequences.

Thus, this study aimed, first, to confirm *Salmonella* strains using *16SrRNA* gene and *S.* Typhimurium using *fli*C by Syber green-based real-time PCR, and second, to differentiate between *S.* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *flj*B1,2 and IS*200* in the *fli*A-*fli*B region using TaqMan real-time PCR.

MATERIALS AND METHODS

Strains

A total of 58 *Salmonellae* isolates recovered from chicken in previous work (Abd El-Lattief, 2014), was identified serologically by slide agglutination test according to White-Kauffmann le minor scheme (Grimont and Weill, 2007) using SIFIN antisera, Berlin, kindly obtained from Serology Unit, Animal Health Research Institute.

Phase inversion method

According to ISO/TR6579 (2014), specific phase inversion antiserum was added to a swarm agar medium (SIFIN) and the *Salmonella* strain was spot inoculated on the plate. The agar medium shall be sufficiently soft for motile *Salmonella* to swarm over the medium. Slide agglutination test was performed from periphery of the plate after incubation at 37C° for 24 hrs.

Duplex Syber green real-time PCR

For the detection of genus *Salmonella* and *S.* Typhimurium, DNA was extracted from the strains according to QIAamp DNA mini kit instructions (Soumet et al., 1999 and Yang et al., 2014). SYBR Green real-time PCR was performed using oligonucleotide primers (Table 1) and Quantitect SYBR green PCR kit containing 1ml 2xQuantiTect SYBR Green PCR Master Mix, 2ml RNase-Free Water**.**

Table 1. Oligonucleotide primers used in this study for detection of genus *Salmonella* using 16*SrRNA* and *fli*C genes

Target gene	Primer sequence (5'-3')	Reference
16*S rRNA*	F: CAGAAGAAGCACCGGCTAACTC	Yang et al., 2014
	R: GCGCTTTACGCCCAGTAATT	
*fli*C	F: CGGTGTTGCCCAGGTTGGTAAT	Soumet et al., 1999
	R: ACTCTTGCTGGCGGTGCGACTT	

F: forward, R: reverse

Table 2. Oligonucleotide primers and probes used for differentiating between biphasic *Salmonella* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *fljB1,2* and *fliB/IS200* in the *fliA-fliB* intergenic region.

Target	Primer sequence (5'-3') and probe	Reference
*flj*B1, 2	F: TGT TAC TAT TGG TGG CTT TAC TGG	Prendergast et al., 2013
	R: CAG CAG GCA TTG TGG TCT TAG	
	FAM- CGC CAG CCG CAA GGG TTA CTG TAC – TAMRA	
fliB/1S*200*	F: GAT CTG TCG ATG ATT CAT CTT CTG AC	
	R: AAC GCT TGT CTT CGG TAT TTG G	
	CY5-TCG GGT GTG CGC TAA GCT CTT TT -BHQ1	

F: forward, R: reverse

Differentiation of *S.* Typhimurium and monophasic 1, 4, [5], 12:i:-by TaqMan real-time PCR

TaqMan real-time PCR was performed according to Prendergast et al. (2013) using oligonucleotide primers and probes presented in table 2, and the Quantitect probe real-time PCR kit (Qiagen).

***fliC* sequencing**

fliC was sequenced using *fliC* primer presented in table 1. A purified PCR product was sequenced in the way of the forward and/ or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA), using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA).

Phylogenetic analysis

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNA Star software Pairwise, which was designed by Thompson et al. (1994) and phylogenetic analyses were done using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

RESULTS

Serotyping of *Salmonella*

The Serotyping of the 58 *Salmonella* strains was confirmed and the result is presented in table 3.

Table 3. Antigenic structure of all *Salmonella* strains recovered using slide agglutination test.

No	Name	Serotyping	No	Name	Serotyping
1-	*S.* Kentucky	8,20 :i:z6	30-	*S.* Washington	13,22 :m,t:–
2-	*S.* Lagos	1,4,5,12:i:1,5	31-	*S.* Newport	6,8,20 :e,h :1,2
3-	*S.* Typhimurium	1,4,[5],12:i:1,2	32-	*S.* Enteritidis	1,9,12 : g,m:–
4-	*S.* Typhimurium	1,4,[5],12:i:1,2	33-	*S.* Rissen	6,7,14 :f,g:-
5-	*S.* Taksony	1,3,19: i: z_6	34-	*S.* Labadi	8,20 :d: z_6
6-	*S.* Derby	1,4,[5],12:f,g:-	35-	*S.* Enteritidis	1,9,12 : g,m: –
7-	*S.* Rissen	6,7,14:f,g:-	36-	*S.* Senftenberg	1,3,19: g,[s],t :–
8-	*S.* Typhimurium	1,4,[5],12:i:1,2	37-	*S.* Cerro	6,14,18 :z4,z23 :[1,5]
9-	*S.* Anatum	3,{10} {15} {15,34} :e,h: 1,6	38-	*S.* Virginia	8: d :1,2
10-	S. Typhimurium	1,4,[5],12:i:1,2	39-	*S.* Papuana	6,7 :r :e,n,z_{15}
11-	*S.* Paratyphi A	1,2,12: a: [1,5]	40-	*S.* Typhimurium	1,4,[5],12:i:1,2
12-	*S.* Paratyphi B	1,4,[5],12 :b: 1,2	41-	*S.* Typhimurium	1,4,[5],12:i:1,2
13-	*S.* Kedougou	1,13,23: i :l,w	42-	*S.* Kentucky	8,20: i: z_6
14-	*S.* Labadi	8,20 :d: z_6	43-	*S.* Typhimurium	1,4,[5],12:i:1,2
15-	*S.* Poona	1,13,22: z: 1,6	44-	*S.* Enteritidis	1,9,12 :g,m: –
16-	*S.* Typhimurium	1,4,[5],12:i:1,2	45-	*S.* Virginia	8:d:1,2
17-	*S.* Kentucky	8,20: i :z_6	46-	*S.* Kentucky	8,20: i :z_6
18-	*S.* Anatum	3,{10}{15}{15,34} :e,h: 1,6	47-	*S.* Washington	13,22 :m,t:–
19-	*S.* Goldcoast	6,8 :r :l,w	48-	*S.* Enteritidis	1,9,12:g,m: –
20-	*S.* Enteritidis	1,9,12:g,m:-	49-	*S.* Newlands	3,{10} {15 ,34}:e,he,n,x:-
21-	*S.* Infantis	6,7,14: r :1,5	50-	*S.* Gallinarum	1,9,12 :–: –
22-	*S.* Gallinarum	1,9,12: – :–	51	*S.* Agama	4,12:i:1,6
23-	*S.* Gallinarum	1,9,12: – :–	52-	*S.* Kentucky	8,20: i: z_6
24-	*S.* Hadar	6,8 :z_{10}:e,n,x	53-	*S.* Kentucky	8,20: i :z_6
25-	*S.* Virchow	6,7,14: r :1,2	54-	*S.* Typhimurium	1,4,[5],12:i:1,2
26-	*S.* Virchow	6,7,14: r :1,2	55-	*S.* Typhimurium	1,4,[5],12 :i :1111,2
27-	*S.* Hadar	6,8 :z_{10} :e,n,x	56-	*S.* Typhimurium	1,4,[5],12:i:1,2
28-	*S.* Bardo	8 :e,h:1,2	57-	Partial identification	1,4,[5],12:i:-
29-	*S.* Montevideo	6,7,14 :g,m,s :-	58-	*S.* Typhimurium	1,4,[5],12:i:1,2

Syber Green real-time duplex PCR

All 58 strains belonged to genus *Salmonella* were positive by SYBER green real-time PCR using *16S rRNA*. The specificity of the reaction was confirmed by melting temperature (Tm) which was consistently specific for amplicon obtained; the mean peaks Tm obtained. The negative control did not show peaks in the Tm when subjected to 40 cycles of amplification. Twenty-one *Salmonella* strains harbored *fliC* gene, including *S.* Typhimurium (12), *S.* Kentucky (6), *S.* Lagos (1), *S.* Kedougou (1) and partial identified strain *S* 1,4, [5],12:i:- which possess first flagellar i antigen (Table 4; figures 1 and 2). A total of 15 strains were positive for *fljB* 1,2. Ten strains of *Salmonella* Typhimurium and serovars Paratyphi A (1) & Paratyphi B (1) & Newport (1) and Virginia (2) harbored *fljB*1,2, while strain no.57 with antigenic formula 1, 4 ,[5], 12:i:- and two strains *Salmonella* Typhimurium lacked this gene. Concerning *fli B/IS200*, the 13 strains possess *fliB/IS200* (12 *S.* Typhimurium and the variant strain S 1, 4 ,[5], 12:i:-) (Table 5 and figure 3).

***fli C* Sequencing**

Individual *Salmonella* serotypes usually alternate between the production of 2 antigenic forms of flagella, termed phase 1 and phase 2, each specified by separate structural genes, *fliC* and *fljB* 1, 2. Sequencing of *fli*C gene based on the nucleotide sequence of *S.*Typhimurium13311 referenced in GenBank illustrated that the biphasic *S.* Typhimurium strain was recorded in GenBank as *S.* Typhimurium Egy 1 with accession number Mk103394 and the monophasic strain as *S.* Typhimurium Egy 2 with accession number MK 103395. The amino acid sequence of the *fliC* gene in the two isolates showing greater than 98% identity.

Table 4. Detection of 16*SrRNA* and *fli*C genes in *Salmonella* serovars using duplex Syber green real-time PCR

No.	Name	16*S rRNA*	*fli*C	No.	Name	16*S rRNA*	*fli*C
1	*S.* Kentucky	+	+	30	*S.* Washington	+	-
2	*S.* Lagos	+	+	31	*S.* Newport	+	-
3	*S.* Typhimurium	+	+	32	*S.* Enteritidis	+	-
4	*S.* Typhimurium	+	+	33	*S.* Rissen	+	-
5	*S.* Taksony	+	-	34	*S.* Labadi	+	-
6	*S.* Derby	+	-	35	*S.* Enteritidis	+	-
7	*S.* Rissen	+	-	36	*S.* Senftenberg	+	-
8	*S.* Typhimurium	+	+	37	*S.* Cerro	+	-
9	*S.* Anatum	+	-	38	*S.* Virginia	+	-
10	*S.* Typhimurium	+	+	39	*S.* Papuana	+	-
11	*S.* Paratyphi A	+	-	40	*S.* Typhimurium	+	+
12	*S.* Paratyphi B	+	-	41	*S.* Typhimurium	+	+
13	*S.* Kedougou	+	+	42	*S.* Kentucky	+	+
14	*S.* Labadi	+	-	43	*S.* Typhimurium	+	+
15	*S.* Poona	+	-	44	*S.* Enteritidis	+	-
16	*S.* Typhimurium	+	+	45	*S.* Virginia	+	-
17	*S.* Kentucky	+	+	46	S. Kentucky	+	+
18	*S.* Anatum	+	-	47	S. Washington	+	-
19	*S.* Goldcoast	+	-	48	S. Enteritidis	+	-
20	*S.* Enteritidis	+	-	49	S. Newlands	+	-
21	*S.* Infantis	+	-	50	S. Gallinarum	+	-
22	*S.* Gallinarum	+	-	51	S. Agama	+	-
23	*S.* Gallinarum	+	-	52	S. Kentucky	+	+
24	*S.* Hadar	+	-	53	S. Kentucky	+	+
25	*S.* Virchow	+	-	54	S. Typhimurium	+	+
26	*S.* Virchow	+	-	55	S. Typhimurium	+	+
27	*S.* Hadar	+	-	56	S. Typhimurium	+	+
28	*S.* Bardo	+	-	57	1,4,[5],12:i:-	+	+
29	*S.* Montevideo	+	-	58	S. Typhimurium	+	+

Table 5. Detection of *fljB1,2* and *fliB*/IS*200* in *Salmonella* serovars using duplex TaqMan real-time PCR

No.	Name	*fljB1,2*	*fliB*/IS*200*	No.	Name	*fljB1,2*	*fliB* /IS*200*
1	*S*. Kentucky	-	ND	30	*S*. Washington	-	ND
2	*S*. Lagos	-	ND	31	*S*. Newport	+	ND
3	*S*. Typhimurium	+	+	32	*S*. Enteritidis	-	ND
4	*S* .Typhimurium	+	+	33	*S*. Rissen	-	ND
5	*S*. Taksony	-	ND	34	*S*. Labadi	-	ND
6	*S*. Derby	-	ND	35	*S*. Enteritidis	-	ND
7	*S*. Rissen	-	ND	36	*S*. Senftenberg	-	ND
8	*S*. Typhimurium	+	+	37	*S*. Cerro	-	ND
9	*S*. Anatum	-	ND	38	*S*. Virginia	+	ND
10	*S*. Typhimurium	+	+	39	*S*. Papuana	-	ND
11	*S*. Paratyphi A	+	ND	40	*S*. Typhimurium	+	+
12	*S*. Paratyphi B	+	ND	41	*S*. Typhimurium	+	+
13	*S*. Kedougou	-	ND	42	*S*. Kentucky	-	ND
14	*S*. Labadi	-	ND	43	*S*. Typhimurium	+	+
15	*S*. Poona	-	ND	44	*S*. Enteritidis	-	ND
16	*S*. Typhimurium	+	+	45	*S*. Virginia	+	ND
17	*S*. Kentucky	-	ND	46	*S*. Kentucky	-	ND
18	*S*. Anatum	-	ND	47	*S*. Washington	-	ND
19	*S*. Goldcoast	-	ND	48	*S*. Enteritidis	-	ND
20	*S*. Enteritidis	-	ND	49	*S*. Newlands	-	ND
21	*S*. Infantis	-	ND	50	*S*. Gallinarum	-	ND
22	*S*. Gallinarum	-	ND	51	*S*. Agama	-	ND
23	*S*. Gallinarum	-	ND	52	*S*. Kentucky	-	ND
24	*S*. Hadar	-	ND	53	*S*. Kentucky	-	ND
25	*S*. Virchow	-	ND	54	*S*. Typhimurium	+	+
26	*S*. Virchow	-	ND	55	*S*. Typhimurium	-	+
27	*S*. Hadar	-	ND	56	*S*. Typhimurium	-	+
28	*S*. Bardo	-	ND	57	*S*. Typhimurium	-	+
29	*S*. Montevideo	-	ND	58	*S*. Typhimurium	+	+

ND: Not detected

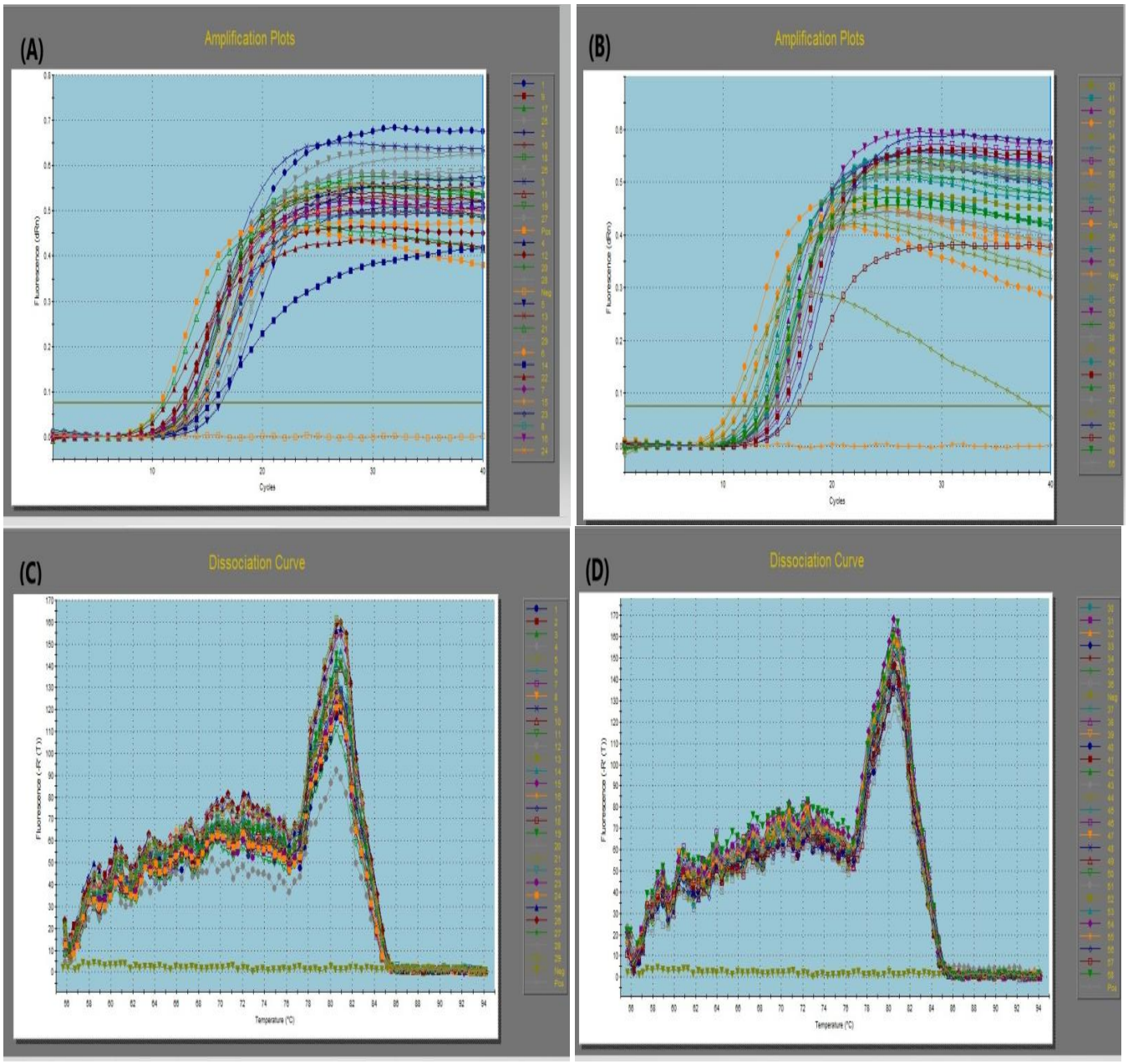


Figure 1. Syber green real-time PCR targeting *16S rRNA* gene for 58 *Salmonella* strains isolated from chickens (fluorescence chart and melting curve). A) Fluorescence chart for strains number 1 to 29. B) Fluorescence chart for strains number 30 to 58. (Amplification plots represent the accumulation of product over the duration of real-time PCR). C) Melting curve for strain number 1 to 29. D) Melting curve for strain number 30 to 58. Melting curve provides representation of the PCR product after the amplification process, A single peak indicates a positive sample. All 58 strains isolated from chickens were positive for *16SrRNA* gene. The specificity of the reaction was confirmed by the melting temperature. The mean peak temperature obtained was 80.55 °C.

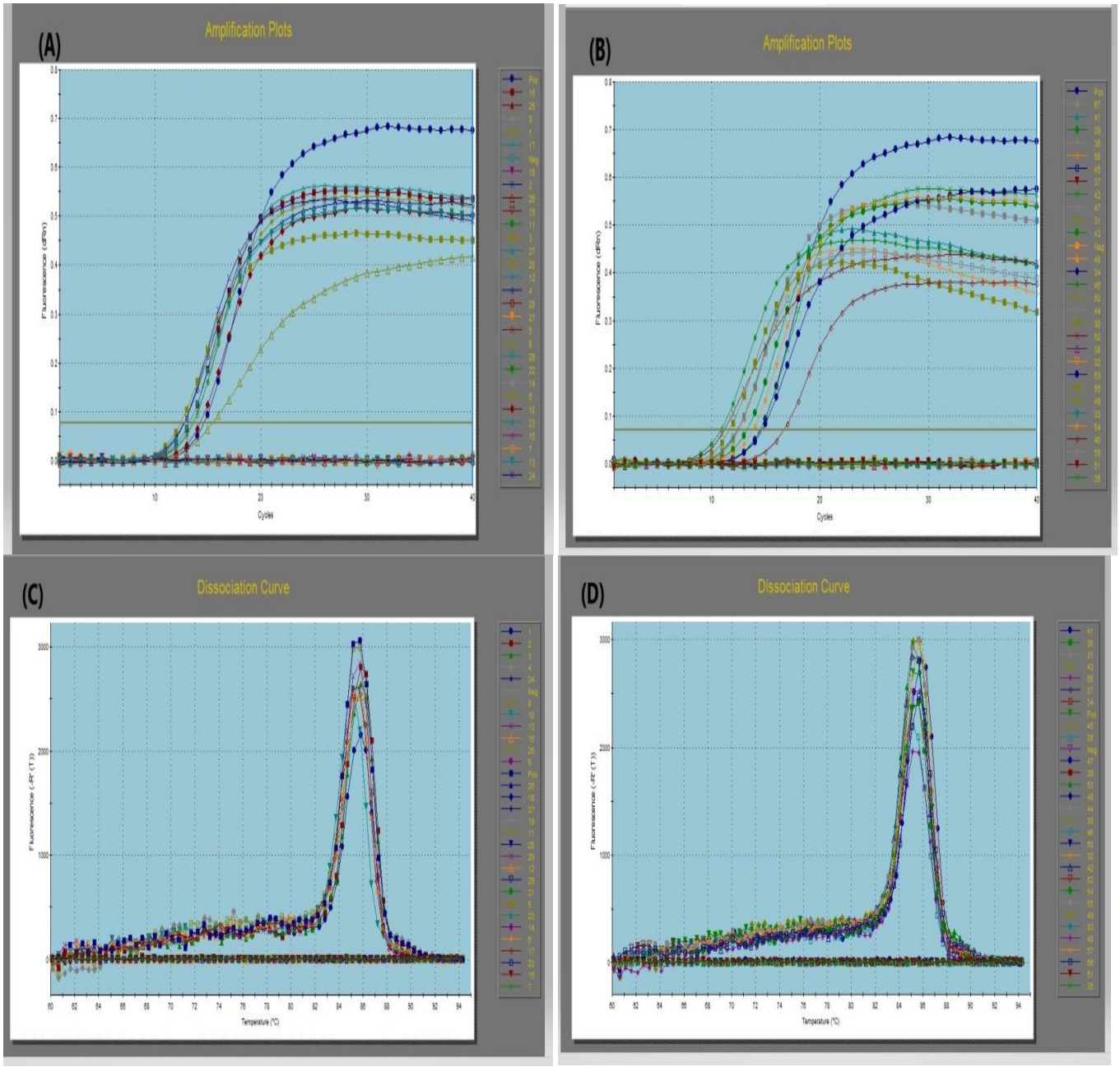


Figure 2. Syber green real-time PCR targeting *fli*C gene for 58 *Salmonella* strains isolated from chicken (fluorescence chart and melting curve). A) Fluorescence chart for strains number 1 to 29. B) Fluorescence chart for strains number 30 to 58 (Amplification plots represent the accumulation of product over the duration of real-time PCR). C) Melting curve for strain number 1 to 29, where strains number 1, 2, 3, 4, 8, 10, 13, 16 and 17 gave positive results. D) Melting curve for strain number 30 to 58, where strains number 40, 41, 42, 43, 46, 52, 53, 54, 55, 56, 57 and 58 gave positive result . (Melting curve provide representation of the PCR product after the amplification process. A single peak indicates a positive sample. Twenty-one *Salmonella* strains harbored *fli*C gene. The specificity of the reaction was confirmed by melting temperature, the mean peak temperature obtained was 85.65 °C.

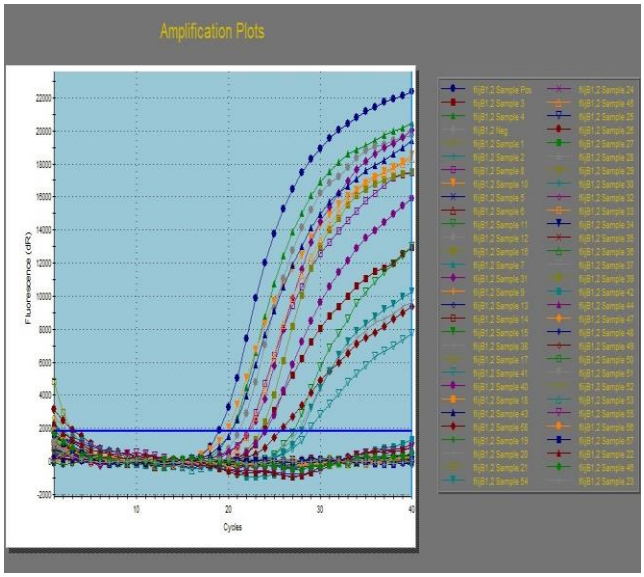


Figure 3. TaqMan real-time PCR amplification chart for *fljB*1,2 gene among 58 *Salmonella* strains isolated from chickens. Typical amplification curves given for positive samples. Fifteen strains (number 3, 4, 8, 10, 11, 12, 16, 31, 38, 40, 41, 43, 45, 54 and 58) gave positive results.

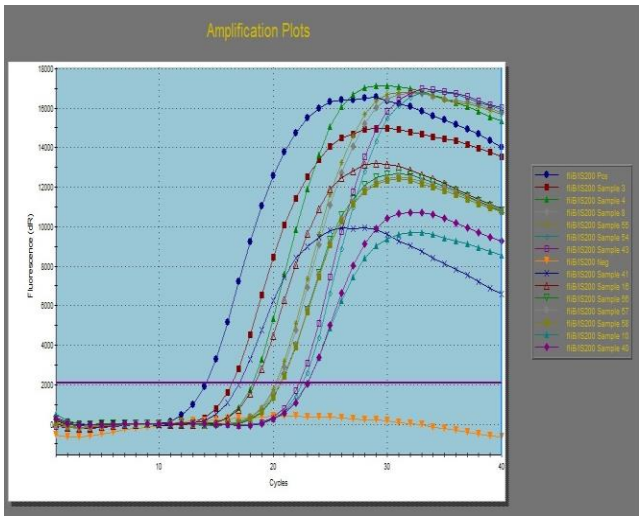


Figure 4. TaqMan real-time PCR amplification chart for *fliB/IS200* gene among 13 *Salmonella* isolates (12 *S.* Typhimurium and the variant strain serotype 1, 4, [5], 12:i:-) (*fliB/IS200* is *a* specific gene for *S*. Typhimurium biphasic and monophasic). Thirteen strains (number 3, 4, 8, 10, 16, 40, 41, 43, 54, 55, 56, 57 and 58) gave positive results.

DISCUSSION

Serological identification of 58 *Salmonella* strains was confirmed by slide agglutination test and the antigenic structure is demonstrated in table 3. Failure to identify the complete antigenic formula prevents the unequivocal identification of serovars even after phase inversion method. The strain was considered monophasic when phase inversion method was repeated at least three times without getting expression of phase 2 flagellar antigen as shown in strain number 57 with antigenic formula S.1, 4, [5], 12:i:-. Grimont and Weil (2007) mentioned that *S*.1, 4, [5], 12:i:- does not appear in the White-Kaufmann-Le Minor scheme and appears to be a monophasic variant of other biphasic serovars, which have lost phase 2 flagellin or the necessary switching mechanism of phase variation. Seven serovars of *S. enterica* subsp. enterica with the same O and phase 1 H antigens are possible ancestors of this serovar, including *S.* Typhimurium, *S.* Lagos, *S.* Agama, *S.* Farsta, *S.* Tsevie, *S.* Gloucester, and *S.* Tumodi. Among these, *S.* Typhimurium monophasic S.1, 4, [5], 12:i:- is commonly isolated from humans, animals, and the environment.

In recent years, many studies try to establish methods that can reduce the time for the detection and identification of salmonellae. Detection of bacteria by conventional methods is time-consuming and allows the detection of viable one only (Kim et al., 2006).

The use of PCR has emerged as an approach to overcome these problems. The exploration of gene targets for evaluation of absence and presence of bacteria is still a matter of importance. Several genes *inv*A, *fim*A, and *ace*K were used for identification of genus *Salmonella* (O'Regan et al., 2008). The duplex Syber green real-time PCR was applied for detection of genus *Salmonella* and the most common serovar *S.* Typhimurium based on melting Temp (TM) and Curve analysis using *16S rRNA* and *fli C* genes respectively. *16 S rRNA* not only allow the presence of bacteria to be proved but also would give information on gene expression .However, the expression of rRNA is tightly depend on physiological status of bacteria (Imre et al., 2005). In this study all 58 *Salmonella* strains harbor *16 S rRNA* (Table 4 and figure 1).

16SrRNA gene sequences contain hypervariable regions that can allow species-specific signature sequences important for identification of bacteria. The *16 SrRNA* gene is used as the standard for classification and identification of bacteria because it is present in most microbes and shows proper changes. *16 SrRNA* gene sequences for most bacteria are available on public databases such as NCBI (Pereira, 2010). Attractive potential uses of *16 S rRNA* gene sequence informatics for providing genus and species identification.

The *fliC* target is specific for the phase-1 flagellar antigen i that encoded by serovars Typhimurium. In the present study twenty one strains possess *fli*C gene serovars Typhimurium (12), Kentucky (6), Kedougou (1), Lagos

(1) and *S* .1,4, [5], 12:i:-.(1) (Table 4 and figure 2). O' Regan et al. (2008) reported that the i antigen is also expressed in uncommon serotypes such as Aberdeen, Bergen, and Kedougou. The structural flagellin gene *fliC* was present in all isolates of serovars Typhimurium and Kentucky (full length) and in all isolates of serovars Heidelberg, Hadar, and Enteritidis (partial length) (Dhanani et al., 2015).

Most *S. enterica* serovar Typhimurium possess two different flagellin proteins, including FliC *(*phase 1) and FljB (phase 2), which are encoded by the genes *fliC* and *fljB*, respectively. European Food Safety Authority (EFSA) (2010) applied a conventional PCR protocol to confirm the absence of 2nd phase antigen. A real- time PCR assay was used to differentiate *S.* Typhimurium monophasic variants from biphasic *S.* Typhimurium and from other variants (Anon, 2010; Tennant et al., 2010).

Fifteen isolates are positive for *fljB1,2 S.* Typhimurium (10), *S.* Paratyphi A(1) , *S.* Paratyphi B(1) , *S.* Newport (1) and *S.* Virginia (2) (Table 5 and figure 3). This result agree with that published by Bugarel et al. (2012) who reported that the second gene codes for the phase 2 flagellar antigen *fljB1,2* is present in *S.* Typhimurium and other serovars such as *S.* Coeln, *S.* Haifa, *S.* Heidelberg, *S.* Paratyphi B, *S.* Saintpaul and *S.* Stanley. This marker is absent in monophasic *S.* Typhimurium. Two serologically identified *S.* Typhimurium strains no. 55 ,56 don't possess *fljB1,2* that could be explained by repeat phase inverted method leads to formation of flakes which may lead to misidentification or wrongly identified strains.

Flagellar phase variation is formed by inversion of the genetic region called the H segment, which have the *hin* gene encoding for DNA invertase and the promoter for the *fljB* gene. The *fljB* constitutes an operon with the *fljA* gene, which encodes a negative regulator of *fliC* expression. FljA binds to the operator region of FliC mRNA and inhibits its translation, leading to the rapid degradation of FliC mRNA. When the H segment is in the "on" state, both *fljB* and *fljA* are transcribed, lead to synthesis of phase 2 flagellin and inhibition of phase 1 flagellin. However, when the H segment is switched to the "off" state, neither *fljB* nor *fljA* are transcribed, resulting in the synthesis of phase 1 flagellin only (Ido et al., 2014).

The location of IS200 between the genes *fliA* and *fliB* can be used as a specific marker for *S.* Typhimurium. The amplicon sizes from the *fliA–fliB* intergenic regions from *S.* Typhimurium and other serovars were expected to be 1000 and 250 bp, respectively. TaqMan real-time PCR could successfully detected *S.* 1, 4, [5], 12:i:- isolates that yield1000-bp amplicon with conventional PCR. These data suggest that *S.* 1, 4, [5], 12:i:- is a monophasic variant of *S.* Typhimurium (Burnens et al., 1997). Also, they reported that within the flagellin gene cluster of *Salmonella*, *S.* Typhimurium carries a conserved IS200 insertion sequence located downstream of the flagellin N-methylase gene (*fli*B) and upstream of the flagellar biosynthesis sigma factor gene (*fli*A). In the present study ten strains yield positive result with *fliC* , *fljB1,2* and *fliB*/ IS200 were biphasic *Salmonella* Typhimurium meanwhile 3 strains harbored the *fliC* and *fliB/ IS200* were monophasic strains *S* 1, 4,[5],12:i:- (Table 6) .

During recent years the cost of sequencing has been reduced dramatically making sequencing based typing more attractive. Some studies have reported DNA sequence for flagellin gene **(**Silverman, 1979; Joys, 1985 and De Vries, 1998). As in 2016, *fliC* sequence (partial coding sequence) has reported in GenBank with accession no DQ095491. This study reported sequencing of *fliC* gene for two strains *S.* Typhimurium and monophasic variant *S* 1, 4,[5],12:i:- with accession no (Mk103394) and (Mk103395), respectively.

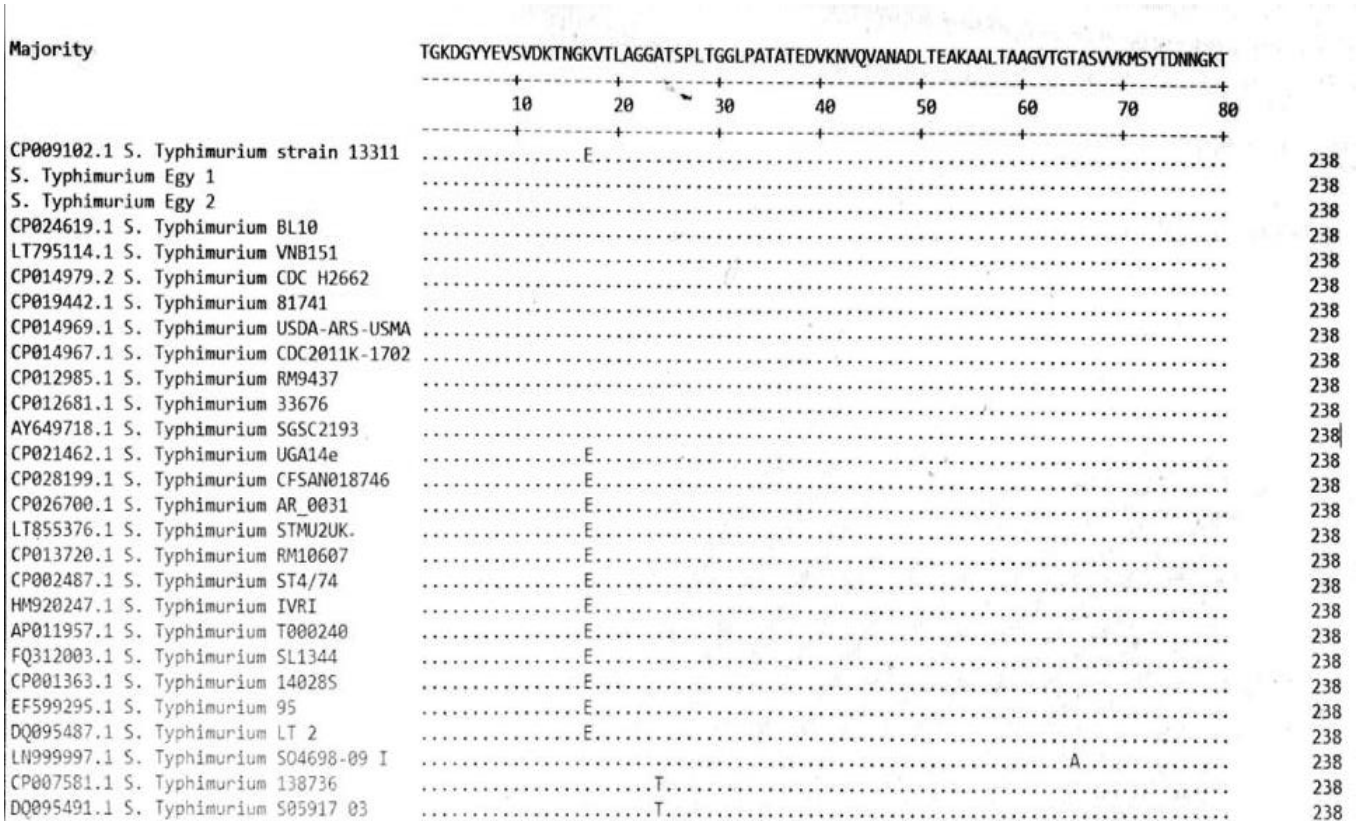
Protein sequence is the practical process of determining the amino acid sequence of all or part of protein or peptide. About 500 naturally occurring amino acids are known, 20 only appear in the genetic code there are termed as codons are always 3 Base pairs (nucleotides). In this study, amino acid sequence were applied for the *fliC* gene. In the location 14-19 sequence TNGKVT was found, which is similar to sequences coded in GenBank with accession no. CP024619, LT795114, CP014979, but in other sequences reported in GenBank with accession no. CP026700, CP021462, CP028199 glutamic acid was found between GK with amino acid sequence TNGEKVT (Figure 5).

In this study, the amino acid threonine was absent at position 24 in *S.* Typhimurium Egy1 and *S.* Typhimurium Egy2, which is similar to sequences recorded in GenBank with accession no. CP014979, CP014967. While the result disagreed with sequences coded in GenBank with accession number CP007581 and DQ09549 which have threonine at position 24 between glycine and alanine.

At position 60-65 found amino acid sequence AGVTGT in *S.* Typhimurium Egy1 and *S.* Typhimurium Egy2, but in sequence coded in GenBank with accession no. LN999997 amino acid alanine at positin 65 between glysine and threonine was found. Alignments show highly degree of identity. There are greater than 98% amino acid sequence identity (Figures 6 and 7). This is according to Sandjong et al. (2007).

Table 6. Comparison between results of conventional serotyping and real-time PCR for *Salmonella* Typhimurium (biphasic and monophasic strains)

No. of isolate	Name of isolate	Conventional serotyping			Real -time PCR		
		O antigen	Phase 1 H antigen	Phase 2 H antigen	*fliC*	*fljB1,2*	*fliB*/IS*200*
10 strains	*Salmonella* Typhimurium (diphasic)	4,[5],12	I	1,2	+	+	+
3 strains	*Salmonella* Typhimurium monophasic)	4,[5],12	I	Not detected	+	-	+
1 strain	Non-*Salmonella* Typhimurium	4,[5],12	I		+	-	-
5strains	Non *Salmonella* Typhimurium	4,[5],12	-	1,2	-	+	-



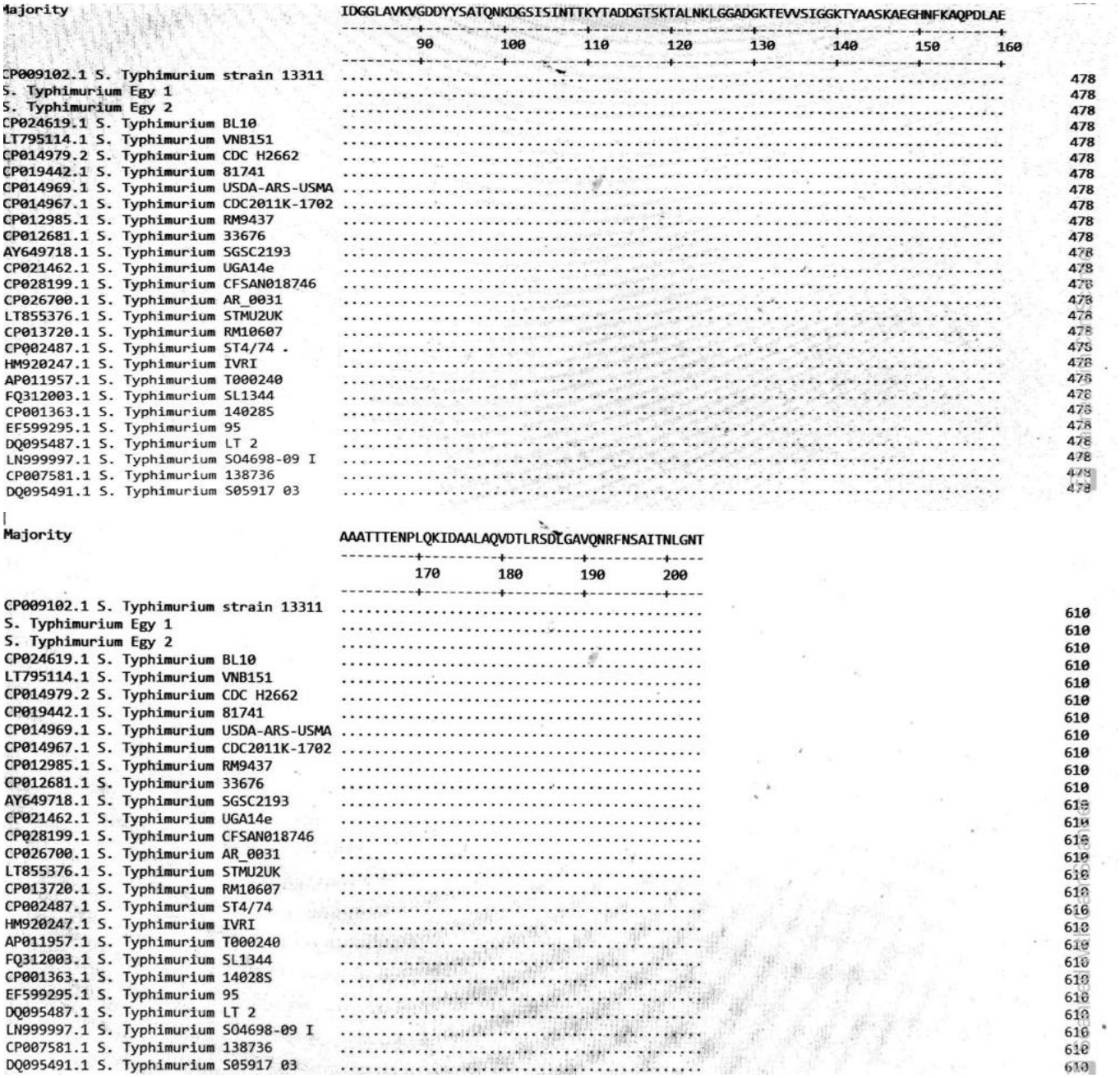


Figure 5**.** Amino acid sequence alignment report for *fliC* gene of two Egyptian *Salmonella* strains recorded in GenBank with accession number Mk103394 and Mk103395 for *S*. Typhimurium Egy 1(biphasic) and *S*. Typhimurium Egy 2 (monophasic), respectively. The sequence alignment of two Egyptian strains is 100% similar to nine strains recorded in GenBank (*S*. Typhimurium BL10, *S*. Typhimurium VNB151 , *S*. Typhimurium CDC H2662 , *S.* Typhimurium 81741 , S. Typhimurium USDA-ARS-USMA, *S*. Typhimurium CDC2011K-1702, *S.* Typhimurium RM9437, *S*. Typhimurium 33676 and *S*. Typhimurium SGSC2193). In the location 14-19, sequence TNGKVT was found for two Egyptian strains that matched sequences of some strains coded in GenBank with accession no. CP024619, LT795114, and CP014979, but in other strains reported in GenBank with accession no. CP026700, CP021462, and CP028199 glutamic acid was found between GK and amino acid sequence was TNGEKVT. The amino acid threonine was absent at position 24 in *S*. Typhimurium Egy1 and *S*. Typhimurium Egy2, but strains recorded in GenBank with accession no. CP007581 and DQ09549 have threonine at position 24 between glycine and alanine. At position 60-65 aminoacid sequence AGVTGT was found in *S*. Typhimurium Egy1 and *S*. Typhimurium Egy2, but in a sequence coded in GenBank with accession no. LN999997 amino acid alanine was found at position 65 between glycine and threonine.

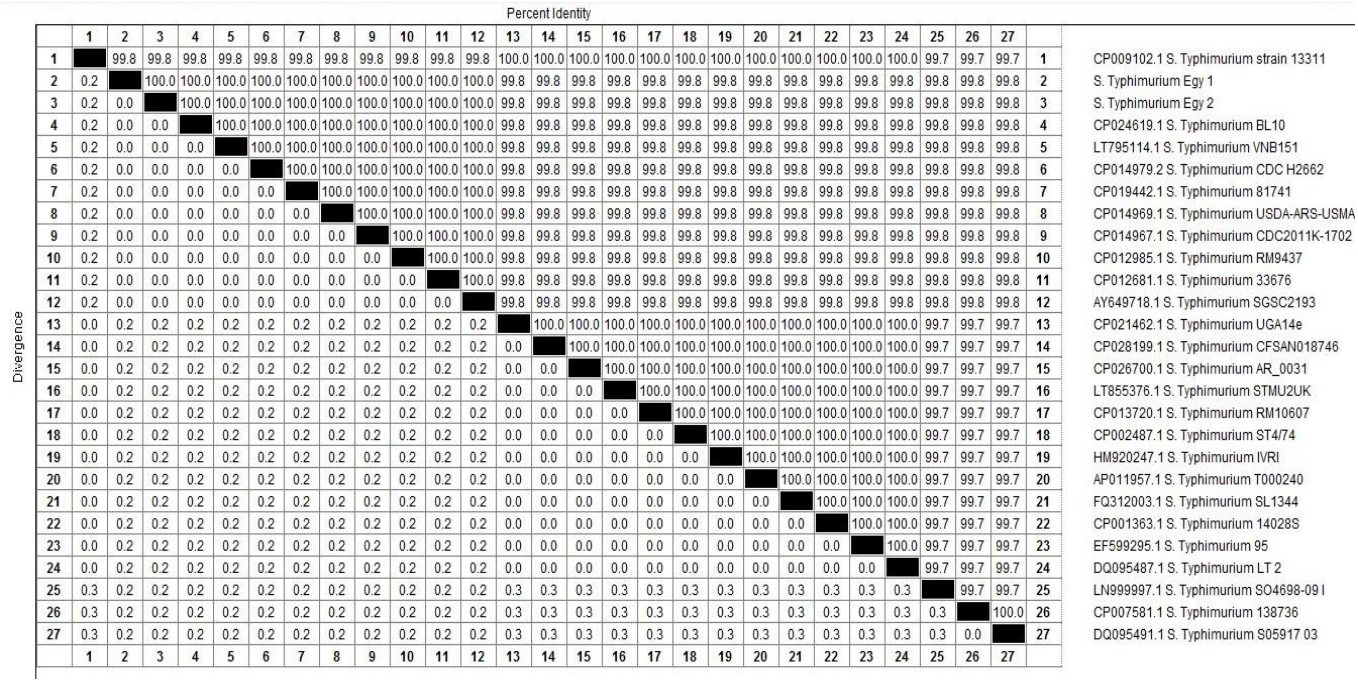


Figure 6. Amino acid sequence distance performed using the CLUSTAL W multiple sequence alignment program and version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise for *fli*C gene among two Egyptian *Salmonella* strains (*S.* Typhimurium Egy 1(biphasic) and *S.* Typhimurium Egy 2 (monophasic)).

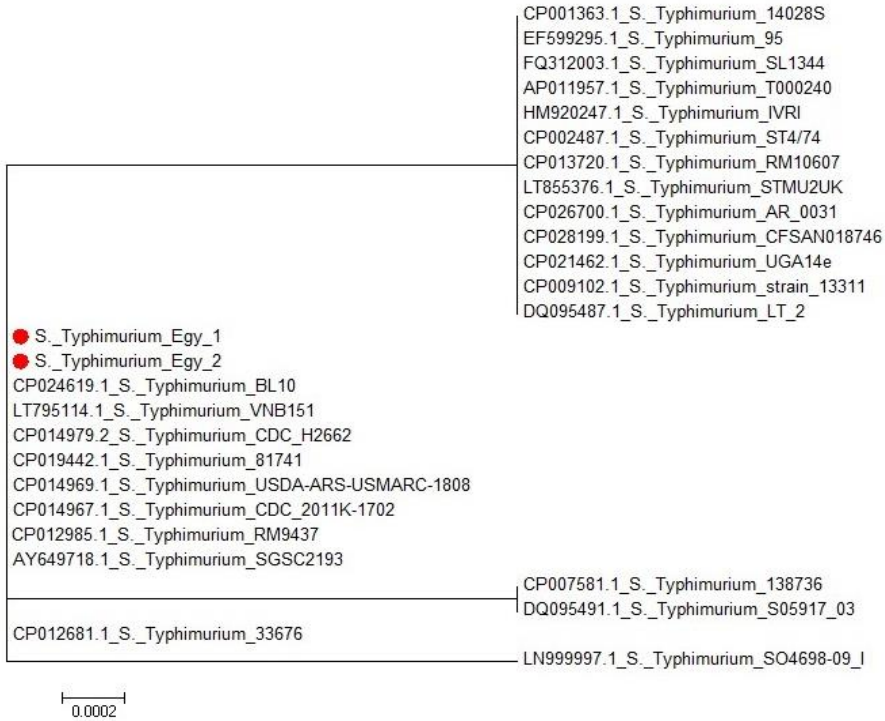


Figure 7. Phylogenetic analysis of *Salmonella* Typhimurium using fliC gene sequence performed by maximum likelihood, neighbor-joining and maximum parsimony implemented in MEGA6. The amino acid sequence of two Egyptian strain (Mk103394 and Mk103395) were closely related to sequences recorded in GenBank (CP024619 for *S.* Typhimurium BL10, LT795114 for *S.* Typhimurium VNB151, CP014979 for *S.* Typhimurium CDC H2662, CP019442 for *S.* Typhimurium 81741, CP014969 for *S.* Typhimurium USDA-ARS-USMA, CP014967 for *S.* Typhimurium CDC2011K-1702, CP012985 for *S.* Typhimurium, CP012681 for *S.* Typhimurium 33676RM9437 and AY649718 for *S.* Typhimurium SGSC2193).

CONCLUSION

The duplex real-time PCR is a rapid and robust method for detection of genus *Salmonella* and can be used for identification and differentiation of *S.* Typhimurium and the most common variant *S.*1, 4, [5], 12:i:-.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists**.**

Authors' contribution

All authors contributed equally to this work

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