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Phenotypic and Genotypic Identification of *Salmonella enteritidis* Isolates from Layers Chicken in Iraq

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Abstract: *Salmonella Enteritidis* is the most common serotype of *Salmonella* isolated from cases of foodborne gastroenteritis's throughout the world. This study aimed to characterize and identified of *Salmonella Enteritidis* bacteria from layer chickens. Intestinal cotton swabs samples have been collected from layer chickens. samples were grown in appropriate culture media and inspected both aerobically and under a microscope. Using the VITEK 2 system and biochemical testing, the diagnosis of the bacterial isolates was verified. The 2019 criteria from the Clinical and Laboratory Standards Institute (CLSI) were followed in interpreting the data. Five isolates of *Salmonella Enteritidis* were characterized using the *16S rRNA* gene and the PCR method. The NCBI received five positive isolates whose sequences were compared to isolates in the bank gene. These isolates were then given accession numbers, which are as follows: PP955421.1, PP955422.1, PP955423.1, PP955424.1, and PP955425.1. Only three Iraqi isolates, according to phylogenetic analyses based on *16SrRNA*, have the highest percent identity (100%), and are most closely related to Canada, China, South Korea, Australia, USA, China, Switzerland, Brazil, Russia, Egypt, Taiwan, and Turkey. The remaining Iraqi isolates have 99% identity. These findings all suggested that the SE clone may be propagated in these farms since the isolates were very genetically related and overwhelmingly dominant.

Keywords: Molecular diagnosis, PCR, *S. Enteritidis*, phylogenetic tree, *16SrRNA*.

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Introduction

Salmonella entritidis (*S. entritidis)* is a serious pathogen that is a global public health concern. It is one of the most common causes of foodborne illness and is frequently linked to eating tainted dairy, eggs, and poultry.

The *S. entritidis* infections can cause gastroenteritis's, which manifests as fever, diarrhea, and cramping in the abdomen. Severe consequences can arise from this illness, particularly in susceptible groups like the elderly, young children, and immunocompromised people (1). According to source attribution studies, infected poultry and poultry products are the main sources of human illness, with chickens being the single greatest reservoir host for *S. entritidis* (2). *Salmonella* spp. are the main foodborne pathogens, and several research have reported that chicken products are the main reservoirs of these bacteria (3, 4). More than 2500 serotypes of *Salmonella* spp. are known to exist; the most commonly implicated serovars are S. *enterica* enteritidis and Typhimurium types (the non-typhoidal *Salmonella* group) being the most frequently implicated serovars in salmonellosis outbreaks (5).

Accurately isolating and identifying *S. entritidis* is essential for conducting efficient outbreak investigations, monitoring food safety, and putting control measures in place. The gold standard for finding this bacterium in environmental and clinical samples is still traditional culture-based approaches including selective enrichment and plating. When combined with biochemical and serological identification approaches, these methods enable the reliable distinction of *Salmonella* from other intestinal bacteria (6).

Recent developments in molecular methods, including polymerase chain reaction (PCR), have improved the efficiency and precision of *Salmonella* detection even more, allowing for the direct identification of particular serotypes from complicated samples. For prompt public health responses and the decrease in the prevalence of *Salmonella* in food products, the integration of traditional and contemporary approaches is essential (7, 8).

The purpose of this study is to present a methodical approach to *S. entritidis* isolation and identification. By using selective enrichment, cautious sample processing, and confirmatory testing, we can identify and reduce the dangers connected to this pathogenic bacterium.

Materials and Methods

Sampling

100 samples were taken from the digestive tract of layer chickens of varying ages. The research was conducted between November 1st and December 1st 2023. All samples obtained by intestinal cotton swabs from Al-Sink Laboratories for Diagnosing Poultry Diseases (Iraq, Baghdad) were transported using non-selective peptone water broth. Every samples was moved right away to the lab for examination while being cooled.

Isolation and Identification

Every sample was cultivated for 24 hours at 37 °C in a non-selective peptone water broth and for 24 hours at 41.4 °C in 10 ml Tetrathionate broth (9). Following that, the cells were grown on a particular broth (Himedia's Selenite F broth) and incubated for 24 hours at 37 °C. On MacConkey agar plates (Oxoid), a loopful of each broth was streaked across the surface. Xylose Lysine was then incubated for 24 hours at 37 °C (9). According to the maker of the Gram's stain kit, Gram's staining was used to ascertain the purity of the culture and the staining characteristics of the isolated bacteria. The isolates were diagnosed morphologically using colony morphology tests, which included the application of pigment formation on various agar media and the shape of the colonies for conformation. Several biochemical assays were also performed to further confirm the diagnosis, including oxidase, catalase, (MR) Methyl red, (VP) Voges – Proskauer test, (KIA) Kliger Iron Agar test, and VITEK 2 (BioMérieux Marcy-l'Étoile, France) (10). The 2019 criteria from the Clinical and Laboratory Standards Institute (CLSI) were followed in interpreting the data (11).

Molecular identification

Total genomic DNA was isolated from the bacterial samples using (Favorgen, Taiwan) kit. The bacterial

Samples produced enough DNA concentration for PCR amplification, according to the DNA extraction results. DNA concentration and purity as determined by Thermo Fisher Scientific's NanoDrop technology.The 1.5% agarose gel electrophoresis was used to detect DNA integrity. Primers were generated using the NCBI

(National Centre for Biotechnology Information PRIMER BLAST Approach) (12). Conventional PCR was used to amplify the particular region *16SrRNA* (13). A ready-to-use PCR master mix (New England Biolabs, UK) containing 12.5 µl made up the 25 µl PCR reaction mix. 3 microliters of template DNA and 1 microliter (10 pmol) of every primer (Table 1). The reverse and forward primers were displayed in Table 2. We used nuclease-free water to fill the remaining capacity. A thermocycler (New England Biolabs, UK) was used to treat the mixes to the following thermal cycling conditions (Table 3).

Table (1): Reaction components of PCR.

Component	25 µL (Final volume)
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	16.5 µl

Table (2): The sequence of primers that used this study .

Gene		Sequence (5′-3′)	Product Size (bp)
16S rRNA	F	5'- AGAGTTTGATCCTGGCTCAG- 3'	1500bp
	R	5'- GGTTACCTTGTTACGACTT- 3’	

Table (3): The thermocycler of detection.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45 Sec	35 cycle
3-	Annealing	56°C	45 Sec	
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	5 min.	1 cycle

DNA sequencing and phylogenetic analysis

The PCR amplification resulted in five PCR products for the identification of the *16SrRNA* gene of some isolates of *S. enterica* from layer chicken samples. These products were stored at -20°C, and 10 µl of the DNA PCR product and primer (Table 2) were sent to the Macrogen Company in Korea to perform nucleotide sequencing using an automated DNA sequencer, the ABI3730XL. The products were separated on a 1.5% Agarose gel electrophoresis after staining with red safe stain (Nucleic acid staining solution). Using the NCBI's Basic Local Alignment Search Tool (BLAST), these DNA sequences were examined and similarities were found. The evolutionary history was deduced and evolutionary studies were carried out utilizing the MEGA 6 method. The evolutionary distances required to calculate the phylogenetic tree's branch lengths were utilized to draw the phylogenetic tree to scale.

Results and Discussion

One hundred intestinal swabs were obtained from Al-Sink Laboratories (Baghdad, Iraq) for the diagnosis of poultry diseases. *S. entritidis* was detected in 17 out of the 100 samples. The culture medium of bacteria on peptone water was murky and stifling when the isolation phase first started. *Salmonella* does not ferment lactose, hence all isolates produced colorless, smooth, pale, and clear colonies on MacConkey agar media; on XLD plates, however, there were little black and pink colonies. The recovered

bacteria were Gram-negative, solitary or paired in Gram's staining, and shaped like small rods. Biochemical analysis was used to identify the growing bacteria, and the results showed that while catalase, MR, KIA, and simmone's citrate were all positive in *Salmonella* , oxidase, indole, VP, and urease were all negative (Table 4). The Vitek2 system, a novel automated method for identifying bacteria, was used to further identify these 17 isolates. The results showed that 17 of the positive isolates were *S. entritidis*. The recent discovery is consistent with the findings of (14)(15). Another studies in Iraq found a similar percentage of 16.66% of *Salmonella entritidis* positive isolates from chicken products (16), and another study found a similar percentage of 16.2% of isolates from chickens in the State of Kuwait (14). According to several investigations, a layer hen that tested positive for *S. entritidis* had a 5.4% infection rate (17). These findings were not as good as those who isolated *S. enterica* from chicken eggs in Iraq and reported a 30% infection rate (18). Additionally, they were less than the 25.67% results obtained in Pakistan after they isolated *S. enterica* from poultry and related food products (19). This study's findings are regarded as superior than those who isolated *Salmonella* spp. from Iranian birds and found 2.8% of the cases (20).

Table (4): Biochemical tests for characterization of *Salmonella Enteritidis*.

Bacteria	Biochemical Tests								
	Catalase	Oxidase	Indole	MR	VP	Citrate	KIA	Urease	H_2S
Salmonella Enteritidis	+	-	-	+	-	+	+	-	+

(+) positive result, (-) negative result, (MR) Methyl red , (VP) Voges –Proskauer test, (KIA) Kliger Iron Agar test.

Molecular identification for *Salmonella* species

In order to corroborate the identification of *Salmonella entritidis*, PCR for the *16S rRNA* gene was used for the molecular identification of *Salmonella* species (13). First, five distinct *Salmonella* isolates from chicken intestinal layers whose genomic DNA had been isolated were identified phenotypically as positive *S. entritidis* isolates. The DNA concentration varied from 0.2 to 1.00 ng/µl, whereas the purity level varied from 1.7 to 2. Sets of primers specifically created for this investigation were used to perform the PCR. The isolates of bacteria were determined to be S. *entritidis* based on the results. As seen in (Figure 1), the whole length of the *16S rRNA* gene is 1250 bp, in comparison to the DNA marker (a 100 bp DNA ladder).

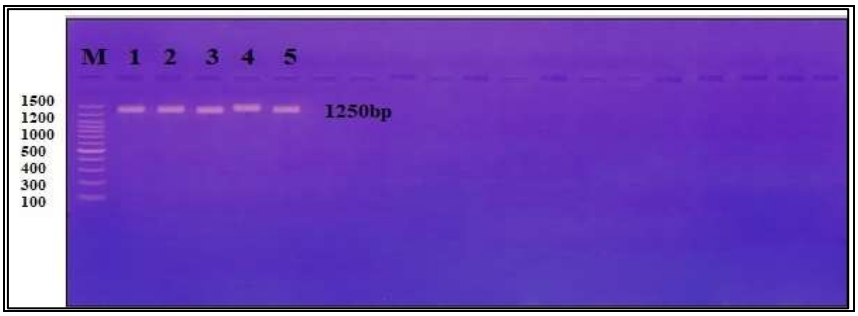

Figure (1): PCR amplicons of *16S rRNA* gene in *S. enterica* serovar Enteritidis isolated from intestine of layers chickens Lane M: 100 bp DNA ladder, Lane (1-5) positive isolates showing a 1250 bp sized PCR product.

Sequences and phylogenetic analysis

Five PCR products that were recovered from the intestine of layers chickens were sequenced in order to validate the diagnosis of *S. enterica.* This was done by amplifying the primer set of the identity *16SrRNA* gene for *S. enterica* serovares (*S. entritidis*). Phylogenetic analyses based on *16srRNA* revealed that the 3,4,2 Iraqi isolates are closely related to Canada, China, South Korea, Australia, China, and the United States. All PCR products that were sent for sequencing were initially registered on the NCBI in 2024 under the accession numbers shown in (Table 5). China, Switzerland Egypt, Brazil, Russia Turkey and Taiwan have the highest percent identity(100%), but isolates 1 and 5 are identical with a percent identity of 99% (Figure 2).

The significant ratio of similarity found in the genomes indicates that *S. entritidis* strains are widely distributed geographically throughout neighboring countries. Additionally Iraqi strains of *S. entritidis* show a significant degree of similarity to global strains, according to the analysis of the phylogenetic tree of *16S rRNA* incomplete nucleotide sequences of the strains and isolates and international strains. Additionally, the high ratio of sequence similarity suggests that *S. entritidis* strains are widely dispersed among the neighboring nations. This indicates that there may be a geographical and epidemiological link between these isolates, which is likely the result of insufficient biosecurity protocols (21).

Table (5): Summarized the symmetrical sequence identity of *Salmonella enteritidis* by NCBI-Blast based on *16S rRNA* gene.

	Source: Salmonella enterica subsp. enterica serovar Enteritidis; 16S ribosomal RNA gene				
	Accession	**Country**	**Isolation Source**	**date of registration**	**Compatibility**
1.	ID: OQ108763.1	Turkey	chicken	2022	99%
2.	ID: CP050716.1	China	Homo sapiens	2020	99%
3.	ID: CP041973.1	South Korea	Homo sapiens	2019	99%
4.	ID: CP045956.1	Australia	Homo sapiens	2019	99%
5.	ID: CP043563.1	South Korea	chicken	2019	99%
6.	ID: CP040646.1	USA	----------	2019	99%
7.	ID: CP033340.1	Canada	----------	2018	99%
8.	ID: CP032851.1	China	chicken	2018	99%
9.	ID: CP025554.1	Switzerland	----------	2018	99%
10.	ID: CP019681.1	Brazil	poultry	2017	99%
11.	ID: CP149435.1	Russia	Homo sapiens	2024	99%
12.	ID: PP430329.1	Egypt	milk	2024	99%
13.	ID: CP100724.1	Taiwan	Homo sapiens	2022	99%
14.	ID: PP955421.1	IRAQ	swab of intestinal tract of the layer chicken	2024	99%
15.	ID: PP955422.1	IRAQ	swab of intestinal tract of the layer chicken	2024	99%
16.	ID: PP955423.1	IRAQ	swab of intestinal tract of the layer chicken	2024	99%
17.	ID: PP955424.1	IRAQ	swab of intestinal tract of the layer chicken	2024	99%
18.	ID: PP955425.1	IRAQ	swab of intestinal tract of the layer chicken	2024	99%

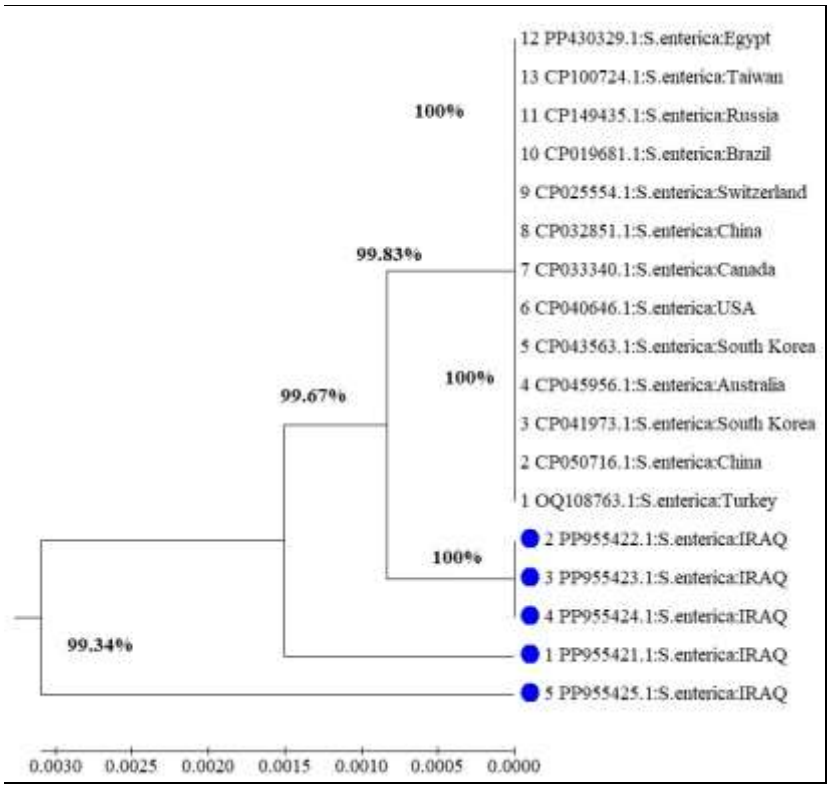

Figure (2): Phylogenetic tree of five isolates *S. entritidis* isolated in this study based on a1250 bp fragment of *16S rRNA* gene using Mega6 with NCBI.

The epidemiological tracing of sources of infections of zoonotic *Salmonella* species and subspecies is critical to avert potential future *Salmonella* outbreak; thus, interspecies transmission can occur in many different ways. Such as wildlife may become infected because of livestock, domestic animals or human wastes, or there may be transmission between wild animals(22). Conclusive characterization of zoonotic *Salmonella* by genetic and molecular modalities has been long sought with inadequate success (23). The elevated occurrence of *Salmonella* spp. in prior research that bears similarities to this investigation may be attributed to inadequate hygiene measures taken throughout the procedures of de-feathering, scalding, evisceration, and carcass cutting. These procedures enable the cross-contamination of clean, healthy birds with sick birds or infected carcasses, and subsequently with humans. Furthermore, if veterinary supervision is lacking, unwell chickens may be killed, which would disseminate the illnesses (24). The primary means of transmission for *Salmonella* spp. is fecal contamination, which can spread both vertically and horizontally. Contamination from handling in the slaughtering, scalding, de-feathering, evisceration, and carcass cutting operations causes the horizontal transfer. Infections can also be contracted by contaminated water and food, which can spread the illness to a whole chicken farm or to individual chickens before reaching humans. This bacteria can spread vertically from one generation of chicken to the next through the egg (25). DNA sequences are essential for both fundamental biological research and a wide range of applied fields, including as biotechnology, forensic biology,

biological applications, and medical diagnosis. The comprehensive sequencing of genomes of various living forms, including as people, animals, plants, and microorganisms, is made possible by the swift advancement and utilization of contemporary DNA sequencing technology (26).

Conclusion

The results of this search showed that the sequences discovered in the NCBI and discovered in Iraq shared similarities with those found in Turkey, China, South Korea, Australia, South Korea, USA, Canada, China, Switzerland, Brazil, Russia, Egypt, and Taiwan. These similarities may be due to the presence of *S. enterica* serovars in the gut of layers chicken products. It additionally illustrates the connection and mode of *S. enterica* bacterial infection as well as the manner in which the illness is spread among these bacteria that have been discovered from chicken goods.

Ethics

We hereby declare all ethical standards have been accepted by the ethics committee of the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

1. Jajere, S. M. (2019). A review of *Salmonella Enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. Veterinary World Journal, 12(4):504.
2. Okolo, J.C. (2017). Mycological quality of fresh and frozen chicken meat retailed within Warri Metropolis, Delta State, Nigeria. Jordan Journal of Biological Science, 10(4): 303-308.
3. Mouttotou, N.; Ahmad, S.; Kamran, Z. and Koutoulis, K. C. (2017). Prevalence, risks and antibiotic resistance of *Salmonella* in poultry production chain. In: Current Topics in *Salmonella* and Salmonellosis, European, 216-234.
4. Antunes, P.; Mourão, J.; Campos, J. and Peixe, L. (2016). Salmonellosis: the role of poultry meat. Clinical Microbiology and Infection, 22: 110-121.
5. Heredia, N. and García, S. (2018). Animals as sources of food-borne pathogens: A review. Animal Nutrition, 4(3): 250-255.
6. Paniel, N. and Noguer, T. (2019). Detection of *Salmonella* in Food Matrices, from Conventional Methods to Recent Aptamer-Sensing Technologies. Foods, 8(9):371.
7. Lobato, I. M. and O'Sullivan, C. K. (2018). Recombinase polymerase amplification: Basics, applications and recent advances. TrAC Trends in Analytical Chemistry, 98:19–35.
8. Fakruddin, M. D.; Mazumdar, R. M.; Chowdhury, A. and Mannan, K. S. B. (2012). Nucleic acid sequence based amplification (NASBA)-prospects and applications. International Journal of Life Science and Pharma Research, 2:106–121.
9. Revolledo, L. (2018). Vaccines and vaccination against fowl typhoid and pullorum disease: An overview and approaches in developing countries. Journal of Applied Poultry Research, 27(3):279-291.
10. Jawad, A. A. and Al-Charrakh, A. H. (2016). Rapid identification and antimicrobial susceptibility testing of *Salmonella* spp. Isolates by vitek 2 system. AL-Qadisiya Medical Journal, 12.
11. Clermont, O.; Christenson, J. K.; Daubie´, A. S. and Gordon, D. M. (2019). Clinical and Laboratory Standards Institute (CLSI). (2019). Performance standards for antimicrobial susceptibility testing, CLSI document M100S27.
12. Kipper, D.; Hellfeldt, R. M.; De Carli, S.; Lehmann, F. K. M.; Fonseca, A. S. K.; Ikuta, N., *et al*. (2019). *Salmonella* serotype assignment by sequencing analysis of intergenic regions of ribosomal RNA operons. Poultry Science, 98(11): 5989–5998.

13. AL Kaabi, H. K. J. and AL-Yassari, A. K. S. (2019). *16SrRNA* sequencing as tool for identification of *Salmonella* spp isolated from human diarrhea cases. Journal of Physics, 1294 (2019): 062041.
14. Alajmi A. D.; Sheet, O. H.; Al-Mahmood, O. A.; Saadeldin, I. M. and Alsanjary, R. A. (2023). Isolation and Identification of *Salmonella* from Chickens Prepared for Slaughter in the State of Kuwait. Journal of Applied Veterinary Sciences, 8 (3): 22-29.
15. Sedeik, M. E.; El-Shall, N. A.; Awad, A. M.; Elfeky, S. M.; Abd El-Hack, M. E.; Hussein, E. O. S., *et al*. (2019). Isolation, conventional and molecular characterization of *Salmonella* spp. from newly hatched broiler chicks. AMB Express, 9(1):136.
16. Al-Shafee, A. A. J. and Abdulwahid, M. T. (2024). Occurrence, antimicrobial resistance, and molecular characterization of *Salmonella Enterica* from chicken products and human in Wasit Governorate of Iraq. Open Veterinary Journal,14(5):1117-1129.
17. Polat, I.; Şen, B. and Onurdağ, F. K. (2024). *Salmonella Enterica* serotypes isolated for the first time in laying hens, and their susceptibility to antibiotics. Poultry Science, 103 (1): 103180.
18. Jaffer, M. R. (2013). Contamination of local laying hen's egg shell with *Salmonella* serotypes. The Iraqi Journal of Veterinary Medicine, 37(1): 13–16.
19. Siddique, A.; Azim, S.; Ali, A.; Andleeb, S.; Ahsan, A.; Imran, M., *et al*. (2021). Antimicrobial resistance profiling of biofilm forming non typhoidal *Salmonella Enterica* isolates from poultry and its associated food products from Pakistan. Antibiotics, 10(7):785.
20. Rahmani, M.; Peighambari, S. M.; Yazdani, A. and Hojjati, P. (2011). *Salmonella* infection in birds kept in parks and pet shops in Tehran, Iran. International Journal of Veterinary Research, 3: 145-148.
21. Zhang, J.; Luo, W.; Liu, G.; Wang, Y.; Geng, S.; Pan, Z., *et al*. (2021). High genetic similarity of *Salmonella Enteritidis* as a predominant serovar by an independent survey in 3 large-scale chicken farms in China. Poultry Science, 100(4): 100941.
22. Sania, T.; Abdul, S.; Muhammad, H.; Areeb, A.; Ayesha, M.; Shehroz, A., *et al*. (2022). *Salmonella* in Poultry; an Overview. International Journal of Multidisciplinary Sciences and Arts, 1: 80–84.
23. Gambi, L.; Ravaioli, V.; Rossini, R.; Tranquillo, V.; Boscarino, A.; Mattei, S., *et al*. (2022). Prevalence of Different *Salmonella Enterica* Subspecies and Serotypes in Wild Carnivores in Emilia-Romagna Region, Italy. Animals (Basel), 12(23):3368.
24. Kanaan, M. H. G.; Khalil, Z. K.; Khashan, H. T. and Ghasemian, A. (2022). Occurrence of virulence factors and carbapenems genes in *Salmonella Enterica* serovar enteritidis isolated from chicken meat and egg samples in Iraq. BMC Microbiology, 22(1):1–8.
25. Kandala, M. F. H. N. J. (2022). Isolation and Identification of Salmonella spp. Using Molecular and Phenotypic Methodes. Iraqi Journal of Biotechnology, *21*(2)..
26. Tang, S.; Orsi, R. H.; Luo, H.; Ge, C.; Zhang, G.; Baker, R. C., *et al*. (2019). Assessment and comparison of molecular subtyping and characterization methods for *Salmonella*. Frontiers in Microbiology, 10: 1591.