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Molecular detection by some virulence genes of *Salmonella enterica* subsp. *enterica* isolated from the stool of children with diarrhea

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Abstract:

Diarrhea is a real disease in childhood which could cause death. Therefore, this study was conducted to isolate Salmonella from 350 stool samples taken from children under five years in age, suffering from diarrhea during the period from March 2019 to March 2020 in Tikrit city / Iraq. The results showed the possibility to isolate ten isolates of *Salmonella enterica* subsp. *Enterica*, an infection rate, represents 2.875% of the total rate of patients who suffer from diarrhea. The virulence genes were investigated for ten isolates of *S. enterica* subsp. *enterica*, the result is that all isolates possessed the genes *stn*, *invA*, *lpfA* with an appearance percentage of 100%, while the percentage of appearance of *pefA* and *fimH* genes was 80% and 60%, respectively. All these isolates as Iraqi detection have been submitted to NCBI then accepted as ten Iraqi strains were in NCBI. All strains were concordance of 99-100% with the *Salmonella enterica* subsp. *enterica* Strains of Taiwan (CP085821.1), the United Kingdom (OU943338.1), China (CP085699.1), South Korea (CP077760.1), Pakistan (OK035700.1) and Hong Kong (CP0823381.1) in addition to four US states.

Keywords: Diarrhea, Salmonella enterica subsp enterica, Virulence factors genes, 16srRNA gene, Phylogenetic tree

Introduction:

Salmonella is a foodborne pathogen that causes three clinical syndromes: typhoid fever, enteritis, and bacteremia. There are more than 2500 serotypes of Salmonella, which infect a variety of hosts. Non-typhoidal Salmonella (NTS) mainly causes gastroenteritis in humans, but it can cause acute bacteremia in young children and immunocompromised patients. It is estimated that around 3.4 million cases of bacteremia due to non-typhoidal Salmonella infection occur each year globally[1]. Reports indicate that 99% of Salmonella infections in humans are associated with serotypes of *Salmonella enterica* subsp. *enterica*[2]. The Salmonella genome contains several sets of genes, referred to as Salmonella pathogenicity islands (SPIs), that code for virulence factors. The genes encoding Salmonella virulence factors can be divided into two main classes, genes located on chromosomes such as the *stn* gene and genes located on the virulence plasmid [3]. Seventeen pathogenicity islands (SPIs) have been identified with Salmonella that contribute to its virulence, as well as many other genes such as Spv operon [4]. SPI-1 is believed to contain genes required for bacterial entry, while SPI-2 is essential for intracellular survival and systemic inflammation [5].

The presence of virulence genes carried on a plasmid was first proposed in 1982, as the virulence plasmid in Salmonella spv (Salmonella plasmid virulence) is important in the process of Salmonella multiplying in the cells of the reticulo-endothelial system in warm-blooded vertebrates [6]. The *invA* gene contains unique sequences specifically for the Salmonella genus, it is located in the pathogenicity island (SPI-1), and is important for host epithelial cell invasion. This gene is present in most salmonella serotypes, so it is used as an important target for the detection of salmonella [7]. Long polar fimbriae (Lpf) is encoded by the *lpfABCDE* operon. These fimbriae are involved in the process of Salmonella adhesion to the surface of M cells in the Peyer's patches, which is the preferred entry port for Salmonella into intestinal epithelial cells [8]. The *stn* gene encodes the enterotoxin of Salmonella, which is a virulence factor that causes diarrhea. Interestingly, the stn gene

has been shown to be present in all *Salmonella spp*. Regardless of their serotypes, this gene is used to identify Salmonella. *Stn* has toxic activity so it is a factor of salmonella virulence factors and is responsible for salmonella enterotoxigenic toxicity [9]. Plasmid-encoded fimbriae (Pef) play an important role in bacterial pathogenesis because they enhance the attachment of bacteria to the surface of the small intestine. These fimbriae are encoded by *pef* operon present in the virulence plasmid. The expression of *pef* genes is regulated by DNA methylation. Plasmid-encoded fimbriae bind to antigens of the Lewis system that are predominant on the surface of human erythrocytes, skin epithelium and mucosal surface [10].

Salmonella possesses another type of fimbriae called Type 1 fimbriae, which are encoded by the fim operon and are assembled by the Chaperone-usher system. Type 1 fimbriae (T1F) are one of the most common organelles in the Enterobacteriaceae, including Salmonella species, and are important virulence factors, helping bacteria to adhere to the host cell surface [11]. Therefore, this study aims to diagnose salmonella by determining the sequencing of *16srRNA* gene and determining some of its virulence factors.

Materials and Methods:

Isolation and Identification

This study was conducted during the period from March 2019 to March 2020 in Tikrit city / Iraq. Salmonella was isolated from 350 stool samples taken from children under five years in age, suffering from diarrhea by using selenite broth and XLD agar. The developing colonies on XLD medium with a red color and black center were isolated. The isolates were identified to genus level by using a Vitek 2 compact system, in addition the isolates were diagnosed to a subspecies level by sequencing the *16srRNA* gene.

Extraction of Genomic DNA

In this study, the Genomic DNA Purification Kit, manufactured by Promega in the United States of America was used to extract DNA.

Primers Preparation

The DNA primers mentioned in Table 1 were manufactured by Alpha Company in Canada as a lyophilized product. All the primers were centrifuged for a few seconds before use, after which the primers were dissolved in sterile deionized water to obtain the final concentration of each primer of 100 µM (stock solution), 10 µl of the stock solution were added to 90 µl of sterile deionized water to obtain the working solution of the primer at a concentration of 10 µM.

Table 1. Primers used in the current study

Genes	Primer sequence (5′-3′)	Product size (bp)	References
16S rRNA	F: GGAACTGAGACACGGTCCAG R: CCAGGTAAGGTTCTTCGCGT	660	[12]
invA	F: GTGAAATTATCGCCACGTTCG GGCAA R: TCATCGCACCGTCAAAGGAAC C	284	
stn	F: TTGTGTCGCTATCACTGGCAACC R: ATTCGTAACCCGCTCTCGTCC	617	[13]
lpfA	F: CTTTCGCTGCTGAATCTGGT R: CAGTGTTAACAGAAACCAGT	250	[14]
pefA	F: TGTTTCCGGGCTTGTGCT R: CAG GGCATTTGCTGATTCTTCC	700	
fimH	F: GGATCCATGAAAATATACTC R: AAGCTTTTAATCATAATCGACTC	1008	[15]

PCR Reaction

All PCR reactions were performed at volumes of 25µl in Eppendorf tube. A Laminar flow cabinet was used to prepare the reaction mixture. All reaction components were frozen separately and used at optimum concentration. Table 2 shows the components of the PCR reactions. The PCR reaction for the genes used in this study was performed according to the reaction conditions as in shown in Table 3

Table 2. Components of PCR reaction

Component	Volume (µl)
GoTaq Green Master Mix (2X)	**12.5**
Nuclease Free Water	**8.5**
DNA Template	**2**
Forward Primer (10 µM)	**1**
Reverse Primer (10 µM)	**1**
Total volume	**25**

Table 3. PCR reaction conditions to detect the genes that used in this study

Virulence genes	Initial denaturation 1 cycle	35 cycles			Final extension 1 cycle	References
		denaturation	annealing	extension		
16srRNA	95 °C / 4 min	94 °C /60 sec	59 °C /30 sec	72 °C /30 sec	72 °C /7 min	[12]
invA	95 °C / 4 min	94 °C /30 sec	64 °C /30 sec	72 °C /45 sec	72 °C /7 min	
stn	95 °C / 4 min	94 °C /60 sec	59 °C /60 sec	72 °C /60 sec	72 °C /7 min	[13]
lpfA	95 °C / 4 min	94 °C /50 sec	58 °C /60 sec	72 °C /45 sec	72 °C /7 min	[14]
pefA	95 °C / 4 min	94 °C /30 sec	55 °C /45 sec	72 °C /45 sec	72 °C /7 min	
fimH	95 °C / 4 min	94 °C /60 sec	56 °C /35 sec	72 °C /60 sec	72 °C /7 min	[15]

Electrophoresis

PCR products were electrophoresis on 2% agarose gel containing red safe stain at a concentration of 5 µl/100 ml of agarose gel. The wells of agarose gel were filled with 5 µl for each well, then the electrophoresis was carried out in two stages. In the first stage, a voltage differences of 2 volts/cm were used for 10 minutes, while in the second stage, a voltage differences of 5 volts/cm were used for 120 minutes.

Results and Discussion:

Isolation

In this study 38 isolates were isolated from the 350 stool samples of children suffering from diarrhea, which are believed to belong to the Salmonella genus. Bacterial isolates were determined on XLD agar medium as in Fig.1, according to the phenotypic shape, as rounded colonies that are 1-2 mm in diameter, red in color and have a black center in the middle, were selected [16].

Figure 1. Salmonella enterica isolates Colonies on XLD agar

Identification

The results showed that only 10 isolates belong to the genus Salmonella, the isolates were diagnosed to the level of the genus by using Vitek 2 compact system. The results of the molecular diagnosis using the *16srRNA* primer according to the reaction conditions, mentioned in Tab.3 showed that all salmonella isolates possess *16srRNA* gene. This gene appeared as a bundle on agarose gel with a size of 660 bp as in Fig.2. Moreover, the sequencing results of *16srRNA* gene of all Salmonella isolates showed a

match of 99-100% with standard gene sequence of *Salmonella enterica* subsp. *enterica* as in Table 4.

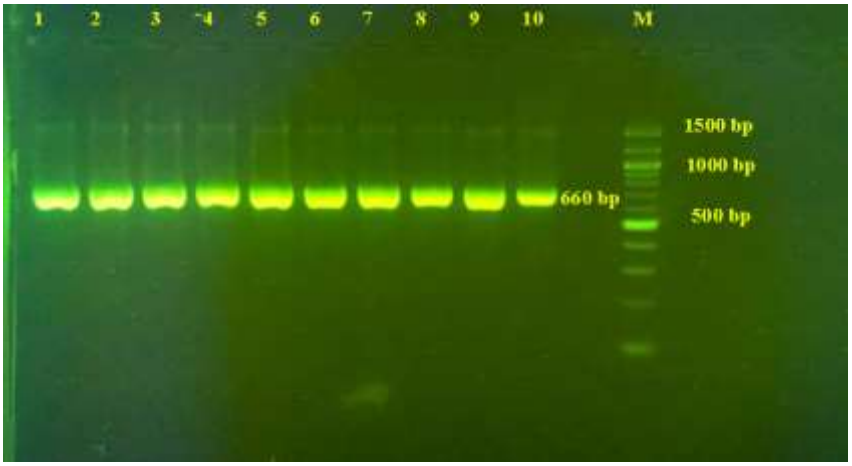

Figure 2. Electrophoresis of PCR products of *16srRNA* gene on agarose gel with concentration 2% at 70 volts/cm for 50 min., The bands in the lanes 1-10 at a size of 660 bp representing the *16srRNA* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Table 4. Identical percentage of Salmonella isolates with standard isolates in NCBI

Source: Salmonella enterica subsp. enterica					
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	**Identities**
1	--------	------	----	ID: CP077704.1	**100 %**
2	--------	------	----	ID: CP077704.1	**100 %**
3	--------	------	----	ID: CP077704.1	**100 %**
4	Transition	463781	A\G	ID: CP077704.1	**99 %**
5	Transition	463890	G\A	ID: CP077704.1	**99 %**
	Transition	463902	A\G		
	Transversion	463909	G\C		
	Transversion	463926	C\G		
	Transversion	464230	A\C		
6	Transition	463781	A\G	ID: CP077704.1	**99 %**
7	Transversion	463780	T\G	ID: CP077704.1	**99 %**
	Transition	464199	T\C		
	Transversion	464329	A\C		
	Transversion	464351	C\G		
	Transition	464354	G\A		
8	--------	------	----	ID: CP077704.1	**100 %**
9	Transition	463781	A\G	ID: CP077704.1	**99 %**
10	Transition	463781	A\G	ID: CP077704.1	**99 %**

Recording of *Salmonella enterica* subsp. *enterica* isolates in NCBI

The ten isolates of *Salmonella enterica* subsp. *enterica* were registered in NCBI based on sequencing of *16srRNA* gene and became a reference for Iraq, the Middle East and the world with the registration numbers: OK120829.1, OK120830.1, OK120831.1, OK120832.1, OK120833.1, OK120834.1, OK120835. 1, OK120836 1, OK120837.1, OK120838.1, OK648469.1

Phylogenetic tree

The ten Iraqi strains recorded in the National Center for Biotechnology Information (NCBI) are in concordance of 99-100% with Taiwan (CP085821.1), the United Kingdom (OU943338.1), China (CP085699.1), South Korea (CP077760.1), Pakistan (OK035700.1) and Hong Kong

(CP0823381.1) in addition to four US states as shown in Table 5. and Fig.3 of the phylogenetic tree of *Salmonella enterica* subsp. *Enterica*

Table 5. Genetic rapprochement of the *16srRNA* gene for the isolates of *Salmonella enterica* subsp. *Enterica*

No.	Accession	Country	Source	Compatibility
1.	ID: CP085821.1	Taiwan	*Salmonella enterica* subsp. *enterica*	99-100 %
2.	ID: OU943338.1	United Kingdom	*Salmonella enterica* subsp. *enterica*	99-100 %
3.	ID: CP085699.1	China	*Salmonella enterica* subsp. *enterica*	99-100 %
4.	ID: CP082641.1	USA:CA	*Salmonella enterica* subsp. *enterica*	99-100 %
5.	ID: CP077760.1	South Korea	*Salmonella enterica* subsp. *enterica*	99-100 %
6.	ID: OK035700.1	Pakistan	*Salmonella enterica* subsp. *enterica*	99-100 %
7.	ID: CP082391.1	USA: IA	*Salmonella enterica* subsp. *enterica*	99-100 %
8.	ID: CP082609.1	USA	*Salmonella enterica* subsp. *enterica*	99-100 %
9.	ID: CP082566.1	USA: MN	*Salmonella enterica* subsp. *enterica*	99-100 %
10.	ID: CP082338.1	Hong Kong	*Salmonella enterica* subsp. *enterica*	99-100 %

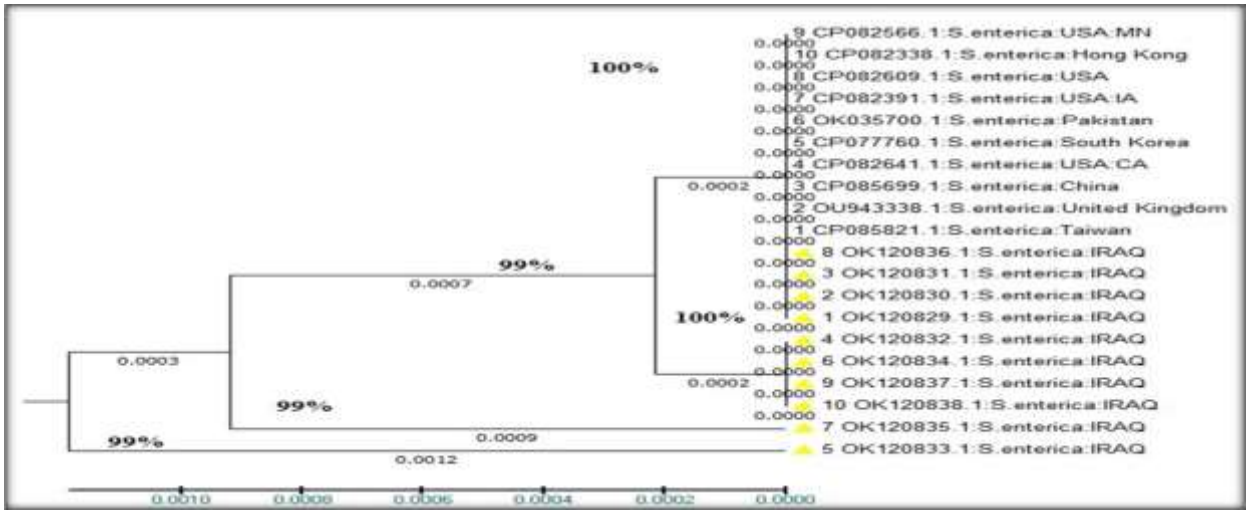

Figure 3. Phylogenetic tree of the ten Iraqi isolates of *Salmonella enterica* subsp. *enterica*

Detection of Virulence Genes

The virulence genes were detected by using the polymerase chain reaction according to the reaction conditions mentioned in Table 3

Detection of *invA* gene

The *invA* gene is an essential gene that is required for the invasion of epithelial cells of the intestines by Salmonella [17]. The *invA* gene in Salmonella contains a unique sequence of this genus, so this gene is also used in the molecular diagnosis of Salmonella [18]. The *invA* gene was amplified using the specialized primer as shown in Table 1 The results of electrophoresis on agarose gel of the PCR products showed that this gene appeared in all Salmonella isolates, with an appearance rate of 100% as shown in Fig.4 The *invA* gene appeared as a bundle on agarose gel with a size of 284 bp as in Fig.5. The results of the current study agreed with the results of Abd El-Tawab and his group [19], as their results showed that the *invA* gene diagnosed in all Salmonella isolates was 284 bp in size. Also, the results of the study conducted by Al-Kaaby *et al*. [17] agreed with the results of the current study, as their results showed that all salmonella isolates possess the *invA* gene. The results of the current study also agreed with the results of the study conducted by Hussain *et al* [20] in Nasiriyah, as their results showed that the percentage of appearance of this gene was 100%. The results of the current study also agreed with the results of the study conducted by El-Sebay *et al* [21] in Egypt.

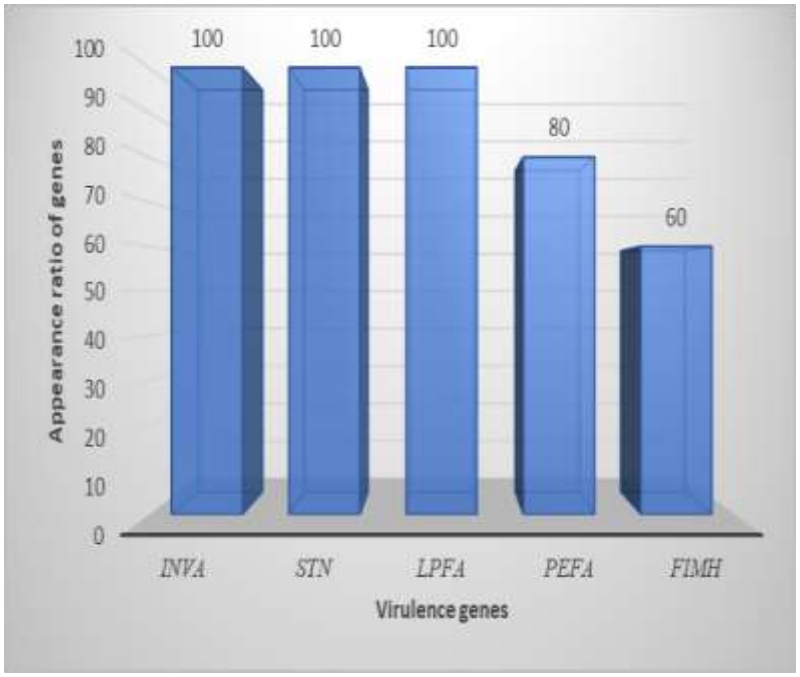

Figure 4. Appearance ratio of virulence genes in isolates of *Salmonella enterica* subsp. *Enterica*

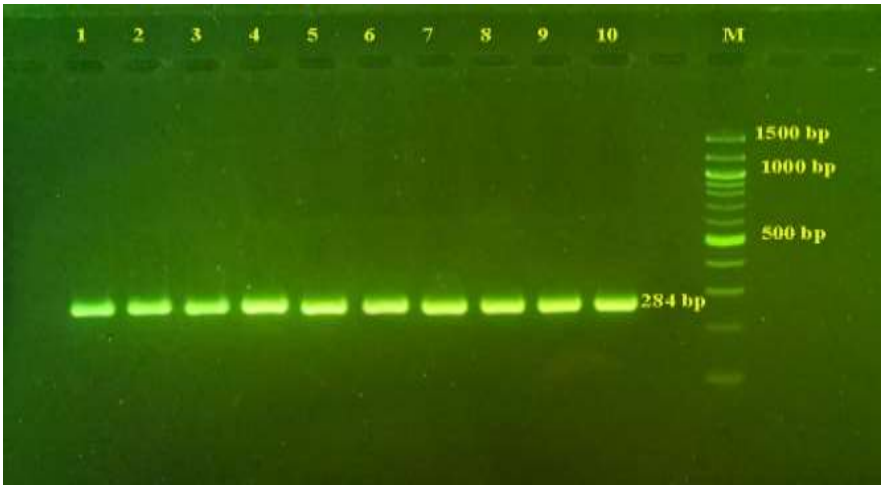

Figure 5. Electrophoresis of PCR products of *invA* gene on agarose gel with concentration 2% at 70 volt/cm for 50 min., The bands in the lanes 1-10 at a size of 284 bp representing the *invA* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Detection of *stn* gene

The results of electrophoresis on agarose gel of PCR products showed the presence of the *stn* gene in all salmonella isolates with a percentage of 100%, as in Fig.4. This gene appeared as a bundle on agarose gel with a size of 617 bp as in Fig.6. The results of the current study agreed with the results of the study conducted by Muthu *et al*. [22], as the results of their study showed that all Salmonella isolates contain *stn* gene with an appearance rate of 100%. The results of the current study did not agree with the results of the study conducted by Ammar *et al*. [23], as they stated that the appearance ratio of this gene was 58.82% of the total number of Salmonella isolates.

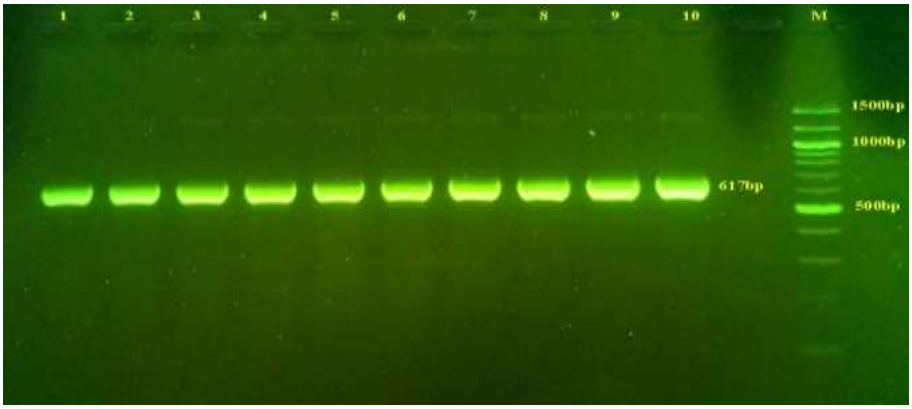

Figure 6. Electrophoresis of PCR products of *stn* gene on agarose gel with concentration 2% at 70 volt/cm for 50 min., The bands in the lanes 1-10 at a size of 617 bp representing the *stn* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Detection of *lpfA* gene

The *lpfA* gene was amplified using the specialized primer *lpfA* as shown in Table 1. The results of electrophoresis on agarose gel of the PCR products showed the presence of the *lpfA* gene in all isolates of Salmonella, with an appearance rate of 100% as shown in Fig.4. This gene appeared as a bundle on agarose gel with a size of 250 bp as in Fig.7 The results of the current study agreed with the results of Webber *et al*. [24], as the results of the study showed that all salmonella contain the *lpfA* gene with an appearance rate of 100%. Long polar fimbriae enhance the adhesion of salmonella to the M cells found in Peyer's patches in the intestine, which increases the virulence of salmonella, because these cells are the preferred entry site for salmonella. The results of the study also agreed with the results of Melo *et al* [25] as the results of their study showed that all salmonella isolates contain the *lpfA* gene with an appearance rate of 100%.

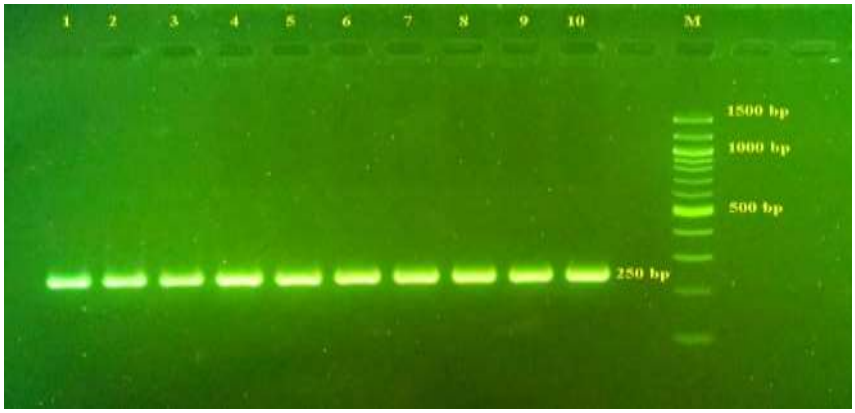

Figure 7. Electrophoresis of PCR products of *lpfA* gene on agarose gel with concentration 2% at 70 volt/cm for 50 min., The bands in the lanes 1-10 at a size of 250 bp representing the *lpfA* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Detection of *pefA* gene

The *pefA* gene was amplified by using the specialized primer *pefA* as shown in Table 1. The results of electrophoresis on agarose gel of PCR products showed the presence of the *pefA* gene in 8 isolates, with an appearance rate of 80%. This gene appeared as a bundle on an agarose gel with a size of 700 bp as shown in Fig. 8. The results of the current study agreed with the results of the study conducted by Abd El-Tawab et al. [19], as the results of the study showed that the appearance ratio of the *pefA* gene in the salmonella was 80%. The results of this study approximately agreed with the results of the study conducted by Shrivastav and his group [26], as they stated in their results that the appearance ratio of this gene was 75%.

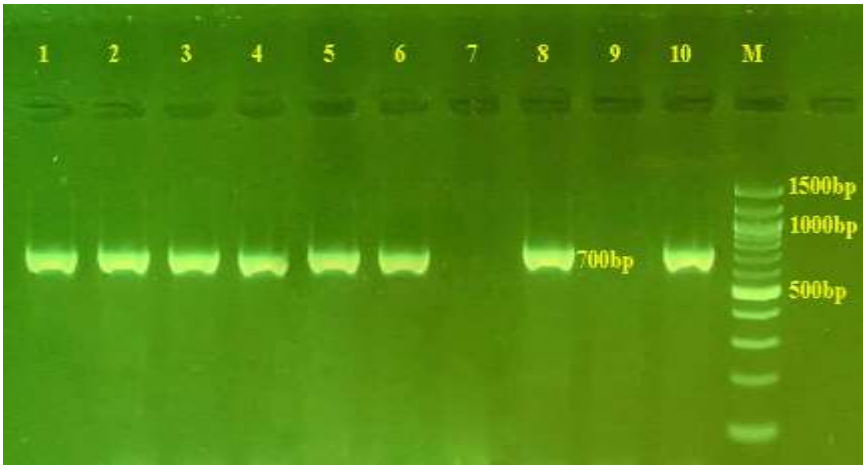

Figure 8. Electrophoresis of PCR products of *pefA* gene on agarose gel with concentration 2% at 5 volt/cm for 50 min., The bands in the lanes 1,2,3,4,5,6,8,9,10 at a size of 700 bp representing the *pefA* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Detection of *fimH* gene

The *fimH* gene was amplified by using the specialized primer fimH as shown in Table 1. The results of electrophoresis on an agarose gel of the products of the polymerase chain reaction showed the presence of this gene in 6 isolates, with an appearance rate of 60%. This gene appeared as a bundle on an agarose gel with a size of 1008 bp as shown in Fig. 9. The results of the current study almost agreed with the results of the study conducted by El-Prince *et al*. [21], as they stated in their results that the rate of appearance of this gene in Salmonella isolates was 50%. The results of this study also differed from with the results of the study conducted by Saad *et al*. [22] in Egypt, as they stated in their results that the appearance rate of this gene was 100%. The results of the appearance of this gene also differed with the results of the study conducted by Ibrahim *et al*. [23] as they mentioned in their results that the appearance rate of this gene in isolates of salmonella was 100%.

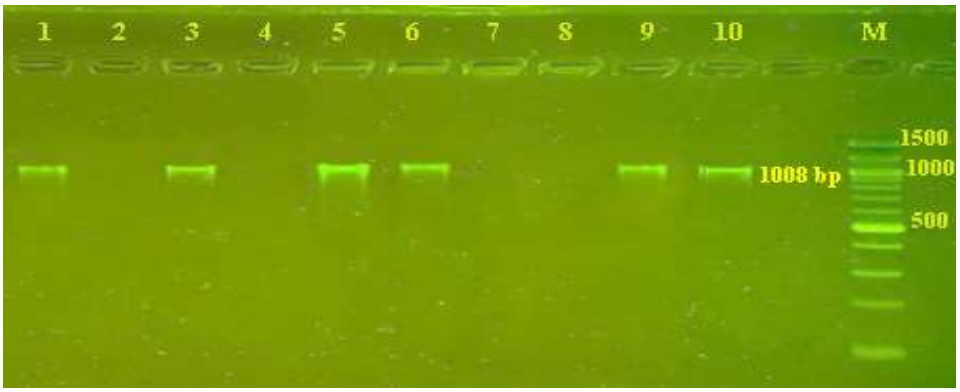

Figure 9. Electrophoresis of PCR products of *fimH* gene on agarose gel with concentration 2% at 5 volt/cm for 50 min., The bands in the lanes 1,3,5,6,9,10 at a size of 1008 bp representing the *fimH* gene of *Salmonella enterica* subsp. *enterica* isolates. M: DNA ladder (100-1500bp)

Conclusion:

This study concludes that 2.875% of diarrheal cases in children are caused by *S. enterica* subsp. *enterica*. All isolates of *S. enterica* subsp. *enterica* possess virulence genes such as *invA*, *stn*, *lpfA*, while the other genes such as *pefA*, *fimH*, are different in terms of appearance rate. The ten Iraqi strains registered in the NCBI it is in concordance with 99-100% with Taiwan (CP085821.1), the United Kingdom (OU943338.1), China (CP085699.1), South Korea (CP077760.1), Pakistan (OK035700.1) and Hong Kong (CP0823381.1) in addition to four US states

Authors' declaration:

-Conflicts of Interest: None.

-We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.

-Ethical Clearance: The project was approved by the local ethical committee in Tikrit University.

Authors Contribution:

AH Dhayea collected the samples and performed all assays that related to the identification of isolates, DNA extraction and investigation of virulence genes: MN Maaroof made a research plan, analyzed all parameters and recording isolates in NCBI.

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التشخيص الجزيئي بواسطة بعض جينات الضراوة لعزلات السالمونيلا المعزولة من براز الأطفال الذين يعانون من الإسهال

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الخلاصة:
الاسهال من الامراض المميتة في مرحلة الطفولة. أجريت هذه الدراسة لعزل السالمونيلا من 350 عينة براز مأخوذة من أطفال دون سن الخامسة يعانون من الإسهال خلال المدة من آذار 2019 إلى آذار 2020 في مدينة تكريت / العراق. أظهرت النتائج إمكانية عزل عشر عزلات تعود لتحت النوع *Salmonella enterica* subsp. *enterica* من 350 عينة براز أي بنسبة إصابة 2.875٪ من إجمالي المرضى الذين يعانون من الإسهال. تم فحص جينات الضراوة لعشر عزلات من *S. enterica* subsp. *enterica* حيث وجد أن جميع العزلات تمتلك الجينات *stn* و *invA* و *lpfA* بنسبة ظهور 100٪ بينما كانت نسبة ظهور الجين *pefA* والجين *fimH* 80% و 60% على التوالي. العزلات العشرة قدمت كعزلات عراقية وقدمت وتم تسجيلها كسلالات عراقية في NCBI. جميع السلالات كانت متوافقة بنسبة 99-100٪ مع سلالات تحت النوع *Salmonella enterica* subsp. *Enterica* في تايوان (CP085821.1) والمملكة المتحدة (OU943338.1) والصين (CP085699.1) وكوريا الجنوبية (CP077760.1) وباكستان (OK035700.1) وهونغ كونغ (CP0823381.1) فضلاً عن ذلك إلى أربع ولايات أمريكية.
الكلمات مفتاحية: اسهال، بكتيريا السالمونيلا المعوية، جينات عوامل الضراوة، الجين *16srRNA* ،الشجرة العرقية.