Current advances in molecular subtyping using multilocus variable number of tandem repeat analysis of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Egyptian chickens

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**Corresponding author:** Hassan WMM, El Tawab AAA, El-Shannat SM (2020) Current advances in molecular subtyping using multilocus variable number of tandem repeat analysis of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Egyptian chickens, *Veterinary World*, 13(10): 2252-2259.

**Received:** 08-06-2020, **Accepted:** 21-09-2020, **Published online:** 28-10-2020

**doi:** www.doi.org/10.14202/vetworld.2020.2252-2259

**How to cite this article:** Hassan WMM, El Tawab AAA, El-Shannat SM (2020) Current advances in molecular subtyping using multilocus variable number of tandem repeat analysis of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Egyptian chickens, *Veterinary World*, 13(10): 2252-2259.

**Abstract**

**Aim:** This study aimed to characterize the genetic diversity, evolutionary level, and prevalence of genotypes of common isolates of *Salmonella* (Enteritidis and Typhimurium). Using one of the most advanced molecular recognition techniques, multilocus variable number of tandem repeat analysis (MLVA), we characterized the genotype and prevalence of *S*. Enteritidis and *S*. Typhimurium.

**Materials and Methods:** One hundred and twenty-five internal organ samples were collected from the major chicken slaughterhouses in Egypt and *Salmonella* species were isolated. PCR was utilized to amplify the IE-1 and Flc-C genes to identify *S*. Enteritidis and *Salmonella* Typhimurium DNA, respectively, from *Salmonella* isolates. MLVA was applied on nine samples of *S*. Enteritidis DNA and three samples of *S*. Typhimurium DNA. Six variable number tandem repeat (VNTR) loci (Sal02, Sal04, Sal06, Sal10, Sal20, and Sal23) were amplified.

**Results:** Of the examined samples (n=125), a total of 12 isolates (9.6%) were either identified as Enteritidis or Typhimurium. PCR-mediated amplification of IE-1 and Flc-C revealed that 75% (n=9) of the 12 *Salmonella* isolates were *S*. Enteritidis and 25% (n=3) were *S*. Typhimurium. The six loci amplified through MLVA had allelic diversity. The most discriminatory heterogenic locus for *S*. Enteritidis was Sal20. Sal04 and Sal23 were the most discriminatory heterogenic loci for *S*. Typhimurium. VNTR allelic profile analysis revealed nine unique genotypes for *S*. Enteritidis and three for *S*. Typhimurium.

**Conclusion:** This study was the first to use MLVA analysis to identify *S*. Enteritidis and *S*. Typhimurium strains isolated from chickens in Egypt. The molecular typing data reported herein allowed us to characterize the genotypes of *S*. Enteritidis and *S*. Typhimurium that are most prevalent in Egyptian chickens. Moreover, this epidemiological information provides valuable insight on how to prevent disease transmission. Moreover, our methods provide an alternative to traditional serotyping techniques that may produce inaccurate strain identifications for organisms with rough lipopolysaccharide structures.

**Keywords:** lipopolysaccharides, multilocus variable number of tandem repeat analysis, *Salmonella*, serotyping.

**Introduction**

*Salmonella enterica* serotypes Enteritidis and Typhimurium are two of the most famous zoonotic strains of *Salmonella* worldwide [1]. The serotype *Salmonella* Enteritidis possesses one of the most prominent risks for severe economic danger in Europe and many countries throughout the world [2]. The serotype Typhimurium is considered a major cause of human and animal salmonellosis [3]. A multi-country outbreak of *S*. Enteritidis confirmed through whole-genome sequencing (WGS) analysis is currently ongoing. There are currently 314 confirmed cases in Austria, France, Ireland, Luxumbourg, and the United Kingdom. Besides, Austria, Belgium, Denmark, the Netherlands, Norway, and the United Kingdom reported collectively 21 probable and 50 historical probable cases. Confirmed cases belong to three closely related genetic clusters. Possible cases have been related to the multilocus variable number tandem repeat (VNTR) multilocus variable number of tandem repeat analysis (MLVA) profiles 2-11-3-3-2 and 2-12-3-3-2. However, 1 additional confirmed isolate has an MLVA profile of 2-9-3-3-2 [4].

Poultry and poultry byproducts are serious vehicles in infection transmission to consumers [5]. Moreover, chickens infected with *S*. Enteritidis play a role in infection spread through trans-ovarian transmission during the stage of egg development [6]. Most reported cases of *S*. Typhimurium infection are also related to infected food products; few cases are reported as a result of direct contact with infected animals or contaminated water or environment [7,8].

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Due to a lack of distinctive genotype properties between S. Enteritidis and S. Typhimurium isolate, it is possible that they have evolved from the same ancestor clone. Determining an accurate clone is complicated by the fact that the *Salmonella* species is not asexual and the final end product of recombination replacement can result in a large number of highly diverse genotypes under the same clonal complex. The clonal diversity of bacteria varies according to the extent of recombination that can vary depending on bacterial species [9]. As MLVA and WGS are not utilized in all EU/EEA countries, other European Union countries may be inadvertently affected by this outbreak. This underscores the need for the use of MLVA analysis to identify comorbidities related to the ongoing outbreak. Performing WGS analysis on isolates associated with the outbreak on MLVA profiles would further confirm their participation in the ongoing outbreak [4].

In Egypt, there is a lack of genetic-relatedness data collected from S. Enteritidis and S. Typhimurium isolated from chickens. It is imperative to use highly advanced molecular subtyping techniques such as MLVA, to accurately identify strains with rough lipopolysaccharide structures.

A combination of MLVA with other molecular techniques, such as pulsed-field gel electrophoresis (PFGE), ribotyping, and phage typing, provide clear data that can accurately identify the outbreak strain and distinguish it from epidemiologically unrelated isolates [10-12].

The three methods previously noted have been proven incompetent in *Salmonella* typing when not used in conjunction with techniques such as MLVA. Many reports demonstrate that PFGE is considered insufficient as a tool for molecular typing of *Salmonella* species, mainly due to poor reproducibility in different laboratories. Ribotyping also has low discrimination power and is not useful in the current epidemiological investigation and surveillance study of the ongoing outbreak [13-16]. The use of phage typing is not ideal because it revealed that some S. Enteritidis strains could not be typed due to phage conversion among strains [17].

For these reasons, MLVA is considered the current method of choice for genotyping *S. enterica* (S. Enteritidis and S. Typhimurium) in most laboratories. MLVA is fast, highly reproducible, cost-effective, can be used with small amounts of DNA, can discriminate between clinical isolates, and allows for systematic analysis as it opens a way for data standardization through processing and development of web-based resources for database query [18,19]. MLVA analyzes the variation in the numbers of tandem repeated sequences in DNA at multiple genomic loci [20]. This means that each target region is individually amplified using specific primers that anneal to the flanks of the repeat-containing region. Determination of the amplicon sizes by electrophoresis on agarose gels enables the number of tandem repeat units present at each locus to be deduced [21-23].

The application of the MLVA technique using Interspersed Repetitive Units-Variable Number of Tandem Repeats for the identification of S. Enteritidis and S. Typhimurium strains isolated from chickens provides accurate data to appropriately identify outbreak strains and distinguish them from epidemiologically unrelated isolates. Furthermore, the MLVA technique can help in determining the prevalence of different *Salmonella* genotypes.

This study aimed to characterize the genetic diversity, evolutionary level, and genotype prevalence of the most predominant isolates of *Salmonella* (S. Enteritidis and S. Typhimurium) in Egyptian chickens. Using the advanced MLVA technique allowed us to decipher the genotypes and spread of S. Enteritidis and S. Typhimurium in Egypt.

**Materials and Methods**

**Ethical approval**

No ethical approval was needed to perform this study. However, the samples were treated according to the national and international criteria.

**Sampling**

One hundred and twenty-five random samples from broiler chickens tissues (internal organs) were collected from different slaughterhouses in Egypt for examination, in the period from December 2017 to October 2019.

The samples were collected following the protocol recommended by the International Commission on Microbiological Specification for Food [24]. The samples were transported on ice to the reference laboratory for quality control on poultry production (Animal Health Research Institute, Dokki, Giza, Egypt). The samples were sealed in sterile bags and stored at −86°C.

**Bacteriological examination**

The samples were treated according to the method described by standard ISO 6579:2002 [25] and CDC manual [26].

**Isolation of *Salmonella* species from chicken tissue samples**

In brief, 5 g of tissue samples, which included intestine, liver, kidney, heart, and spleen, were thawed at room temperature under aseptic conditions. The samples were then macerated into fine pieces using sterile blades, homogenized using a sterile pestle and mortar, added to 45 mL of buffered peptone water (HiMedia, India), and then incubated at 37°C for 18 h. After the incubation, either 0.1 mL or 1 mL of pre-enriched aliquots were transferred into 10 mL Rappaport and vassiliadis broth for the enrichment and incubated at 42°C for 24 h (HiMedia, India). Finally, the enriched aliquot samples were seeded on MacConkey and xylose-lysine-deoxycholate (XLD) agar [27] and incubated at 37°C for 24-48 h for the observation of the growth of typical *Salmonella* colonies (HiMedia, India), (i.e., pink colonies with a black center on XLD plates and colorless colonies on MacConkey plates).

*Veterinary World, EISSN: 2231-0916* 2253

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**Extraction of Salmonella genomic DNA**

Genomic DNA was obtained from *Salmonella* subcultures from XLD (HiMedia, India), plates according to manufacturer’s instructions from the QIAamp Blood and Tissue Kit for Gram-Negative Bacteria (Qiagen®, Hilden, Germany). Briefly, colonies (2-3) were harvested in a microcentrifuge tube by centrifuging for 10 min at 5000× g (7500 rpm). Supernatants were subsequently discarded and the remaining pellet was resuspended in 180 μL buffer ATL. 20 μL of proteinase K was added and the mixture was incubated for 2 h at 56°C to allow for extensive bacterial lysis. DNA was eluted from the QIAamp mini spin columns with 100 μL of buffer to increase the concentration of the recovered DNA. The concentration of DNA was measured using UV absorption with a spectrophotometer (BMG Labtech, Germany) at a wavelength of 260 nm. The DNA was stored at −20°C for further analysis.

**Molecular identification of *S. Enteritidis* and *S. Typhimurium* DNA based on IE-1 and Flic-C genes**

PCR was used to amplify the IE-1 and Flic-C genes, which are specific for *S. Enteritidis* and *S. Typhimurium*, respectively. A 316- and 432-bp fragment containing IE-1 and Flic-C gene sequences was amplified by PCR. Primers specific for IE-1 were: IE-1/F (5' - AGT GCC ATA CTT TTA ATG AC -3') and IE-1/R (5' - ACT ATG TCG ATA CGG TGG G -3'). Primers for Flic-C were: Flic-C/F (5' - CCC GCT TAC AGG TGG ACT AC -3) and Flic-C/R (5' - AGC GGG TTT TCG GTG GTT GT-3). These primers have been found to be specific and more reliable for the differentiation of *S. Typhimurium* from other members of the *Salmonella* species [28]. DNA samples were subjected to two differential amplification reactions in two separate tubes. The *S. Enteritidis* specific amplification reaction mixture was identical to those of *S. Typhimurium* with the exception of the primers targeting IE-1 instead of Flic-C. PCR reactions were carried out in 25 μL containing 5 μL of 5× PCR master mix (Jena bioscience®, Germany), 3 μL of each primer (forward and reverse) at a working concentration of 10 pmol/μL, (Metabion International AG®, Germany) 2 μL of DNA template, and 12 μL ddH2O. For MTBC identification, the primer set IE-1/F, IE-1/R, and Flic-C/F, Flic-C/R was used according to the following parameters: Initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 30 s, and a final extension at 72°C for 7 min [29]. The amplification products were resolved by electrophoresis on a 1.5% agarose gel, followed by ethidium bromide staining (0.5 μg/mL) and examination under UV light.

**MLVA**

Literature was extensively reviewed to identify primer sequences for *S. enterica* loci with potential to distinguish *S. Enteritidis* and *S. Typhimurium* strains. Six polymorphic tandem repeat loci, which vary in size from 107 to 250 bp, were expected to have potential to distinguish the two serovars [30]. In this study for MLVA analysis, six VNTR (0681, 0764, 0789, 2053, 4301, and 4774) primer pairs were used (Table-1) [31]. The PCR reaction was performed in a total volume of 25 μL per reaction with the following reagent concentrations: 5 μL of 5× PCR master mix (Jena bioscience®, Germany), 3 μL of each primer (forward and reverse) at a working concentration of 10 pmol/μL, (Metabion International AG®, Germany) 12 μL ddH2O, and 2 μL of genomic DNA as a template. PCR was conducted (Biometra, Germany) with the following cycling parameters: Initial denaturation at 96°C for 5 min, followed by 30 cycles at 96°C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

Positive and negative controls were included in each set of reactions and consisted of the same reaction mixtures with genomic DNA of *S. Enteritidis* and *S. Typhimurium* reference strains as the template for the positive control and no template DNA in the negative control. The PCR fragments were analyzed by gel electrophoresis using 2% agarose stained with ethidium bromide for each locus. Sizes of amplicons were estimated by comparison with 50 bp markers (Jena bioscience®, Germany), and the number of repetitive units was determined. The genomic sequences of *S. Enteritidis* and *S. Typhimurium* (ATCC 13076 and ______________________________________________________

| VNTR locus | VNTR locus alias | VNTR locus size (bp) | PCR Primer sequences (5'-3') |
|------------|------------------|----------------------|-----------------------------|
| 0681       | Sal 02           | 6                    | (F) GGA AAG ACT GGC GAA CAA AT (R) TCG CCA ATA CCA TGA GTA CG |
| 0764       | Sal 04           | 20                   | (F) TCG CAC AGA TGA CCA ATT TT (R) GAT CGA GGC TCA CTG C TT |
| 0789       | Sal 06           | 6                    | (F) TTG TCC CGGGGACTATTAAGT (R) GTCGCTGGTACGGCGAGT |
| 2053       | Sal 10           | 12                   | (F) GCGTCTGACGCGTGAGTTT (R) TGGAAATGATGGCATCAGC |
| 4301       | Sal 20           | 3                    | (F) CACCGCCACCAACTTACAG (R) ACTGACCGTGCGGAC |
| 4774       | Sal 23           | 12                   | (F) CCCCCGACAGAGGAGAGAGA (R) ACCGGGTTGCGCTACCAT |

List of primers used for *S. Enteritidis* and *S. Typhimurium* strains typing obtained from [31]. A total of six loci were selected for amplification of DNA from *Salmonella* isolates.

**Table-1:** Primer sequences and the size of the repeat units of the VNTR loci in this study.

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Table-2: Description of each VNTR locus, repeat unit size, flank size, tandem repeat copy number, number of alleles, and fragments length size.

| Locus | Repeat unit size (bp) | No. of alleles | Tandem repeat coy number | Fragment length (bp) | Flank size (bp) | Description of each VNTR locus* |
|-------|------------------------|---------------|--------------------------|----------------------|----------------|--------------------------------|
| Sal 02 | 6                      | 4 2           | 1, 2, 3, 4, 5            | 95, 101, 107, 113    | 89            | (6×1)+89, (6×2)+89... etc.     |
| Sal 04 | 20                     | 3 3           | 1, 2, 3                  | 174, 194, 214        | 154           | (20×1)+154, (20×2)+154...etc.  |
| Sal 06 | 6                      | 2 2           | 2, 3                     | 156, 162             | 144           | (6×2)+144, (6×3)+144            |
| Sal 10 | 12                     | 3 2           | 1, 2, 3                  | 184, 196, 208        | 172           | (12×1)+172, (12×2)+174...etc.  |
| Sal 20 | 3                      | 6 2           | 8, 10, 11, 12, 13, 14   | 169, 175, 178, 181, 184, 187 | 145 | (3×8)+145, (3×10)+145...etc.   |
| Sal 23 | 12                     | 3 3           | 3, 4, 5                  | 250, 262, 274        | 214           | (12×3)+214, (12×4)+214...etc.  |

*Each VNTR locus was described as (repeat unit size×copy number) + size of the partial repeat. SE=Salmonella Enteritidis, Tm=Salmonella Typhimurium

ATCC 14028, respectively) were also used as reference strains and were evaluated to obtain the amplicon for each locus [32].

Determination of tandem repeats

The number of tandem repeats in each amplified MLVA locus was estimated based on the amplified MLVA loci size and the size of the flanking region using the formula Table-2.

PCR assays for the six loci were repeated and compared within and between gels to ensure consistent estimation of size and tandem repeat copy number.

Results

PCR-mediated detection of S. Enteritidis and S. Typhimurium DNA from isolates based on the presence of IE-1 and Flic-C genes 12 Salmonella isolates (9.6% of the samples collected) were identified in this study. All DNA extracted from bacterial isolates was subjected to PCR amplification of target sequences specific for S. Enteritidis (IE-1) and S. Typhimurium (Flic-C) (Figure-1). PCR based IE-1 gene and Flic-C identification for Salmonella isolates revealed that 9/12 of the Salmonella isolates were S. Enteritidis and 3/12 were S. Typhimurium (Table-3).

Genotyping of S. Enteritidis and S. Typhimurium using MLVA

MLVA was applied to 12 samples of Salmonella DNA which was classified into two groups. Based on the amplification of the six VNTR loci, the first group consisted of nine samples which were classified as S. Enteritidis and the second group consisted of three samples that were classified as S. Typhimurium. These six polymorphic loci identified 12 unique MLVA genotypes (Table-4). Nine unique genotypes were specific to S. Enteritidis (A, B, C, D, E, F, G, H, and I) while three unique genotypes (J, K, and L) were specific to S. Typhimurium.

Data for all six loci (0681, 0764, 0789, 2053, 4301, and 4774) were found to be polymorphic in both S. Enteritidis and S. Typhimurium DNA. A representative agarose gel is shown in Figures-2 and 3. All MLVA types were comprised single isolates with unique genotypes (Table-4) and yielded the results (Table-2), we expected based on a previous report [31]. The length of the yielded amplicon in bp must be calculated as number of repeats (Table-2).

Discussion

In recent decades, the S. enterica serovars enteritidis and Typhimurium have been considered the main source of outbreaks in Egyptian poultry farms [33]. The use of effective and accurate molecular typing techniques has significantly benefited epidemiological studies. To prevent and detect potential outbreaks, further exploration of transmission methods is necessary. For this purpose, MLVA was recently introduced in Egyptian laboratories to investigate outbreaks of both S. Enteritidis and S. Typhimurium serovars. However, some economical and technical obstacles, including economic instability, higher cost of advanced molecular genotyping techniques, and the lack of adequate laboratory equipment for characterization of S. Enteritidis and S. Typhimurium, prevent widespread use of MLVA in Egypt.
This study documented the occurrence of *S. Enteritidis* and *S. Typhimurium* in Egyptian chickens. The findings are of public health concern, especially in the realm of poultry farming. The results revealed that 75% of the isolates submitted for PCR analysis were *S. Enteritidis* while the remaining 25% were *S. Typhimurium*. The serovars were distinguished through the amplification of two distinct primer sets. The enteritidis specific primer (IE-1) was reported previously [31,34]. The Typhimurium specific primer (Flic-C) amplified a gene that participates in flagellin synthesis and was chosen from a whole-genome sequence database for the *S. Typhimurium* strain AY649720 retrieved from GenBank [29,35]. The specificity of primer pairs was confirmed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov). The results of PCR amplification showed sharp bands of a 316-bp fragment (diagnostic for *S. Enteritidis*) and a 432-bp fragment (diagnostic for *S. Typhimurium*).

MLVA was assessed as a molecular tool for genotyping 12 samples of *Salmonella* DNA. Of those 12 samples, nine samples were *S. Enteritidis* and three were *S. Typhimurium*. DNA was extracted from isolates based on traditional epidemiological tracing information using six VNTR markers. These markers comprised Sal02, Sal04, Sal06, Sal10, Sal20, and Sal23 from a previously reported MLV A scheme developed for *S. enterica* [30].

There were 12 unique MLV A genotypes (A, B, C, D, E, F, G, H, and I) that represented *S. Enteritidis*. 

Figure-1: PCR- identification of *Salmonella* Enteritidis and *Salmonella* Typhimurium DNA based IE-1 sequences and allele-specific Flic-C gene. (a) DNA extraction from *Salmonella* isolate. Amplicons obtained with IE-1 (lanes 2, 5) with the size of 316 bp. Lanel: 100 base pair ladder, (Jena bio science®). (b) DNA extraction from *Salmonella* isolate. Amplicons obtained with Flic-C (lanes 2, 3) with the size of 432 bp. Lanel: 50 base pair ladder, (Jena bio science®).

Figure-2: The results of multilocus variable number of tandem repeat analysis typing, based on DNA extracted from grown *Salmonella* Enteritidis isolates. (a) MLVA typing of DNA from grown *S. enteritides* isolates using the 0681 locus (Lane 2, 3, and 4), with a tandem repeat size of 6 base pairs. Lane1: 50 base pair ladder; Lane 2, 3 and 4: Amplicon obtained based on 0681 locus with fragment length of 113 bp (4tr); Lane 5, and 6: negative and positive control respectively. (b) MLVA typing of DNA from grown *S. enteritides* isolates using the 2053 locus (Lane 2, 3, and 4), with a tandem repeat size of 12 base pairs. Lane1: 50 base pair ladder; Lane 2, 3 and 4: Amplicon obtained based on 2053 locus with fragment length of 208 bp respectively; Lane 5, and 6: negative and positive control respectively. (c) MLVA typing of DNA from grown *S. enteritides* isolates using the 0764 locus (Lane 2, 3, and 4), with a tandem repeat size of 20 base pairs. Lane1: 50 base pair ladder; Lane 2, 3 and 4: Amplicon obtained based on 0764 locus with fragment length of 174 bp (1tr); Lane 5, and 6: negative and positive control respectively. (d) MLVA typing of DNA from grown *S. enteritides* isolates using the 4774 locus (Lane 2, 3, and 4), with a tandem repeat size of 12 base pairs. Lane1: 50 base pair ladder; Lane 2, 3 and 4: Amplicon obtained based on 4774 locus with fragment length of 250 bp (3tr) ; Lane 4, and 5: negative and positive control respectively. (e) MLVA typing of DNA from grown *S. enteritides* isolates using the 4301 locus (Lane 2, 3, and 4), with a tandem repeat size of 3 base pairs. Lane 1: 50 base pair ladder; Lane 2, 3 and 4: Amplicon obtained based on 4301 locus with fragment length of 175 bp (10tr), 175 (10tr), and 181 bp (12tr) respectively; Lane 5, and 6: negative and positive control respectively. (f) MLVA typing of DNA from grown *S. enteritides* isolates using the 2053 locus (Lane 1, 2, and 3), with a tandem repeat size of 12 base pairs. Lane 6: 50 base pair ladder; Lane 1, 2 and 3: Amplicon obtained based on 2053 locus with fragment length of 196 bp (2tr), 184 bp (1tr), and 208 bp (3tr) respectively; Lane 4, and 5: negative and positive control respectively.
Alternatively, three unique genotypes (J, K, and L) represented S. Typhimurium and Enteritidis genotypes were dispersed throughout the region of study area.

All six loci had polymorphisms in both S. Enteritidis and S. Typhimurium. The analysis of S. Enteritidis DNA revealed that five loci (0681, 0764, 0789, 2053, and 4774) were involved in only a 1-step change in TR copy number. The locus 4301 did exhibit a change in TR copy number, which varied from 8 to 14 copies with a 1- or 2-step change in the TR copy number. S. Typhimurium had a 1-step change only in the TR copy number in all loci examined.

This data could not be compared with other Egyptian studies as there have been no previous studies on the genotypes of S. Enteritidis and S. Typhimurium in Egypt. However, our results are similar to those reported from S. enterica isolated from blood and stool samples from symptomatic patients in France [30]. In the French study, loci Sal04, Sal06, Sal10, Sal20, and Sal23 showed higher diversity in tandem repeat copy number than in our study. Notably, there was a complete difference in the tandem repeat copy number of Sal 02. Another study based on the typing of Heidelberg isolates [36] revealed that tandem repeat copy numbers for Sal02,
Sal10, and Sal20 are similar to our reported results for S. Enteritidis. This suggests the possibility that not all Sal loci are informative for the identification of S. enterica serovars. Therefore, the formation of a locus standardization model for each serovar is an urgent necessity. The data from molecular typing have allowed us to discern the genotypes of S. Enteritidis and S. Typhimurium that are prevalent in Egyptian chickens. Moreover, this epidemiological information gives us insight into how to prevent disease transmission of salmonellosis [37].

Conclusion

MLVA typing is an effective and beneficial tool for investigating and identifying early warning signs of Salmonella outbreaks and providing epidemiological surveillance for S. Enteritidis and S. Typhimurium infections.

Authors' Contributions

SME created the research and experimental design, performed the laboratory experiment, data analysis, and wrote the manuscript. AAE supervised the experiment, checked the data analysis, and revised the manuscript. WMHH supervised the experiment, helped in the laboratory work, and revised the manuscript. All authors contributed to drafting and revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are thankful to Reference Laboratory for Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt, for providing the necessary laboratory facilities for this study. The authors did not receive any funds for this research.

Competing Interests

The authors declare that they have no competing interests.

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