Phylogenetic analysis and comparative investigation in Al-Diwaniyah City and worldwide isolates from genebank for Shigella species

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Abstract

Between November 2020 and May 2021, 30 samples from diverse patients admitted to the general hospital, the teaching hospital for women and children, and the Al-Diwaniyah teaching hospital yielded a total of 20 Shigella species isolates. The isolates, numbered No.1 to No.8, were identified using standard methods and the polymerase chain reaction methodology. The entire genomic DNA of twenty isolates, numbered 1 to 8, isolated from various geographic locations of Iraq, was retrieved. Pathogenic Shigella spp. 16S ribosomal RNA gene sequencing was done, and the locations of these genes on the isolates' genomes were determined.

Sequencing of the 16S ribosomal RNA gene from pathogenic Shigella spp. was done, and the locations of these genes on the isolates' genomes were identified. Using the MEGA 6 software, a phylogenetic tree analysis was completed, and the local Shigella spp. was revealed by the comparison of the current study isolates with the worldwide Shigella strains in the NCBI-Gen bank.

Keywords: *Phylogenetic tree, phylogenetic origin, 16S ribosomal RNA, Shigella spp., DNA sequencing, and PCR.*

Introduction

Shigella spp. are facultative anaerobes, non flagellated, gram-negative, rodshaped, nonspore-forming bacteria. They induce acute diarrhea that can progress into bacillary dysentery, often known as bloody mucoid diarrhea (or shigellosis),which is more commonly seen (Kahsay and Muthupandian, 2016). Shigella remains a major pathogen responsible for increased rates of morbidity and mortality from dysentery each year around the world, particularly in children under the age of five in poor countries, and is the most common cause of diarrhea (Puzari et al., 2018; Mukhopadhyay et al., 2020). The Shigella genus is divided into four species based on serological characteristics: Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei. Many serotypes are created for each species based on the 'O' antigen of the lipopolysaccharide layer, with S. with S. dysenteriae having fifteen serotypes, S. boydii having eighteen serotypes, S. flexneri having six serotypes and fifteen subtypes, and S. sonnei only having one serotype (Kotloff et al., 2018).

Shigellosis can manifest itself as a random illness, an epidemic, or a pandemic. Because

to its modest infectious dose of 10-100 organisms—as opposed to 105-108 for Salmonella and Vibrio—it poses a major public health risk (Kotloff et al., 2018). Also, a study that estimated the burden of disease worldwide found that it was the second most common cause of death related to diarrhea across all age categories (Khalil et al., 2018). Children who lack access to clean water, practice poor hygiene and sanitation, and have inadequate nourishment are more likely to contract shigellosis.

Shigellosis is more common in children who do not have access to safe drinking water, have poor hygiene and sanitation, or are malnourished.

Shigella causes large intestine inflammation and ulcers by attacking the colonic epithelium. Diarrhea symptoms can range from minor to severe, and might include bloody feces, stomach pain, and a high fever (Mukhopadhyay et al., 2020). Shigella species secrete a variety of virulence factors in the colon that generate considerable inflammation and mild enterotoxic consequences, culminating in the onset of characteristic watery diarrhea. As a result, the pathogenicity of a given isolate is determined by the expression of several virulence genes involved in colonization, invasion/penetration, and toxinmediated illness (Zhang et al., 2014).

Shigellosis was caused by chromosomal or large pathogenic inv plasmids that contained a variety of virulence factors (Shen et al., 2013). In addition to virulence factors expressed on the virulence plasmid, Shigella also manufactures toxins such as Shigella enterotoxin 1 (ShET-1), Shigella enterotoxin 2 (ShET2), and shiga toxin (Stx).

The triple mutations on chromosomes gyrA and parC cause resistance to evolve over time. Horizontally transmitted components may also help shape and establish resistant clones throughout development. A phylogenetic tree is a tree diagram that displays the evolutionary histories or links of distinct biological groups or other categories based on physical or genetic similarities and differences (Hu et al., 2020). It is utilized in a discipline of biology that examines morphological data matrices and DNA sequencing data to determine how different groups of animals evolved over time. The phylogenetic tree is significant because it has been used to investigate biodiversity, evolution, genetics, and ecology among organism groups (Quiroz-Morales et al., 2022). A single evolutionary tree representing all life on Earth depicts a common ancestry (Felsenstein, 2004). As a result, the current study focused on the phylogenetic origins of Shigella Spp isolates in Iraq.

Materials and Methods Gathering of Samples

Thurty participants of various ages (adults, adolescents, and children), including both sexes, had their stools sampled. Between November 2020 and May 2021, stools samples were taken from patients with diarrhea who were admitted to general hospitals, teaching hospitals for women and children, and Al-Diwaniyah teaching hospitals.

Identification and Isolation

Using a sterile loop and blood agar (Himedia), the specimens were grown for 24 hours at 37 C. Pristine colonies were maintained at 4 C° in nutritional broth with glycerol (Himedia) (Jawetz et al., 2019). Bacteria were cultivated on MacConkey agar, blood agar, and nutritional agar (Himedia), and their form, size, texture, and colony structure were examined. The isolates were identified using the polymerase chain reaction

method according to MacFaddin's (2000) descriptions of the morphological properties (for cells and colonies) and biochemical properties of testes . A single colony of the isolates was Gram stained and examined under a 100x oil-emersion light microscope (Suwaidian, and Naji, 2020).

Extraction of DNA

Shigella spp. molecular identification required the manufacturer standard for whole genomic DNA extraction (Favorgen, Taiwan).

Primer		Sequence (5----------›3)	Amplicon size (bp)	Conditions (D, A and E)	Cycle No.
16S RNA gene Shigella sp.	F	CGCAGGCGGTTTGTTAAGTC	515	94˚C/1min 57˚C/1min 72˚C/2 min	35
	R	ACATTCGAGCAACACGGGG			

Abbreviations: D stands for denaturation, A for annealing, E for extension, F for forward primer, and R for reverse primer.

Producer of PCR Reaction Mixtures

According to the manufacturer's instructions, the PCR reaction mixture was carried out in 12 l of PCR MasterMix (Bioneer, South Korea). The reaction took place in a total volume of 25 l, made up of 2 l from each primer (forward and reverse), 3 l of DNA, and the remaining 6 l from free nucleases in deionized water. All of the aforementioned information is present in the negative control, but no DNA template was employed. The amplification processes were carried out in a thermocycler machine that was automated (Clever Scientific, UK).

Agarose Gel Electrophoresis Gel Agarose PCR results were subjected to electrophoresis on a 1% horizontal agarose gel and dyed with red safe dye for an hour at 75 volts. 5 l of amplification products and 1 l of loading dye were added to the gel's wells. A 100-1500 bp DNA ladder was used to measure the size of amplified gene fragments (Promega, USA). The gel documentation system (Biometra-Germany) was reportedly utilized to take pictures of the DNA bands, according to Lin et al., (2012).

Based on 16S ribosomal RNA gene pathogenic Shigella spp. isolates, DNA sequencing was used to explore the confirmative genetic identity and genetic variation (substitution Mutations) investigation. DHL (DHL Home - Global Logistics and International Shipping) transported the PCR products to the Macrogen Company in Korea in ice bags so that Applied Biosystems could sequence the DNA on them. Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0), partial metE and partial metF gene-based ClustalW alignment analysis, and the Maximum Composite Likelihood approach via phylogenetic tree UPGMA method were used to carry out the DNA sequencing investigation. The identification of homologous sequences and analysis of mutations using NCBI BLAST. The genes were finally entered into the NCBI-Genbank database for retrieval.

Phylogenetic Relationship

The phylogenetic tree was constructed using the neighbor method and the MEGA4 software program (Tamura et al., 2007), as well as the neighbor-joining phylogeny tree (Saitou and Nei, 1987). The evolutionary distances were calculated using the maximum composite likelihood approach, and the tree's reliability was assessed using a 1000 data set bootstrap similar to (Tamura et al., 2004)

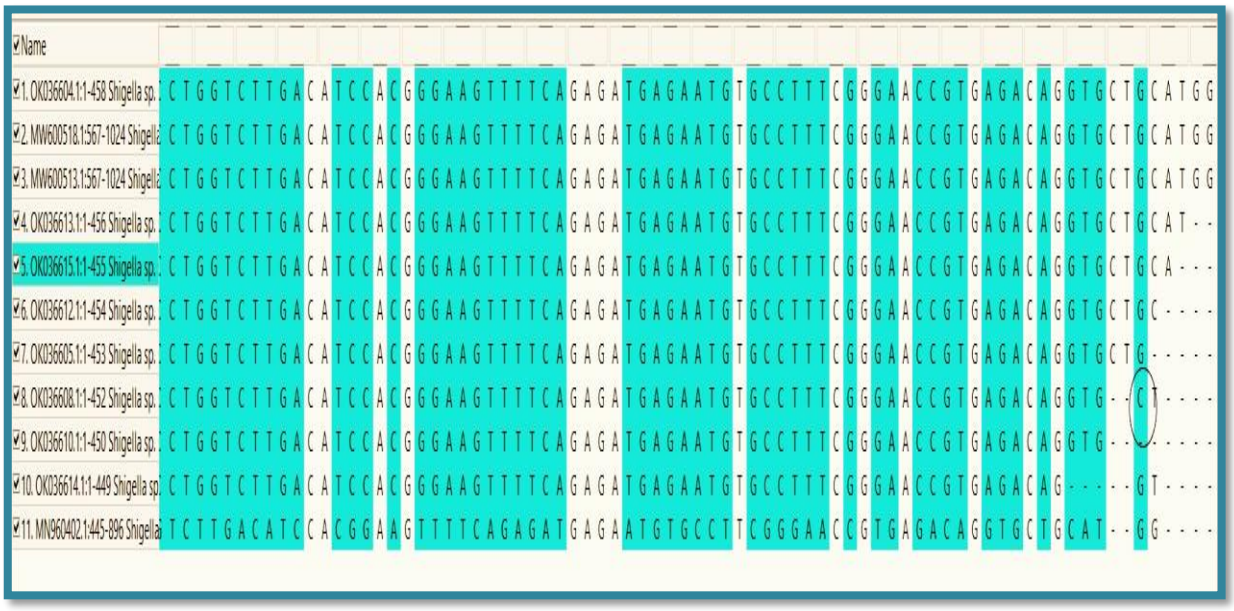
Result and Discussion:

Using molecular typing, epidemiological research has successfully tracked the origins and genetic relationships of dangerous

microorganisms (Sheikh et al., 2019). Hospital acquired infections caused by multidrug resistance bacteria are becoming a significant problem for patients. Understanding pathogen relatedness is crucial for determining the epidemiology of nosocomial infections and assisting in the development of rational pathogen control approaches. Pathogen typing is used to determine whether genetic links exist between epidemiologically related isolates. Novel DNA-based technologies or molecular analysis are the methods of choice for establishing the molecular relatedness of isolates for epidemiologic study. DNA-based molecular technologies include pulsed-field gel electrophoresis (PFGE), PCR-based typing procedures, and multilocus sequence analysis (Singh et al., 2006).

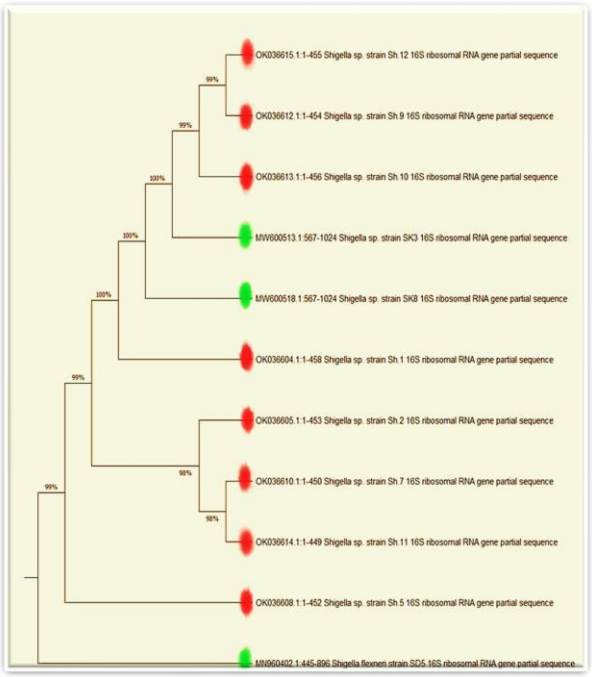
Table 1: The percentage of homologous sequence identity (%) between a local Shigella spp. isolate (IQ-No.1 to IQ-No.8) and a related Shigella spp. isolate submitted to NCBI-BLAST.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Shigella sp. strain Sh.1 16S ribosomal RNA gene, partial sequence	Shigella sp.	846	846	100%	0.0	100.00%	458	OK036604.1
Shigella sp. strain SK8 16S ribosomal RNA gene, partial sequence	Shigella sp.	846	846	100%	0.0	100.00%	1147	MW600518.1
Shigella sp. strain SK3 16S ribosomal RNA gene, partial sequence	Shigella sp.	846	846	100%	0.0	100.00%	1146	MW600513.1
Shigella sp. strain Sh.10 16S ribosomal RNA gene, partial sequence	Shigella sp.	843	843	99%	0.0	100.00%	456	OK036613.1
Shigella sp. strain Sh.12 16S ribosomal RNA gene, partial sequence	Shigella sp.	841	841	99%	0.0	100.00%	455	OK036615.1
Shigella sp. strain Sh.9 16S ribosomal RNA gene, partial sequence	Shigella sp.	839	839	99%	0.0	100.00%	454	OK036612.1
Shigella sp. strain Sh.2 16S ribosomal RNA gene, partial sequence	Shigella sp.	837	837	98%	0.0	100.00%	453	OK036605.1
Shigella sp. strain Sh.5 16S ribosomal RNA gene, partial sequence	Shigella sp.	835	835	98%	0.0	100.00%	452	OK036608.1
Shigella sp. strain Sh.7 16S ribosomal RNA gene, partial sequence	Shigella sp.	832	832	98%	0.0	100.00%	450	OK036610.1
Shigella sp. strain Sh.11 16S ribosomal RNA gene, partial sequence	Shigella sp.	830	830	98%	0.0	100.00%	449	OK036614.1
Shigella flexneri strain SD5 16S ribosomal RNA gene, partial sequence	Shigella flexneri	802	802	100%	0.0	98.47%	1231	MN960402.1



Shigella sp. alignment reveals a modest number of mutations, indicating that there are only minor changes between isolates. Nonetheless, the same locus and overall function of the gene remain, and this function may be expressed differently as it is vital for the bacterium, therefore the impact will be resistance or sensitivity depending on the mutation present. The results agreed with the researchers' presentation of the draft genome sequence of a strain identified from India, which exhibits large levels of resistance and a high level of mutation.

Figure 2. A phylogenetic tree based on the partial sequencing of the 16S ribosomal RNA gene was used to examine the genetic relationships between Shigella spp. (IQ-No.1 to IQ-No.8) isolates from the local area. The phylogenetic tree was made using UPGMA and the inferred evolutionary history (MEGA6).



Shigella spp. from the local area (IQ-No. 1 to IQ-No. 8) and the NCBI-BLAST Shigella sp. were shown to be genetically related.

The study's chromosomal gene sequences were used to create a phylogenetic tree. Isolates are classified according on their serotype and species. The key nodes of the network all have bootstrap values more than 50%, which aids in identifying the three primary Shigella genus clusters and subclusters. Shigella's gene map was analyzed for comparison. The PCR study used to identify the most common genetic features in table 1 revealed striking commonalities. Shigella is a bacteria in the enterobacteriaceae family that causes diarrhoea, fever, and stomach pain in persons who are infected. Shigella dysenteria, S. sonnei, and the recently discovered S. boydii are clinically important Shigella species. Shigellosis is more commonly connected with children.

This technique employs a large number of housekeeping gene segments to identify microbial species based on their different allelic patterns (Wang et al., 2019). The sequence type is defined by the alleles at each location (ST). Using BLAST homology analysis, this study discovered two distinct sequence classes in Shigella species. The disease is especially common in children under the age of five who live in impoverished nations where shigellosis is rampant. According to a number of specialists, people whose stool cultures for Shigella species were positive should receive treatment to reduce the length of their clinical symptoms and minimize the amount of faeces discharge produced by the organism, hence lowering the risk of transmission (Rahman and Sarker, 2021).

Shigella sonnei was the most common Shigella serotype in the study of Madhavan et al.,. (2018). (62.5 per cent). In India, no epidemics of S. sonnei of this magnitude have been seen. S. sonnei is more common in industrialized countries with a slower rate of development. The prevalent species in Thailand, Vietnam, and Sri Lanka has shifted from S. flexneri to S. sonnei, most likely as a result of these countries' improved socioeconomic conditions. According to Madhavan et al.,. (2018), a similar scenario is also playing out in India's southern states. Shigella infections are typically caused by a lack of sanitation and access to adequate drinking water.

Conclusion

This study proved that 16S rRNA gene PCR can be utilized to reliably diagnose Shigella spp. Regular travel to India was a major factor in the spread of the isolates to Iraq. The results revealed a small number of mutations, indicating that the differences across isolates are limited. Yet, the same locus and overall function of the gene remain, and while this function is critical for the bacterium, it may be expressed differently, resulting in resistance or sensitivity depending on the mutation present.

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