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Molecular diagnosis for *Shigella sonnei* bacteria from stools of children under five years

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Abstract
This study was conducted to isolate *Shigella* SPP. from (150) stool samples of children with both sex suffering from diarrhoea whose ages ranged from (1-60) months during the period from October 2021 to March 2022 in Mosul / Iraq. The results showed that (17) isolates belong to *Shigella sonnei*, representing (12.59%) of the total number of isolates, The age group (49-60) months was in isolating to *Shigella sonnei* which diagnosis by morphological and biochemical.
The sequence analysis of the DNA was carried out, especially for the ribosomal 16SrRNA gene, it was relied upon because it is a fixed sequence that does not change over the years for bacterial species and genera. The DNA extract was sent to the Microgene Company to target the diagnostic gene 16SrRNA by adding the special Forward and Reverse primer genes and amplification and doubling polymerase chain reaction (PCR), It was found the sequence of gene for all the (17) samples was present in all strains. When comparing the duplicated bands, it was found that the bands were (1460) base pairs in size and at the same level for all types of strains. The results of these strains were compared for the genetic sequence with the gene sequence of the standard strains installed in the database Basic global Alignment Search Tool (BLAST) within National Center for Biotechnology Information (NCBI). The diagnosis was confirmed with a cause similarity ranging from (98-100%).Found that the size of the nitrogenous bases reaches (1460) base pairs when electrophoresis after 16SrRNA gene amplification when electroporation after 16SrRNA gene amplification by polymerase chain reaction (PCR).
Conclusion: Bacteria were diagnosed by PCR as a confirm test for *Shigella sonnei*.

Keywords: *Shigella sonnei*, children under five years, 16SrRNA gene, PCR and sequence analysis of the DNA

Introduction

Every year, diarrhea kills two million children under the age of five worldwide, with two years of life accounting for 80% of these deaths. Even though the number of children dying from diarrhea has significantly decreased, it remains a serious public health concern, particularly in underdeveloped nations (Shati *et al*., 2020) [1]. One of the main causes of diarrheal infections and the deaths that result from them is shigellosis, a serious issue for human health on a global scale. Shigella spp. is bacteria that spread from person to person by contaminated food and water, infect epithelial cells, and grow there to cause ulcers, inflammation, and hemorrhage (Al-Masudi *et al*., 2020). *Shigella* accounted for 212.438 deaths (or 95%) and roughly 13.2% of all diarrhea-related deaths in 2016, making it the second most common cause of diarrheal mortality across all age groups. *Shigella* spp. caused 63.713 deaths in children under the age of five and was commonly linked to diarrhea across all adult age categories, with an increased incidence in the elderly and widespread geographic distribution (Martin and Tang, 2022) [16, 21]. In 2016, Enterotoxigenic Escherichia coli (ETEC) accounted for 51186 deaths (26 757-83 064) and around (3.2)% (1.8-4.7) of diarrhea deaths, making it the ninth most common cause of diarrhea mortality across all age groups. Approximately (4.2)% (2.2–6.8) of children under the age of five who died from diarrhea were affected by ETEC (Khalil *et al*., 2018) [4].
Shigella spp. bacteria were first identified in 1898 by the Japanese doctor and bacteriologist Kiyoshi Shiga, who isolated and described them (Trofa *et al*., 1999) [5]. *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* are the four types of the genus *Shigella* SPP. based on the serous features. Each type is split into different serotypes by the lipopolysaccharide (LPS) specific O antigen. (15) serotypes of *Shigella* dysenteriae are present. *Shigella flexneri has six serotypes and fifteen subtypes, Shigella boydii has eighteen serotypes, and Shigella sonnei has just one, placing it third among bacteria linked to food-related diseases behind Salmonella and Campylobacter* (Muthuirulandi *et al*., 2017) [6]. *Shigella* spp. bacteria, which are members of the Enterobacteriaceae family and are gram-negative, rod-shaped, facultatively anaerobic bacteria that cause ulcers and inflammation in the large intestine, infiltrate the epithelium of the colon (Alwan and Maaroof, 2021) [7]. The signs and symptoms of diarrhea range from moderate bloody diarrhea to severe dysentery, which is marked by bloody stools and abdominal cramps. The infection dose is typically between (10-100) CFU/cell, which is sufficient to produce the disease (Kotloff *et al*., 2013) [8]. *Shigella* species have a type III secretion system that is tightly linked to the pathogenesis of the organism and is controlled by several genes associated with the

freshness of the food (Medeiros, *et al*., 2018) [9]. Different enterotoxins, such as enterotoxin ShET-1 encoded by the gene set and enterotoxin ShET-2 encoded by the gene set, are produced by *Shigella* spp (Li *et al*., 2020) [10]. The determinants of main virulence are encoded by a circular virulence plasmid and one circular chromosome in the Shigella spp. genome (Seribeilli *et al.,* 2016) [11].

Materials and Methods

Stool samples (150) were collected from children suffering from diarrhea of both sexes, under five years old were attended Mosul city Hospitals, Al-Hamdaniya Hospitals and health centers, during the period from October to March 2022 using clean and sterile gloves. About (1-2) gram of stool were taken and were placed in boxes clean and sterile with a tight lid, the names of the patients and the date were written on the boxes, all of them were tightly closed to prevent spillage and contamination of them, then were transplanted immediately.

Shigella spp. bacteria were isolated according to what was reported in (England Public Health, 2018) [12].

A set of tools was used to purify genomic DNA manufactured by the Presto™ Mini g DNA Bacteria Kit is optimized for genomic and viral DNA purification from Gram (-) negative and Gram (+) positive bacterial cells, whole blood and biological fluids. Agarose gel electrophoresis were used after PCR amplification to analyze the amplicons according to, (Green and Sambrook, 2019) [13]. In PCR technique, Forward and Revers primers (27) forward and (1492) reverse were selected from (Jiang *et al*., 2006) were used to detect (1500) bp of universal 16S rRNA gene.The primers provided as lyophilized form (Macrogen/ Korea).

Purified PCR DNA fragment from the amplification of 16S rRNA gene for both forward and reverse PCR products were sent directly for (Sanger Sequencing) using (ABI3730XL automated DNA sequences) by the corporation of Macrogen Company in Korea. Then, the study results were obtained by (Email) and analyzed using (Geneious Software), the edited sequence were compared with data base using BLAST (Basic Local Alignment Search Tool) to obtain the closed relation with submitted sequences.

Results and Discussion

DNA was extracted from all strains (17) and electrophoresis was carried out on an agarose gel at a concentration of 1% to ensure the presence of DNA in the extract After the migration, the presence of luminous bundles was observed upon examination under UV rays, and this indicates the presence of the genetic material as showed in Figure (1).

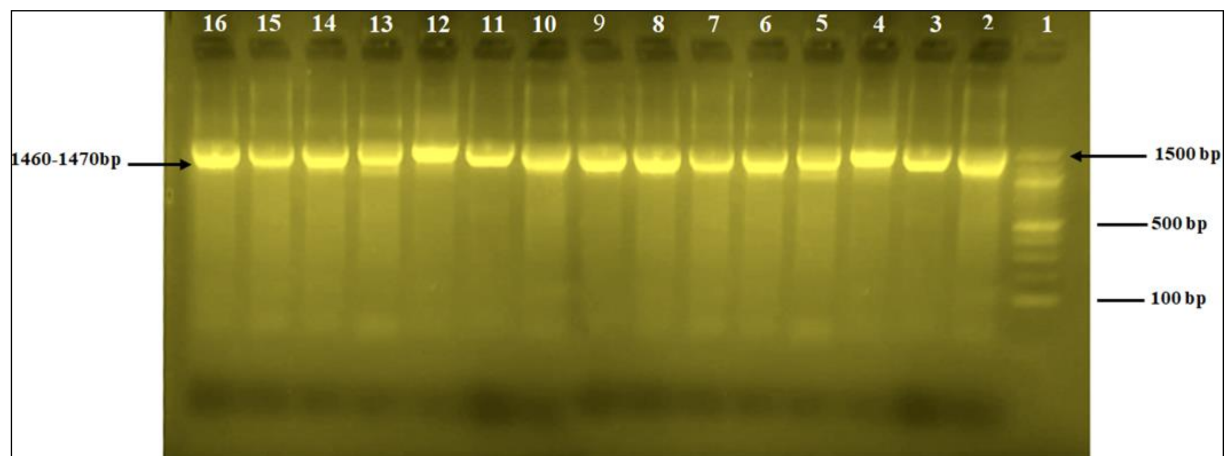

Fig 1: Detection of *Shigella sonnei* 16SrRNA gene (1460-1470bp). Line (1) marker (100bp). Lines (2-16) represent positive results. Electrophoresis conditions are 5v /cm$^{2.}$

The sequence analysis of the DNA was carried out, especially for the ribosomal 16SrRNA gene, it was relied upon because it is a fixed sequence that does not change over the years for bacterial species and genera. The DNA extract was sent to the Microgene Company to target the diagnostic gene 16SrRNA by adding the special Forward and Reverse primer genes and amplification and doubling polymerase chain reaction (PCR), It was found the sequence of gene for all the (17) samples is present in all isolates. When comparing the duplicated bands, it was found that the bands were (1460) base pairs in size and at the same level for all types of strains. The results of these isolates were compared for the genetic sequence with the gene sequence of the standard strains installed in the database Basic Local Alignment Search Tool (BLAST) within NCBI The diagnosis was confirmed with a cause similarity ranging from (95- 98%). Nisa *et al*. (2020) [14], found that the size of the nitrogenous bases reaches (1460) base pairs when electrophoresis after 16SrRNA gene amplification when electroporation after 16SrRNA gene amplification by polymerase chain reaction (PCR).

The use of the gene 16SrRNA is one of the best methods used in diagnosing bacteria, because of the speed of diagnosis in a short time this gene does not change and is not affected for long periods time, as well as contains fixed and variable regions as these regions will be targeted when diagnosing It is considered one of the most accurate tests, with a diagnosis rate of (98%) compared to other tests (Saima SA *et al* 2018) [15].

The 16SrRNA gene was amplified in 3 PCR-positive products before sequencing using the Sanger technique. The BLAST program was then used to compare these sequences to the reference *Shigella sonnei* strains that are readily available in NCBI. All isolates were found to belong to the *Shigella sonnei* strains according to the results of the present sequence alignment, which showed a high similarity ratio of 100% between the query sequence and the subject.

Many studies have been conducted worldwide to isolate *Shigella sonnei* from diarrheal cases of children under five years old and using PCR to detect the 16S ribosomal RNA gene as well as sequencing alignment for genetic analysis (Pizzato *et al*., 2022; Torrez Lamberti *et al*., 2022; Nochi *et al*., 2009;Chakravorty *et al*., 2007) [16, 21, 17, 18, 19].

Sequencing alignment is a gold standard confirmative tool for bacteria because it is difficult to diagnose, in addition, to the ability to distinguish between many bacteria belonging to the same family. Furthermore, it plays a critical role in the detection of similarity and distance indices of bacteria that belong to the same species (Church *et al*., 2020) [20].

Ten of our isolates and four global gene bank isolates were included in the phylogenetic tree, which was created using the largest amount of sequence similarity (Figure 2).

Molecular analysis of the *Shigella sonnei*'s evolutionary relationship revealed two main branches and a secondary branch connected by high bootstrap values (98%), and these branches were divided into subgroups with bootstrap values ranging from (98% - 100%). Sequences 1 and 3 showed the similarity rate among our isolates (98%), while sequences 1 and 3 showed the similarity (100%) with sequences 2. On the other hand, the results revealed a genetic relationship between our isolates and global isolates was (100%).

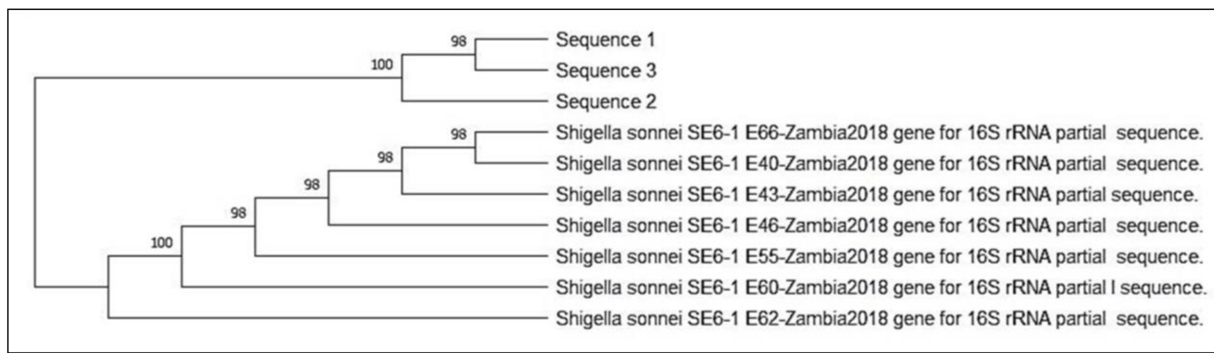

Fig 2: Phylogenetic tree analysis for *Shigella sonnei* isolates under study using the Mega X program.

By creating a phylogenetic tree based on the nucleotide sequences of the 16SrRNA gene, studies have shown phylogenetic linkages and wide genetic variation within *Shigella sonnei* isolates (Pizzato *et al*., 2022; *Deutsch-Nagy et al., 2018;* Watts *et al*., 2017; Suardana., 2014; Cilia & Christen., 1996) [16, 18, 22, 23, 24, 25]

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