Salmonella Typhimurium in Iran: Contribution of molecular and IS200 PCR methods in variants detection

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Abstract

Salmonella Typhimurium, a zoonotic pathogen, is regarded as a major health and economic concern worldwide. Recently, monophasic variants of this serovar have been significantly associated with human gastroenteritis outbreaks globally, making its accurate identification essential for epidemiological and control purposes. We have identified and analyzed 150 S. Typhimurium from 884 Salmonella genus isolated from humans, domestic animals, poultry, food items and abattoirs origins. The Salmonella isolates were obtained from Iranian National Veterinary Reference Laboratories of 9 provinces during 2007–2016, and from five hospitals in Tehran in 2015. The isolates were evaluated biochemically, serologically, and by PCR amplification of invA, mdh, STM4492, fliC, fljA, fljB, hin genes, IS200 and DT104. invA and mdh genes were used to confirm the S. Typhimurium serotype, fliC and fljB genes for determination of monophasic variants and amplification of IS200 to discriminate the monophasic variants from the closely related serotypes. We identified 78.6% (118/150) as classical S. Typhimurium (fliC, fljB and IS200 positive), 12.6% (19/150) were IS200 negative from all isolates. DT104 is another marker for S. Typhimurium serovar typing. Contrary to EFSA guidelines 20.6% (19/29) of human isolates that lacked IS200 insertion sequence, were confirmed as S. Typhimurium. Compared to the North American/European isolates the low prevalence of fljB negative 6% (9/150) and the high abundance of fliC negative 23.3% (35/150) isolates also were indicative of a different regional atypical population. Studies have shown that the prevalence of monophasic (fljB') S. Typhimurium worldwide is promoted by the Swine industry. Thus, one reason for this high number of different atypical strains could be inhibition of swine breeding system (house hold and industry) in Iran. These results demonstrate a need for a modified identifying protocol to overcome the regional differences.
Introduction

Non-typhoidal Salmonellae are a common cause of salmonellosis in humans and animals. Despite improvements in hygiene and food safety standards, the infection remains a common public health concern worldwide [1]. The spreading of infectious human Salmonella serovars occurs through consumption of contaminated food and water, contact with infected animals, international food trades and travelling by infected people within and among countries [2]. The burden of the infection is not limited to the associated morbidity and mortality costs of humans and animals, but it also includes the loss of trade and the consequent socioeconomic problems [2, 3]. Salmonella, enterica and bongori, are the two species of the genus with the former containing six subspecies enterica, salamae, arizonae, diarizonae, houtenae and indica. The most clinically important is S. enterica sub species enterica due to its common association with humans and warm-blooded animals and includes approximately 1500 serovars [4, 5]. Although potentially, all serovars of this subspecies could be pathogenic for humans, only about 80 of them account for nearly 99% of Salmonella infections in humans and domestic animals. Salmonella Typhimurium ranks among the top-five serovars, isolated from pigs, poultry, red meat and food-borne outbreaks in the European Union and the United States [4–8]. In Iran, the latest available data by the Center for Communicable Diseases and Control in 2011 showed that from 1038 reported food-borne diseases outbreaks, 5.1% were caused by Salmonella isolates, all serovars taken together [9].

Culture-based identification of Salmonella utilizes specific media and biochemical tests, followed by serotyping using special sera to determine the antigenic attributes of the isolated strains. This type of identification is based on the presence of somatic (O) and flagellar (H) antigens, according to the White-Kauffmann-Le minor scheme [10, 11]. The variable polysaccharide component of the cell wall specifies the O antigen of various serogroups and its presence, and variable expression of two flagellar antigens are determinants of the serovars [10, 11]. Alternative expression of fliC (H1) and fljB (H2) genes, controlled by hin locus, leads to phase-variation in biphasic motility in most of Salmonellas serovars [10–12]. fljB gene is located upstream of fljA, and FljBA mRNA codes both proteins. FljA protein represses the expression of phase I flagellar antigen, FliC [13]. The "phase variation" mechanism that controls both flagellar antigens in Salmonella spp., is an "on" and "off" switch, involving the reversible inversion of the DNA segment containing the fljB promoter and hin gene that codes the main DNA invertase regulator of phase variation. At "on" position, both FljA and FljB proteins are expressed, inhibiting fliC gene transcription, therefore, allowing the second flagellar phase to be expressed. Whereas, when the switch is at "off" position, neither genes are expressed, permitting fliC to be transcribed [14]. Determination of the antigenic formula of over 2600 serovars of Salmonella has been made possible by combining 46 different somatic and 114 different H antigens, identified in the White-Kauffmann-Le minor scheme [10–12, 15]. The antigenic formula for classic strains of S. Typhimurium is 1,4,[5],12:i,1,2 indicates the biphasic nature of the flagellar antigen, as specified by serotyping. However, variants lacking phase 1 (1,4,[5],12:i,1,2), phase 2 (1,4,[5],12:i:-) or both 1,4,[5],12:i:- are called "Salmonella Typhimurium-like" strains, as described by the European Food Safety Authority (EFSA). Except for the flagellar monophasic variant of 1,4,[5],12:i:- the other two variants have not been commonly the cause of salmonellosis in either humans or animals [10]. On the other hand, since 1990s, the monophasic serovar 1,4,[5],12:i:-, has reportedly been associated with a significant number of human salmonellosis outbreaks in different countries, and has been isolated from domestic animals, poultry as well as food items [10, 15].

Serotyping by slide agglutination using polyclonal and monoclonal antisera for differentiation of a variety of organisms, has been a valuable tool for surveillance and epidemiological
investigation of food-borne diseases [15]. However, this method is costly, time consuming and labor-intensive, while the procedure for detection of phase variation is technically demanding. Therefore, various DNA-based serotyping methods have been developed as alternative/complementary procedures to conventional sero-agglutination [10, 16]. Furthermore, the emergence and prevalence of Salmonella isolates lacking expression of phase 1 and/or phase 2 flagellar antigens have made the use of molecular methods essential [10]. PCR-based methods for identification and serotyping of S. Typhimurium are easy to perform, relatively inexpensive and reproducible [15, 17]. For serovar identification, amplification of Salmonella-specific invA, and for phase 1 and 2 antigens, fliC, fljA, fljB, fliB-fliA and hin genes have been proposed [18, 19]. Although, recently STM 4492 coding for a putative cytoplasmic protein, and malic acid dehydrogenase (mdh) genes have been suggested as more suitable targets for Typhimurium serovar identification [20, 21].

Salmonella Typhimurium DT (definitive type) 104 carries multiple drug resistance genes on an integrated DNA segment in the genomic island 1. Salmonella Typhimurium receptor for phage type 104 has been detected since 1994 and reported worldwide due to increasing consumptions of related antibiotics. The receptors are flagella such as FliC and FljB on the bacterial surface [22] and identification of this phage is now one of the diagnostic tools for subtyping S. Typhimurium serovar and increasing antibiotic resistance trait [23].

To discriminate the S. Typhimurium variants from their closely-related serovars that share the same antigenic formula and phase one flagellar antigen, amplification of fliB-fliA intergenic region which carries a serovar specific copy of IS200 insertion element and produces a 1000-bp amplicon instead of a 250-bp product is recommended by EFSA [10, 14, 19, 21, 23, 24].

Bacterial transposons are mobile genetic elements that promote DNA rearrangements and horizontal gene transfer of the host organism. Ellis et al. have shown that IS200 encodes (707bp gene size) transposase mRNA (tnpA). The sRNA coded in mRNA, starts in 5’ UTR of the gene, has a trans-acting effect repressing the expression of genes encoded within Salmonella Pathogenicity Island 1 (SPI-1) mRNA such as invF. However, despite its negative impact on the invasion of the bacteria into the non-phagocytic cells, it is recognized as a pathogenic marker [25].

In Iran, no published data are available on isolation of S. Typhimurium variant strains. Therefore, this study is aimed to investigate the variant strains among the isolates collected from humans, domestic animals, poultry, slaughter houses, red meat, chicken paste and non-domesticated birds, using sero-agglutination and PCR methods.

Materials and methods

Sample collection

The National Veterinary Reference Laboratories (NVRL), based in different provinces of Iran (S1 Fig), sent 819 Salmonella samples to the Iran Veterinary Organization from 2007–2016. The NVRL network is responsible for supervision and surveillance of farm animals’ health and diseases as well as the hygiene of the dependent industries in Iran. The samples had been identified as Salmonella at genus level through routine and random sampling using biochemical tests [26], and had been stored for further studies. Therefore, no live animals were used in our research. The origin of animals related samples were: feces from industrial animals [cattle, sheep, poultry (broiler, layer)]; fecal droppings from garden birds (sparrow, geese, duck, canary, parrot); the unprocessed food portions such as, red meat and chicken; the processed foods such as chicken paste; and water effluent from the slaughterhouses. There were no humans or human samples directly involved in this study. Sixty five Salmonella samples were
isolated from outpatients in microbiology laboratories of five different hospitals during 2015. The isolated *Salmonella* samples were collected by Microbiological Laboratory of the hospital and handed to us with no information or codes regarding the patient’s identity.

**Ethical approval**

Although no human samples or live animals were involved in this study, this proposal has been reviewed and approved by the committee on the ethics of animal experiments of the Pasteur Institute of Iran (permit number: IR.PII.REC. 1394.83).

**Cultivation and seroagglutination**

All *Salmonella* isolates were confirmed at the genus level using selective media, biochemical and molecular analysis [10, 19]. The isolates were cultivated overnight from transferred media into the Nutrient Broth, and subsequently subcultured on XLD selective media. A single colony from each plate was used for seroagglutination using polyclonal antibody against O antigen for B serogroup antigen (Mast, UK). The positive isolates were tested by polyclonal antibody against O4 antigen using purchased antisera (Mast, UK).

**Molecular identification and serotyping**

A single colony from all the 884 isolates, irrespective of seroagglutination results, was sub-cultured in Luria Bertani broth (Merck, Germany) and DNA extracted using a PCR template extraction kit (11796828001 Roche, Germany), according to the manufacturer’s protocol. Purity and concentration of the extracted DNA was determined by spectrophotometer (NanodropX100, USA), and 10–20 ng was used as PCR templates.

Specific primer pairs for *invA*, *mdh*, *STM4492* and *DT104* genes were used for identification and subtyping and 150 *Salmonella* of the 884 isolates were confirmed as *S*.Typhimurium (Table 1). Seroagglutination of phase 1 antigen was performed on the 150 confirmed *Salmonella* isolates using anti H(i) antisera (Mast, UK) (Table 1). Phase 2 flagellar antigen was tested only for the *S*.Typhimurium isolated from humans and STM4492PCR-negative isolates using antisera from Mast, UK.

*fliC*, *fliA*, *fljB*, and *hin* genes, as well as *flia–fliB* intergenic region were amplified to identify the *S*.Typhimurium serovar and its variants (Table 2). Amplifications were carried out in 20 μl using 2X master mix (Fermentas, Lithuania) with denaturation at 94˚C for 5 min and 30 cycles of 94˚C for 60 sec, annealing for each primer pair was as specified in the Table 2 for 30 sec and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 10 min. Genomic DNA

| Sample Province | Human | Slaughter-house | Cattle | Sheep | Red meat | poultry | Chicken paste | Gees | Duck | Canary | Parrot | Sparrows | Total |
|-----------------|-------|----------------|--------|-------|----------|---------|---------------|-----|------|--------|--------|---------|-------|
| Tehran          | 29    | 1              | 1      | 12    | 41       | 14      | 1             | -   | -    | 1      | 1      | 1       | 109   |
| Qazvin          | -     | -              | -      | -     | -        | 2       | -             | -   | -    | -      | -      | -       | 2     |
| Qom             | -     | 3              | -      | -     | -        | 1       | -             | -   | -    | -      | -      | -       | 4     |
| Mazandaran      | -     | -              | -      | -     | -        | 6       | -             | 1   | 1    | -      | -      | -       | 8     |
| Gilan           | -     | -              | -      | -     | -        | 2       | -             | -   | -    | -      | -      | -       | 2     |
| E. Azarbyjan    | -     | -              | -      | -     | -        | 9       | -             | -   | -    | -      | -      | -       | 9     |
| W. Azarbyjan    | -     | -              | -      | -     | -        | 7       | -             | -   | -    | -      | -      | -       | 7     |
| Sistan          | -     | -              | 5      | 1     | 2        | -       | -             | -   | -    | -      | -      | -       | 8     |
| Fars            | -     | -              | -      | -     | 1        | -       | -             | -   | -    | -      | -      | -       | 1     |
| Total           | 29    | 4              | 6      | 12    | 42       | 44      | 1             | 1   | 1    | 1      | 1      | 1       | 150   |

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from S. Typhimurium (ATCC14028) and S. Enteritidis (ATCC13076) were used as positive and negative controls, respectively. Amplifications were repeated at least twice and each round of PCR contained a tube with no template DNA to monitor the potential contamination in the PCR components.

Results

Out of 819 animals and 65 human Salmonellas isolates, 121 and 29 were positive for O4 (group B) antigen and PCR positive for invA and mdh genes respectively and were identified as S. Typhimurium (Table 1). The STM4492 gene was not detected in 6.6% (10/150) of the strains. One of the STM4492 negative strains belonged to human isolates (Table 3), three to red meat (Table 4), one to sheep (Table 5), four to chicken and one to chicken paste isolates (Table 6). Sero-agglutination tests for phase 1 and 2 flagellar antigens were repeated on these isolates, confirming the original identification and phase 2 serotyping was positive for one human, one red meat and one sheep sample.

Presence of flagellar genes was investigated with primers recommended by the EFSA [10], Amplification and expression of phase one flagellar antigen was observed in 79.3% (23/29) Salmonella Typhimurium from patients (Table 3). Of the remaining 6 isolates, 4 contained phase two flagellar gene (fliB) but serotyping results were negative and two were both genetically and phenotypically negative. The concomitant presence of each fliB and fliC genes and positive seroagglutination occurred in 86.2% (25/29) and 100% (29/29), respectively (Table 3).

Identification of DT104 subtype of S. Typhimurium showed that 43.3% (65/150) of S. Typhimurium isolates belonged to DT104 subtype. The results from five hospitals has shown that 95.0% (19/20) of positive DT104 isolates were collected from one hospital with three different flagellar genotype variants. The most prevalent clone of human patient isolates are diphasic isolates 58% (17/29) and the prevalence of negative phase one or two was similar in the remaining isolates (20.6% (6/29) Table 3).

The fliB gene was detected in 92.8% (39/42) of isolates from red meat and the PCR product for intergenic region (fliA-fliB) in all of these strains was the expected 1000 bp, except two
isolates which produced a 250 bp amplicon and 11.9% (5/42) of the isolates from red meats sold in shops were of DT104 subtype (Table 4). But 100% (18/18) of the Salmonella strains from live cattle and sheep were of DT104 phage type (Table 5).

In Sistan province, 87.5% (7/8) of isolates in 2011 showed 250-bp amplicon for fliA-fliB, which is the highest, and had different genetic pattern for flagellar genes. Out of these isolates one was from red meat (Table 4), five from cattle (Table 5), and one from chicken (Table 6).

Table 5 shows that 100% of the isolates from cattle, sheep and slaughter house carried fljB gene and 86.3% (19/22) were positive for DT104, which was the highest among the sample groups.

The most variation of S. Typhimurium isolates in genotype and in distribution belonged to the chicken group. The gene, fljB, was not found in 28.8% (13/45) of the isolates of poultry and chicken paste, but 23% (3/13) of these isolates showed 250bp amplification products for fliA-

| Human Isolate | Year | Province | invA | STM 4492 | mdh | Serotyping— (H:i) | fliC | Serotyping (H:1 &2) | fljB | fljA | hin | fliA-B IS200 bp | Phage type DT104 | Hospital |
|---------------|------|----------|------|----------|-----|-----------------|-----|-----------------|-----|-----|-----|----------------|-----------------|----------|
| H8            | 2015 | Tehran   | +    | +        | -   | -               | +   | +               | +   | -   | +   | 1000           | +               | Milad     |
| H9, H11, H12, H13 | 2015 | Tehran   | +    | +        | +   | +               | -   | +               | +   | +   | +   | 1000           | +               | Milad     |
| H10, H14, H15, H16, H17, H20, H21, H22, H23, H25, H26, H27, H28 | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 1000           | +               | Milad     |
| H31           | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 250            | -               | Emam      |
| H32           | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 250            | -               | Baghiyatalah |
| H18, H19      | 2015 | Tehran   | +    | +        | -   | -               | +   | +               | +   | -   | +   | 1000           | -               | Milad     |
| H29           | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 250            | -               | Milad     |
| H33, H34      | 2015 | Tehran   | +    | +        | -   | -               | +   | +               | +   | +   | +   | 250            | -               | Baghiyatalah |
| H24           | 2015 | Tehran   | +    | +        | +   | +               | -   | -               | -   | +   | +   | 1000           | +               | Milad     |
| H30           | 2015 | Tehran   | +    | +        | +   | +               | -   | -               | +   | +   | +   | 250            | -               | Milad     |
| H96           | 2015 | Tehran   | +    | -        | +   | +               | +   | +               | +   | +   | +   | 1000           | -               | kodakan   |
| H97           | 2015 | Tehran   | +    | +        | +   | -               | +   | +               | +   | +   | +   | 1000           | +               | Baghiyatalah |

Table 4. Slide-agglutination of H(i) flagellar antigen and PCR results of Salmonella samples, isolated from red meat.

| Red meat Isolate | Year | province | invA | STM 4492 | mdh | Serotyping— (Hi) | fliC | Serotyping (H:1 &2) | fljB | fljA | hin | fliA-B IS200 bp | Phage type DT104 PCR |
|------------------|------|----------|------|----------|-----|-----------------|-----|-----------------|-----|-----|-----|----------------|----------------------|
| R1               | 2013 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 1000           | -                     |
| R65              | 2011 | Sistan   | +    | +        | +   | +               | +   | +               | -   | -   | -   | 250            | -                     |
| R73, R76, R78, R82, R84, R83, R88, R90, R94, R102, R120, R98, R99, R100, R101, R104, R105, R106, R119 | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 1000           | -                     |
| R77, R80        | 2015 | Tehran   | +    | +        | +   | -               | -   | -               | +   | +   | +   | 1000           | -                     |
| R79, R138, R141, R142 | 2015 | Tehran   | +    | +        | +   | +               | +   | +               | +   | +   | +   | 1000           | +                     |
| R81, R85, R86, R87, R89, R91, R92, R93 | 2015 | Tehran   | +    | +        | -   | +               | +   | +               | +   | +   | +   | 1000           | -                     |
| R95             | 2015 | Tehran   | +    | -        | +   | -               | +   | +               | +   | +   | +   | 1000           | -                     |
| R118            | 2015 | Tehran   | +    | -        | +   | +               | +   | +               | +   | +   | +   | 1000           | -                     |
| R121            | 2015 | Tehran   | +    | -        | +   | +               | +   | +               | +   | +   | +   | 1000           | +                     |
| R136, R137      | 2015 | Tehran   | +    | +        | -   | -               | +   | +               | +   | +   | +   | 1000           | -                     |
| R139, R140      | 2015 | Tehran   | +    | +        | -   | -               | +   | +               | +   | +   | +   | 250            | -                     |
from all provinces in different years. The remaining 71.1% (32/45) of the isolates were fljB positive and only two of them produced a 250-bp product for fliA-fljB region (Table 6). The majority of the genetically non motile S.Typhimurium [77.7% (7/9)] were found in the poultry group and the remaining two genetically non motile isolates belonged to the red meat (R77 and R80). The expression of phase one flagellar antigen was not observed in 42.2% (19/45) of the isolates; however, there is a discrepancy where one isolate (C3) gave positive agglutination results, even though the gene for ‘i’ antigen was not detected by PCR (Table 6). The presence of DT104 gene cluster and the lack of IS200 were detected in 42.2% (19/45) and 11.1% (5/45) of the isolates, respectively (Table 6).

Slide agglutination on 47 bacteria isolated from chicken samples with antisera for phase one showed negative result in 42.5% (20/47) of the isolates (Table 6) which was the highest among all the categories.

All 12 Salmonella samples in wild birds group, that were isolated in 2009 were positive for both phase 1 and 2 antigen genes by PCR and the phase 1 by slide agglutination. All the isolates had fliA-fljB region of 1000bp (Table 7)

The results of testing different markers and genes produced 11 genetic groups of variants. The most common isolated Salmonella variant (group I) was genetically positive for all the tested genes (Table 8). Followed by VII variants (23.3% (35/150) lacking fliC gene and protein) (Table 8).

Almost 62.6% (94/150) of S.Typhimurium were genotypically diphasic, 32% (48/150) were genotypically monophasic, and 6% (9/150) did not contain any flagellar genes. Classical S. Typhimurium accounted for 78.6% (119/150) of the isolates (fljB and IS200 positive), and 8.0% (12/150) as EFSA monophasic variants (no fljB in II,III,IV,X,XI variants). Among all S.Typhimurium 25.3% (38/150) were genotypically monophasic for phase one antigen: no fliC (V, VII, VIII,IX). Out of 150 S. Typhimurium, 19 failed to produce IS200 insertion sequence which are distributed in six variant groups of I,II,IV,VII,X,XI (Table 8) of which, almost half belonged to the monophasic variant VII, 47% (9/19), lacking fliC gene and flagellar H(i) antigen.

### Discussion

Effective surveillance and control of pathogens require accurate identification and strain characterization. In this study, 150 out of 884 Salmonella isolates were identified as S.Typhimurium based on conventional and PCR methods, of which 121 were of non-human origin sent to the National Veterinary Laboratory and 29 from human diarrheal cases.

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Table 5. Slide-agglutination of H(i) flagellar antigen and PCR results of Salmonella samples, isolated from cattle, sheep and slaughter houses.

| Isolate | Source variety | Year | province | invA | STM 4492 | mdh | Serotyping — (H:i) | fliC | fljB | fljA | hin | fljA-BIS200 | Phage type | DT104 |
|---------|----------------|------|----------|------|---------|-----|-------------------|------|------|------|-----|------------|------------|-------|
| Ca66    | Cattle         | 2011 | Sistan   | +    | +       | +   | +                 | +    | +    | +    | +   | +         | 250        | +     |
| Ca67, Ca68, Ca 69, Ca70 | Cattle | 2011 | Sistan   | +    | +       | +   | -                 | -    | +    | +    | +   | +         | 250        | +     |
| Ca75    | Cattle         | 2014 | Tehran   | +    | +       | +   | -                 | -    | +    | +    | +   | +         | 1000       | +     |
| S124, S125, S127, S128, S129, S130, S131, S132, S133, S134, S135 | Sheep | 2015 | Tehran   | +    | +       | +   | +                 | +    | +    | +    | +   | +         | 1000       | +     |
| S126    | Sheep          | 2015 | Tehran   | +    | -       | +   | +                 | +    | +    | +    | +   | +         | 1000       | +     |
| S-H7    | Slaughter House | 2012 | Tehran   | +    | +       | +   | -                 | -    | +    | +    | +   | +         | 1000       | +     |
| S-H112, S-H113, S-H114 | Slaughter House | 2015 | Qom      | +    | +       | +   | +                 | +    | +    | +    | +   | +         | 1000       | -     |

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Table 6. Slide-agglutination of H(i) flagellar antigen and PCR results of *Salmonella* isolated from poultry.

| Isolate name | Source variety | Year | province | invA | STM 4492 | mdh | Serotyping—(H: i) | fliC | fljB | fljA | hin | fliA-B IS200 | Phage type
|--------------|---------------|------|----------|------|----------|----|------------------|------|------|------|-----|-------------|-----------|
| C71         | Chicken       | 2011 | Sistan   | +    | +        | -   | -                | -    | -    | -    | +   | 1000        | DT104     |
| C2          | Chicken       | 2013 | Tehran   | +    | +        | +   | -                | -    | -    | -    | +   | 250         | -         |
| C3          | Chicken       | 2013 | Tehran   | +    | +        | +   | +                | -    | -    | +    | -   | 1000        | -         |
| C72         | Chicken       | 2014 | Tehran   | +    | +        | -   | -                | -    | -    | -    | +   | 1000        | -         |
| C103        | Chicken       | 2015 | Tehran   | +    | +        | -   | -                | +    | +    | +    | +   | 1000        | +         |
| C107        | Chicken       | 2015 | Tehran   | +    | +        | -   | -                | +    | +    | +    | 250 | +           | -         |
| C108        | Chicken       | 2015 | Tehran   | +    | +        | -   | -                | +    | +    | +    | 1000| +           | -         |
| C109, C110  | Chicken       | 2015 | Tehran   | +    | +        | -   | -                | +    | +    | +    | 1000| -           | +         |
| C111, C115, C116 | Chicken | 2015 | Tehran | +    | +        | +   | +                | -    | -    | -    | -   | 1000        | +         |
| C117        | Chicken       | 2015 | Tehran   | +    | +        | +   | +                | -    | -    | -    | -   | 1000        | -         |
| C122        | Chicken       | 2015 | Tehran   | +    | -        | +   | +                | +    | 1000| +    | -   | -           | +         |
| C123        | Chicken       | 2015 | Tehran   | +    | -        | +   | -                | -    | -    | -    | -   | 1000        | -         |
| C149, C150  | Chicken       | 2015 | Tehran   | +    | +        | +   | +                | +    | -    | +    | +   | 1000        | +         |
| L35, L40    | Layer         | 2008 | Mazandaran | +  | +        | +   | +                | -    | +    | +    | +   | 1000        | -         |
| L37         | Layer         | 2008 | Mazandaran | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| L36, L38, L39 | Layer     | 2008 | Mazandaran | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| L6          | Layer         | 2011 | Qom      | +    | +        | -   | -                | +    | +    | +    | -   | 1000        | -         |
| L4, L5      | Layer         | 2012 | Qazvin    | +    | +        | -   | -                | +    | +    | +    | 1000| -           | +         |
| L62         | Layer         | 2013 | Gilan     | +    | +        | -   | -                | +    | 1000| +    | -   | +           | -         |
| L63         | Layer         | 2013 | Gilan     | +    | +        | +   | -                | +    | 1000| +    | -   | -           | +         |
| L74         | Layer         | 2014 | Fars     | +    | +        | -   | +                | +    | 1000| +    | -   | -           | -         |
| B41, B42, B43, B44, B45, B46, B47 | Broiler | 2007 | E. Azarbyjan | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| B48         | Broiler       | 2007 | E. Azarbyjan | +  | +        | +   | +                | -    | +    | +    | +   | 1000        | -         |
| B49         | Broiler       | 2007 | E. Azarbyjan | +  | +        | +   | +                | +    | +    | +    | 250 | +           | -         |
| B64         | Broiler       | 2011 | Sistan    | +    | +        | +   | +                | -    | +    | +    | +   | 250         | +         |
| B144, B146, B147, B148 | Broiler | 2016 | W. Azarbyjan | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | +         |
| B145        | Broiler       | 2016 | W. Azarbyjan | +  | +        | -   | +                | +    | 1000| +    | -   | +           | -         |
| Cp143       | Chicken paste | 2015 | Tehran   | +    | -        | -   | -                | +    | 1000| +    | -   | -           | +         |

C: chicken, L: Laying chicken, B: Broiler, Cp: Chicken paste

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Table 7. Slide-agglutination of H(i) flagellar antigen and PCR results of *Salmonella* samples, isolated from wild birds.

| Isolate | Source variety | Year | province | invA | STM 4492 | mdh | Serotyping—(H: i) | fliC | fljB | fljA | hin | fliA-B IS200 | Phage type
|---------|----------------|------|----------|------|----------|----|------------------|------|------|------|-----|-------------|-----------|
| Sp50, Sp51, Sp52, Sp 54, Sp55, Sp56, Sp57 | Sparrow | 2009 | Tehran | +    | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| Sp53    | Sparrow        | 2009 | Tehran | +    | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| G58     | Gees           | 2009 | Mazandaran | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| D59     | Duck           | 2009 | Mazandaran | +  | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| Cy60    | Canary         | 2009 | Tehran | +    | +        | +   | +                | +    | +    | +    | +   | 1000        | -         |
| P61     | Parrot         | 2009 | Tehran | +    | +        | +   | +                | +    | +    | +    | +   | 1000        | +         |

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In this study, *mdh* gene was amplified in all 150 isolates whereas, *STM4492* which had been suggested by Saeki *et al.* to be able to differentiate *S. Typhimurium* from 22 other *Salmonella* serovars failed to produce an amplicon in 6.6% of isolates [21]. All the isolates identified as *S. Typhimurium*, were tested for the presence of structural (*fliC* and *fljB*) and regulatory genes (*fljA* and *hin*) by PCR and phase one flagellar antigen (H(i) using seroagglutination [13]. The results of serology and PCR for phase one antigen were the same except for 1.3% (2/150) of the isolates differing from Bugarel *et al.* who reported a 12% (3/25) similarity between the results of the two tests (serology and gene absence for phase one) for S 1,4,[5],12:-:1,2 strains [19].

Based on the molecular results, 11 variants were identified. Similar to other studies Variant I was the most prevalent group. Although, monophasic 1,4,[5],12:i:- variant of *S. Typhimurium* has emerged as an important cause of salmonellosis, and it has been observed in many countries worldwide, this is the first report concerning their identification in Iran. As the prevalence of genotypically negative *fljB* was 12% (18/150) (variants II, III, IV, X, XI) of which 66.66% (12/18) were positive for IS$_{200}$ (Table 8), therefore, 8% (12/150) could be monophasic as recommended by EFSA.

In our study, following classical isolate, the monophasic serotypes of *fliC* was the second most prevalent group followed by *fljB*. The latter result is not in agreement with that reported by Bugarel *et al.* which showed that in France the genotypically negative *fljB* 28.9% (74/256) is the second most prevalent variant following the classical group [19].

Phase switching of flagellar protein expression is a common feature of *Salmonella* species [13]. In two isolates of variants III and IV that *fljA* gene is not detectable, the expression of *fliC* is in on position, while the *fljB* could still be subject to the phase variation (Table 8). For IV, VI and VIII variants that the *hin* gene is not present if the promoter for *fljB* gene is active, the expression of H(1,2) is locked in on or off position.

In this study, four out of 29 isolated *S. Typhimurium* 1,4,[5],12:i:- were from hospital patients that carried *fljB* gene, similar to that reported by Bugarel *et al.* [19] as “inconsistent” variant. The reason for this could be mutation(s) in the *fljB* gene out of the primer-binding site affecting protein structure and consequently inhibiting the serological detection or deletion of promoter of *fljB* gene [27]. These isolates were genotypically considered as Typhimurium-like

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**Table 8. Different variants of Salmonella isolates regarding Slide-agglutination and PCR results.**

| Variants | Total count | Serology (H:i) | fliC | fljB | fljA | Hin | IS200 | DT104 | flagellar phase prediction based on genotype |
|----------|-------------|----------------|------|------|------|-----|-------|-------|-----------------------------------------------|
|          |             | Serology | fljB | fljA | IS200 | DT104 |       |       | Non motile absence of both genes | Diphasic presence of both genes | Monophasic presence of either fliC gene or fljB |
| I        | 93          | +        | +    | +    | +     | 0    | 250 bp | +1000 bp | - | + | 93 | 0 |
| II       | 6           | +        | -    | +    | +     | 0    | 24 | 4 | 2 | 4 | 6 | 0 | 0 |
| III      | 1           | +        | -    | +    | -     | 0    | 1  | 1  | 1  | 1  | 0 | 0 |
| IV       | 2           | +        | +    | -    | -     | 2    | 0  | 0  | 1  | 0  | 2 | 0 | 0 |
| V        | 1           | +        | +    | +    | -     | 0    | 1  | 1  | 1  | 1  | 1 | 0 | 0 |
| VI       | 1           | -        | +    | -    | +     | 0    | 4  | 2  | 4  | 4  | 4  | 0 | 0 |
| VII      | 35          | -        | +    | -    | +     | 9    | 26 | 24 | 11 | 35 | 0 | 0 |
| VIII     | 1           | -        | -    | +    | -     | 0    | 0  | 0  | 0  | 0  | 0 | 0 |
| IX       | 1           | -        | -    | -    | +     | 0    | 1  | 1  | 1  | 1  | 0 | 0 | 0 |
| X        | 5           | -        | -    | -    | +     | 1    | 1  | 1  | 1  | 1  | 0 | 0 | 0 |
| XI       | 4           | -        | -    | -    | -     | 1    | 1  | 1  | 1  | 1  | 0 | 0 | 0 |
| Sub total|             | 19       | 131  | 85   | 65    | 48   | 93  | 0  | 0  | 9  | 0  |
| Total    |             | 150      | 150  | 150  | 150   | 150  | 150 | 150| 150| 150| 150|

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strains with some flaw in gene expression [19]. Similar events in \textit{fliC} gene could explain the results obtained with the \textit{fliC} positive, H:i negative isolate of group V. Moreover, mismatched nucleotides in primer annealing sites of \textit{fliC} gene could have produced the \textit{fliC} negative but H:i positive strain of the variant VI (Table 8).

Bugarel \textit{et al.} have shown that 28.9\% (74/256) and 1.1\% (3/256) of the isolated strains from various origins (except humans) (Table 2) were genotypically \textit{fljB} and \textit{fliC} negative, respectively [19, 28]. Whereas, in our study using the same primers, the \textit{fljB} and \textit{fliC} negative isolates from various origins were 10.6\% (16/150) and 27.3\% (41/150), respectively. This result could represent a new pattern for prevalence of \textit{S}.\textit{Typhimurium} variants in Iran.

One of the parameters of a serotype spread in a community is the animal industry, studies have shown that the first isolates of monophasic \textit{fljB} negatives were from poultry (1986) and later detected in pig industry (2003) [15]. Moreover, different studies have shown that the majority of the monophasic \textit{Salmonella} strains (1,4,[5],12:i:-) were isolated from pigs and pork industry in Spain (1999) [28], beef and pork products in China (2015) [29] and pigs, and turkey in Great Britain [30]. In Iran, the meat industry is mostly based on poultry producing 2.2 million tons yearly being the seventh largest producer of chicken meat in the world [31]. On the other hand, pig is not bred in house-hold or in industry and there is no record of importing the pork products. This condition could eliminate the propagation and circulation of the well adapted monophasic \textit{S}.1,4,[5],12:i:- variants of pork origin in Iran. To our knowledge there is no publication on prevalence of monophasic variants from other Islamic countries for comparison.

The profile of the genetic markers used for identification of monophasic \textit{S}.\textit{Typhimurium} have also been considered as indicators of the clonal lineage of these isolates [13, 19, 25, 32]. The “Spanish” and the “USA clone” of monophasic \textit{S}.\textit{Typhimurium} have been reported negative for \textit{fljA} as well as \textit{fljB}, which are a genotypic combination observed in only one monophasic \textit{S}.\textit{Typhimurium} (1,4,[5],12:i:-) isolated from human (Table 3) and in 2/7 genotypically monophasic \textit{fljB} negative animal isolates (Tables 4 and 6). Therefore, most of the Iranian monophasic \textit{fljB} negative isolates lacked only the \textit{fljB} gene (6/9) (Table 8) and were not similar to Spanish/French (\textit{fljA}, \textit{fljB}, \textit{hin}) and USA (\textit{fljA}, \textit{fljB}) clones [19].

In this study, 6\% (9/150) of the isolates (X, XI) were genotypically non-motile. Two were from red meat and seven from chicken. These isolates were mostly from Tehran (8/9) that were collected in 2015. In the study by Bugarel \textit{et al.}, the rate of non-motile was close to 6.6\% (17/256), but none of the isolates were genotypically negative for both genes [19].

Some of the factors that affect the severity and prognosis of the disease induced by \textit{S}.\textit{Typhimurium} are the presence of DT104 prophage [33], flagellar antigens and IS200 [25]. \textit{S}.\textit{Typhimurium} DT104 is a human food-borne and animal pathogen that carries DT104 phage. In our study, 150 samples were tested for DT104, the highest isolation rate was observed in samples from cattle (86.3\% (19/22) Table 5), highlighting the importance of these in dissemination of DT104/U302 subtypes with possible phage-associated resistance genes. A review in 1995 from UK has shown that the prevalence of \textit{S}.\textit{Typhimurium} DT104 in cattle/sheep, swine and poultry was 70\%, 36\%, and 52\%, respectively [34]. However, in our study, the prevalence of DT104 for cattle/sheep, human and chicken were 86.3\%, 68.8\%, and 42.2\%, respectively. Almost half the \textit{S}. \textit{Typhimurium} isolates (45.1\% (42/93) in group I or in classic group belonged to this subtype (Table 8).

Flagella play an important role as receptor for DT104 [22] and among the 65 confirmed DT104 positive isolates 64.6\% (42/65) carried both flagellar genes; 18.6\% (12/65) were positive for \textit{fljB} and negative for \textit{fliC} gene and 9.2\% (6/65) were \textit{fliC} positive and \textit{fljB} negative (Table 8). This is in accordance with Shin \textit{et al.} that showed the presence of both flagella is important as receptor for DT104 phage that belongs to the group FI and FII of phage families [22].
One of the important markers of *S. Typhimurium* is IS200 sequence located in *fliA*-B region, downstream of *fliC* gene [35]. According to the EFSA guidelines for *S. Typhimurium* identification, both classical and *fljB*-monophasic variants carry IS200 in *fliB*-fliA intergenic region by which *S.*Typhimurium and its variant are distinguished from other *Salmonella* serovars namely: S. Lagos, S. Agama, S. Farsta, S. Tsevie, S. Gloucester, and S. Tumodi [10]. Here in human category (Table 4), 3/13 classical and 1/6 monophasic S.1,4,[5],12:i:- of confirmed *S.*Typhimurium in this study (Table 8) lacked IS200 and thus did not support the EFSA suggestion that IS200 should be present in all variants of *S.*Typhimurium. Bugarel et al has also shown that IS200 is absent in 0.4% (1/256) of the European isolates [19].

Beuzone et al. have shown that IS200 could rearrange chromosome up to several kb near its insertion site when it transposes [35]. The proximity (<1.5 kb) of the *fliC* gene and IS200 insertion site, may also affect the presence of *fliC* gene when IS200 transposes. In our study, the comparison of the ratio of *S.*Typhimurium lacking IS200 among variant I (4.3%) and variant VII (25.7%) showed 5.97 times increase in the absence of IS200 in variant VII that lacks *fliC* gene (Table 8).

**Conclusion**

Our results showed that *S. Typhimurium* with IS200 deletion is a fairly common serotype among the Iranian *Salmonella* isolates of human and non-human origins. Therefore, with the procedure recommended by EFSA some of the variants will not be correctly identified and therefore missed. In addition, with 34.3% prevalence of DT104 subtype that could carry resistant genes, among the atypical strains highlight the importance of adaptation of new guidelines for identification of all the variants.

**Supporting information**

S1 Fig. Location of provinces that *Salmonella* samples were obtained.
(TIF)

S1 File. The raw data of *Salmonella* samples. Molecular detection of genes involved in the study.
(XLSX)

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References

1. Ido N, Lee K-i, Iwabuchi K, Iizumiya H, Uchida, Kusumoto M, et al. Characteristics of Salmonella enterica serovar 4,[5], 12:i:- as a monophasic variant of serovar Typhimurium. PLoS One. 2014; 9(8): e104380. https://doi.org/10.1371/journal.pone.0104380 PMID: 25093666

2. Forshell LP, Wierup M. Salmonella contamination: a significant challenge to the global marketing of animal food products. Rev sci tech Off int Epiz. 2006; 25(2):541–54.

3. Tadesse G, Tessema TS. A meta-analysis of the prevalence of Salmonella in food animals in Ethiopia. BMC microbiology. 2014; 14(1):270.

4. de Freitas Neto O, Penha Filho R, Barrow P, Berchieri Junior A. Sources of human non-typhoid salmonellosis: a review. Revista Brasileira de Ciência Avícola. 2010; 12(1):01–11.

5. Ranieri ML, Shi C, Switt AIM, Den Bakker HC, Wiedmann M. Comparison of typing methods with a new procedure based on sequence characterization for Salmonella serovar prediction. Journal of clinical microbiology. 2013; 51(6):1786–97. https://doi.org/10.1128/JCM.03201-12 PMID: 23554194

6. Desai P, Porwollik S, Long F, Cheng P, Wollam A, Bhonagiri-Palsikar V, et al. Evolutionary genomics of Salmonella enterica subspecies. mBio 4 (2): e00198-13. https://doi.org/10.1128/mBio.00198–13 Erratum; 2013.

7. Zdragas A, Mazarakis K, Vafeas G, Giantzi V, Papadopoulos T, Ekateriniadou L. Prevalence, seasonal occurrence and antimicrobial resistance of Salmonella in poultry retail products in Greece. Letters in applied microbiology. 2012; 55(4):308–13. https://doi.org/10.1111/j.1472-765X.2012.03298.x PMID: 22943611

8. Boore AL, Hoekstra RM, Iwamoto M, Fields PI, Bishop RD, Swerdlow DL. Salmonella enterica infections in the United States and assessment of coefficients of variation: a novel approach to identify epidemiologic characteristics of individual serotypes, 1996–2011. PloS one. 2015; 10(12):e0145416. https://doi.org/10.1128/JCM.03201-12 PMID: 26701276

9. Asi HM, Gouya MM, Soltan-dallal MM, Aghili N. Surveillance for foodborne disease outbreaks in Iran, 2006–2011. Medical journal of the Islamic Republic of Iran. 2015; 29:285. PMID: 26913248

10. Panel EB. Scientific opinion on monitoring and assessment of the public health risk of “Salmonella Typhimurium-like” strains. EFSA Journal. 2010; 8(10):1826.

11. Grimont PA, Weill F-X. Antigenic formulae of the Salmonella serovars. WHO collaborating centre for reference and research on Salmonella. 2007; 9:1–166.

12. Silverman M, Zieg J, Hilinen M, Simon M. Phase variation in Salmonella: genetic analysis of a recombinational switch. Proceedings of the National Academy of Sciences. 1979; 76(1):391–5.

13. Yamamoto S, Kutsukake K. FljA-mediated posttranscriptional control of phase 1 flagellin expression in flagellar phase variation of Salmonella enterica serovar Typhimurium. Journal of bacteriology. 2006; 188(3):958–67. https://doi.org/10.1128/JB.188.3.958-967.2006 PMID: 16428400

14. Herrera-León S, McQuiston JR, Usera MA, Fields PI, Bishop RD, Wiedmann M. Emergence, distribution, and molecular and phenotypic characteristics of Salmonella enterica serotype 4, 5, 12:i:–. Foodborne pathogens and disease. 2009; 6(4):407–15. https://doi.org/10.1089/fpd.2008.0213 PMID: 19292687

15. Shi C, Singh P, Ranieri ML, Wiedmann M, Moreno Switt AI. Molecular methods for serovar determination of Salmonella. Critical reviews in microbiology. 2015; 41(3):309–25. https://doi.org/10.3109/1040841X.2013.837862 PMID: 24228625

16. Braun SD, Ziegler A, Methner U, Slickers P, Keiling S, Monecke S, et al. Fast DNA serotyping and antimicrobial resistance gene determination of Salmonella enterica with an oligonucleotide microarray-based assay. PLoS one. 2012; 7(10):e46489. https://doi.org/10.1371/journal.pone.0046489 PMID: 23056321

17. Maurischat S, Baumann B, Martin A, Malorny B. Rapid detection and specific differentiation of Salmonella enterica subsp. enterica Enteritidis, Typhimurium and its monophasic variant 4,[5], 12:i:– by real-time multiplex PCR. International journal of food microbiology. 2015; 193:8–14. https://doi.org/10.1016/j.ijfoodmicro.2014.10.004 PMID: 25462917

18. Bugarel M, Vignaud ML, Mouri F, Fach P, Brisabois A. Molecular identification in monophasic and non-motile variants of S almonella enterica serovar T yphimurium. Microbiologiyopen. 2012; 1(4):481–9. https://doi.org/10.1002/mbo3.38 PMID: 23233427

19. Boland C, Van Hessche M, Mahillon J, Wattiaux P. A liquid bead array for the identification and characterization of fljB-positive and fljB-negative monophasic variants of Salmonella Typhimurium. Food Microbiology. 2017.
21. Saeki EK, Alves J, Bonfante RC, Hirooka EY, de Oliveira TCRM. Multiplex PCR (mPCR) for the Detection of Salmonella spp. and the Differentiation of the Typhimurium and Enteritidis Serovars in Chicken Meat. Journal of food safety. 2013; 33(1):25–9.

22. Shin H, Lee J-H, Kim H, Choi Y, Heu S, Ryu S. Receptor diversity and host interaction of bacteriophages infecting Salmonella enterica serovar Typhimurium. PloS one. 2012; 7(8):e43392. https://doi.org/10.1371/journal.pone.0043392 PMID: 22927964

23. Pritchett LC, Konkel ME, Gay JM, Besser TE. Identification of DT104 and U302 Phage Types among Salmonella enterica Serotype Typhimurium Isolates by PCR. Journal of clinical microbiology. 2000; 38(9):3484–8. PMID: 10970411

24. Echeita MA, Herrera S, Usera MA. Atypical, fljB-Negative Salmonella enterica subsp. enterica Strain of Serovar 4, 5, 12:i− Appears To Be a Monophasic Variant of Serovar Typhimurium. Journal of clinical microbiology. 2001; 39(8):2981–3. https://doi.org/10.1128/JCM.39.8.2981-2983.2001 PMID: 11474028

25. Ellis MJ, Carfrae LA, Macnair CR, Trussler RS, Brown ED, Haniford DB. Silent but deadly: IS200 promotes pathogenicity in Salmonella Typhimurium. RNA biology. 2018; 15(2):176–81. https://doi.org/10.1080/15476286.2017.1403001 PMID: 29120256

26. Vandepitte J, Verhaegen J, Engbaek K, Rohner P, Piot P, Heuck C, et al. Basic laboratory procedures in clinical bacteriology: World Health Organization; 2003.

27. Boland C, Bertrand S, Mattheus W, Dierick K, Jasson V, Rosseel T, et al. Extensive genetic variability linked to IS26 insertions in the fljB promoter region of atypical monophasic variants of Salmonella Typhimurium. Applied and environmental microbiology. 2015:AEM. 00270–15.

28. Echeita MA, Aladueña A, Crucchaga S, Usera MA. Emergence and spread of an atypical Salmonella enterica subsp. enterica serotype 4, 5, 12:i− strain in Spain. Journal of clinical microbiology. 1999; 37(10):3425–-. PMID: 10488227

29. Yang X, Wu Q, Zhang J, Huang J, Guo W, Cai S. Prevalence and characterization of monophasic Salmonella serovar 1, 4,[5], 12:i−, an emerging Salmonella serotype that represents multiple distinct clones. Journal of clinical microbiology. 2009; 47(11):3546–56. https://doi.org/10.1128/JCM.00546-09 PMID: 19741087

30. Mueller-Doblies D, Speed K, Kidd S, Davies R. Salmonella Typhimurium in livestock in Great Britain—trends observed over a 32-year period. Epidemiology & Infection. 2018; 146(4):409–22.

31. Shirdelian A. Iran’s Red Meat Output Meets 90% of Demand, Financial Tribune 2017.

32. Soyer Y, Switt AM, Davis M, Maurer J, McDonough P, Schoonmaker-Bopp D, et al. Salmonella enterica serotype 4, 5, 12:i−, an emerging Salmonella serotype that represents multiple distinct clones. Journal of clinical microbiology. 2009; 47(11):3546–56. https://doi.org/10.1128/JCM.00546-09 PMID: 19741087

33. Leekitcharoenphon P, Hendriksen RS, Le Hello S, Weill F-X, Baggesen DL, Jun S-R, et al. Global genomic epidemiology of Salmonella Typhimurium DT104. Applied and environmental microbiology. 2016: AEM. 03821–15.

34. Agriculture Mo. Data from Salmonella in Livestock Production, 1995, Ministry of Agriculture Fisheries and Food, a Veterinary Laboratory Agencies, UK). 1995.

35. Beuzón CR, Chessa D, Casadesús J. IS200: an old and still bacterial transposon. International Microbiology. 2004; 7(1):3–12. PMID: 15179601