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 fliB1, 2 and fliB/IS200 in the fliA-fliB intergenic region. Ten out of twelve S. Typhimurium harbored fliB 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 fliB1,2 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, fli 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 antiserum 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 fliC 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 fljB1.2 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 fljA-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 (fljB) 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 fliB 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 fliC by Syber green-based real-time PCR, and second, to differentiate between S. Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using fljB1,2 and IS200 in the fliA-fliB 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 37°C 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 16SrRNA and fliC genes**

| Target gene | Primer sequence (5'-3') | Reference |
|-------------|-------------------------|-----------|
| 16S rRNA    | F: CAGAAGAACGAGCCGCTAACTC | Yang et al., 2014 |
|             | R: GCGCTTACAGCCGCTAATT   |           |
| fiIC        | F: CAGTGTGCGCCAGGTTGTAAT  | Soumet et al., 1999 |
|             | R: ACTCCTGTGCGGCTGCGACTT  |           |

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 |
|-------------|-----------------------------------|-----------|
| fljB1,2     | F: TGT TAC TAT TGG TGG CCC TTA TAC TGG | Prendergast et al., 2013 |
|             | R: CAG CAG GCA TTG TGG TCT TAG      |           |
|             | FAM - CGC CAG CAA GGG TTA CTG TAC - TAMRA |         |
| fljB/IS200  | F: GAT CTG TCG ATG ATT CAT CTT CTG AC |           |
|             | R: AAC GCT TGT CTT CGG TAT TGG G    |           |
|             | CYS-TGG GGT GTG GCC 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).

**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:-- |
| 5  | S. Taksony   | 1,3,19: i: z6 | 34 | S. Labadi    | 8,20: d: z6 |
| 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 :z4,23: [1,5] |
| 9  | S. Anatum    | 3,[10] [15] [15,34]:e:h: 1,6 | 38 | S. Virginia  | 8: d :1,2 |
| 10 | S. Paratyphi A| 1,2,12: a: [1,5] | 39 | S. Papuana   | 6,7: r: e,n,z5 |
| 11 | S. Paratyphi B| 1,4,5,[12]: b: 1,2 | 40 | S. Typhimurium| 1,4,[5],12:i:1,2 |
| 12 | S. Kedougou  | 1,13,23: i: l,w | 41 | S. Typhimurium| 1,4,[5],12:i:1,2 |
| 13 | S. Kentucky  | 8,20: i: z6  | 42 | S. Kentucky  | 8,20: i: z6 |
| 14 | S. Labadi    | 8,20: d: z6  | 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: z6  | 46 | S. Kentucky  | 8,20: i: z6 |
| 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: z6 |
| 24 | S. Hadar     | 6,8: z10,e,n,x | 53 | S. Kentucky  | 8,20: i: z6 |
| 25 | S. Vichow    | 6,7,14: r: 1,2 | 54 | S. Typhimurium| 1,4,[5],12:i:1,2 |
| 26 | S. Vichow    | 6,7,14: r: 1,2 | 55 | S. Typhimurium| 1,4,[5],12:i:1,2 |
| 27 | S. Hadar     | 6,8: z10: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:1,2 |
| 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 *fljC* 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 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*, while strain no.57 with antigenic formula 1, 4 ,[5], 12:i:- and two strains *Salmonella* Typhimurium lacked this gene. Concerning *flj B*IS200, the 13 strains possess *fljB*IS200 (12 *S. Typhimurium* and the variant strain S 1,4 ,[5], 12:i:-) (Table 5 and figure 3).

*flj 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 *fliB* 1, 2. Sequencing of *fliC* gene based on the nucleotide sequence of *S.Typhimurium*1331 referenced in GenBank illustrated that the biphasic *S. Typhimurium* strain was recorded in GenBank as *S. Typhimurium* Egy 1 with accession number M103394 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 16SrRNA and *fljC* genes in *Salmonella* serovars using duplex Syber green real-time PCR

| No. | Name      | 16S RNA | *fljC* | No.  | Name      | 16S RNA | *fljC* |
|-----|-----------|---------|--------|------|-----------|---------|--------|
| 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. Pappuana | +       | -      |
| 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/IS200* in *Salmonella* serovars using duplex TaqMan real-time PCR

| No. | Name           | *fljB1,2* | *fliB/IS200* | No.  | Name           | *fljB1,2* | *fliB/IS200* |
|-----|----------------|-----------|--------------|-----|----------------|-----------|--------------|
| 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. Pauana      | -         | 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 16S rRNA 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 \textit{fliC} gene for 58 \textit{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 results. (Melting curve provide representation of the PCR product after the amplification process. A single peak indicates a positive sample. Twenty-one \textit{Salmonella} strains harbored \textit{fliC} gene. The specificity of the reaction was confirmed by melting temperature, the mean peak temperature obtained was 85.65°C.)
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 invA, fimA, and aceK 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 fliC 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).

16S rRNA gene sequences contain hypervariable regions that can allow species-specific signature sequences important for identification of bacteria. The 16 S rRNA gene is used as the standard for classification and identification of bacteria because it is present in most microbes and shows proper changes. 16 S rRNA 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 fliC gene serovars Typhimurium (12), Kentucky (6), Kedougou (1), Lagos...
(1) and S. 1,4, [5], 12:i:-i (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 FlhC (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. 55,65 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 fljA and fljB can be used as a specific marker for S. Typhimurium. The amplicon sizes from the fljA–fljB 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 (fljB) and upstream of the flagellar biosynthesis sigma factor gene (fljA). In the present study ten strains yield positive result with fliC, fljB1,2 and fljB/IS200 were biphasic Salmonella Typhimurium meanwhile 3 strains harbored the fliC and fljB/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 DQ95491. 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 TNGKVT (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 DQ95495 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 position 65 between glycine 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 | fljB/IS200 |
| 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 | + | - | - |
| 5 strains      | Non-*Salmonella* Typhimurium | 4,[5],12 | - | 1.2 | - | + | - |
Figure 5. Amino acid sequence alignment report for \textit{fliC} gene of two Egyptian \textit{Salmonella} strains recorded in GenBank with accession number Mk103394 and Mk103395 for \textit{S. Typhimurium} Egy 1 (biphasic) and \textit{S. Typhimurium} Egy 2 (monophasic), respectively. The sequence alignment of two Egyptian strains is 100% similar to nine strains recorded in GenBank (\textit{S. Typhimurium} BL10, \textit{S. Typhimurium} VNB151, \textit{S. Typhimurium} CDC H2662, \textit{S. Typhimurium} 81741, \textit{S. Typhimurium} USDA-ARS-USMA, \textit{S. Typhimurium} CDC2011K-1702, \textit{S. Typhimurium} RM9437, \textit{S. Typhimurium} 33676 and \textit{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 \textit{S. Typhimurium} Egy1 and \textit{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 \textit{S. Typhimurium} Egy1 and \textit{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 fliC gene among two Egyptian *Salmonella* strains (*S. Typhimurium* Egy 1 (biphasic) and *S. Typhimurium* Egy 2 (monophasic)).

| Percent identity |
|------------------|
| 1                |
| 2                |
| 3                |
| 4                |
| 5                |
| 6                |
| 7                |
| 8                |
| 9                |
| 10               |
| 11               |
| 12               |
| 13               |
| 14               |
| 15               |
| 16               |
| 17               |
| 18               |
| 19               |
| 20               |
| 21               |
| 22               |
| 23               |
| 24               |
| 25               |
| 26               |
| 27               |

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, CP014967 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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