Isolation and Identification of *Shigella Sonnei* Producing Shiga Toxin from Children with Bloody Diarrhea and Evaluation of the Inhibition Effectiveness of Zinc oxide Nanoparticles

**Hayfa Rajab Alwan**¹, Shaymaa Naji Dahham², Ahmed Hussein Dhayea³, Mohammed Nadhir Maaroof⁴

¹Department of Biology, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq
²Department of Chemistry, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq
³Salah Al-Din Education Directorate, Ministry of Education, Tikrit, Iraq
⁴Department of Microbiology, College of Medicine, Tikrit University, Tikrit, Iraq

**ABSTRACT**

*Shigella* infection strains producing shiga toxin results in bloody diarrheas a dangerous symptom in children under five years of age that can even lead to death. Therefore, this study was conducted to isolate *Shigella* strains producing shiga toxin from 150 samples which were taken from bloody stool of children under the five years of age. They were suffering from the diarrhea during the period from March 2021 to March 2022 in Tikrit city, Iraq. The results showed the possibility to isolate six isolates of *Shigella sonnei* at ratio 4% of the total samples. The isolates producing shiga toxin were identified by using *stx1* gene. The results showed two isolates of *Shigella* possessing *stx1* gene, at ratio 33.33 of total *S. sonnei* strains. The two isolates producing shiga toxin have been submitted to NCBI, then accepted as Iraqi strains in NCBI under the registration numbers OK127759.1 and OK127760.1. The Iraqi strains registered in NCBI showed agreement with a global strain of 99-100% that were recorded by registration numbers in Malaysia (CP060117.1), China (CP000038.1), United Kingdom (CP066810.1), Hungary (CP019689.1), Somalia (CP023645.1), Spain (CP022672.1), Italy (CP035008.1), India (CP041322.1), Australia (CP045932.1), Nigeria (CP046286.1), Switzerland (CP049183.1), USA (CP053751.1) and South Korea (CP055292.1). As for the sensitivity test, the results showed that the strain OK127759.1 was resistant to antibiotics azithromycin, cefazidime, cefotaxime, ceftriaxone, augmentin and tetracycline. And it was sensitive to gentamicin and intermediate sensitive to ampicillin, chloramphenicol and nalidixic acid. As for the strain OK127760.1, the results showed that this strain was intermediate sensitive to gentamicin and resistant to the other antibiotics which were used in the test. ZnO-NPs with a concentration of 200 μg/ml showed the highest inhibitory effectiveness against *S. sonnei* strains OK127759.1 and OK127760.1 with inhibition diameter of 22 mm and 21 mm respectively.

**Keywords**: Bloody diarrhea, Phylogenetic tree, *Shigella sonnei* strains producing shiga toxin, *stx1* genes, ZnO nanoparticles.

**Email**: hyafaass@tu.edu.iq

---

** длинным**

**Isolation and Identification of *Shigella Sonnei* Producing Shiga Toxin from Children with Bloody Diarrhea and Evaluation of the Inhibition Effectiveness of Zinc oxide Nanoparticles**

**Hayfa Rajab Alwan**¹, Shaymaa Naji Dahham², Ahmed Hussein Dhayea³, Mohammed Nadhir Maaroof⁴

¹Department of Biology, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq
²Department of Chemistry, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq
³Salah Al-Din Education Directorate, Ministry of Education, Tikrit, Iraq
⁴Department of Microbiology, College of Medicine, Tikrit University, Tikrit, Iraq

**ABSTRACT**

*Shigella* infection strains producing shiga toxin results in bloody diarrheas a dangerous symptom in children under five years of age that can even lead to death. Therefore, this study was conducted to isolate *Shigella* strains producing shiga toxin from 150 samples which were taken from bloody stool of children under the five years of age. They were suffering from the diarrhea during the period from March 2021 to March 2022 in Tikrit city, Iraq. The results showed the possibility to isolate six isolates of *Shigella sonnei* at ratio 4% of the total samples. The isolates producing shiga toxin were identified by using *stx1* gene. The results showed two isolates of *Shigella* possessing *stx1* gene, at ratio 33.33 of total *S. sonnei* strains. The two isolates producing shiga toxin have been submitted to NCBI, then accepted as Iraqi strains in NCBI under the registration numbers OK127759.1 and OK127760.1. The Iraqi strains registered in NCBI showed agreement with a global strain of 99-100% that were recorded by registration numbers in Malaysia (CP060117.1), China (CP000038.1), United Kingdom (CP066810.1), Hungary (CP019689.1), Somalia (CP023645.1), Spain (CP022672.1), Italy (CP035008.1), India (CP041322.1), Australia (CP045932.1), Nigeria (CP046286.1), Switzerland (CP049183.1), USA (CP053751.1) and South Korea (CP055292.1). As for the sensitivity test, the results showed that the strain OK127759.1 was resistant to antibiotics azithromycin, cefazidime, cefotaxime, ceftriaxone, augmentin and tetracycline. And it was sensitive to gentamicin and intermediate sensitive to ampicillin, chloramphenicol and nalidixic acid. As for the strain OK127760.1, the results showed that this strain was intermediate sensitive to gentamicin and resistant to the other antibiotics which were used in the test. ZnO-NPs with a concentration of 200 μg/ml showed the highest inhibitory effectiveness against *S. sonnei* strains OK127759.1 and OK127760.1 with inhibition diameter of 22 mm and 21 mm respectively.

**Keywords**: Bloody diarrhea, Phylogenetic tree, *Shigella sonnei* strains producing shiga toxin, *stx1* genes, ZnO nanoparticles.

**Email**: hyafaass@tu.edu.iq

---

**References**


1. Email: hyafaass@tu.edu.iq
INTRODUCTION

*Shigella dysenteriae* serotype 1 is considered a unique species among *Shigella* species in producing a potent toxin which is known as a shiga toxin (Stx). This, however, does not mean that there are no other species that have the ability to produce shiga toxin [1]. Only a few strains of *Shigella sonnei* produce shiga toxin and this ability is due to Stx phages acquisition. Phage acquisition can occur in different environments as independent events. Horizontal transfers are also responsible for the emergence of Stx phages in *S. sonnei* strains [2]. Shiga toxin belongs to a large family of ribosome-inactivating proteins (RIPs). There are two groups of shiga toxin, Stx1 and Stx2. Shiga toxin molecule is the bi-terminal molecule made up of one coenzyme A subunit and five B subunits [3]. The deep damage of bowel epithelium is promoted by cytotoxic action of bacterial Shiga toxin which is encoded by chromosomal *stx* gene. Maximal toxin production is essential for virulence of *S. dysenteriae* type 1. The toxin action pattern is very similar to enterohemorrhagic *E. coli* (EHEC) verotoxins [4]. As in EHEC, Stx toxin is composed from A and B subunits. Several receptors of B-subunits bind to cellular receptor glycolipid Gb3. Exotoxin internalization is then followed by A-subunit cleavage. Toxic A1 fragment possesses RNA N-glycosidase activity and thereby cleaves N-glycosidic bond within 28S ribosomal RNA which leads to the termination of protein synthesis and death of host cells.
[5]. Infection with *Shigella* producing shiga toxin results in bloody diarrhea that may develop into extra-intestinal complications which threaten the life. Shiga toxin induces apoptosis of epithelial cells, endothelial cells, leukocytes, lymphoid cells and neuronal cells. The genes encoding shiga toxin are carried by phages [6, 7]. Hemolytic uremic syndrome (HUS) is a well-described process known to cause severe renal dysfunction, thrombocytopenia and anemia. HUS is usually associated with a shiga toxin which is found in *Shigella* strains [8]. Zinc oxide has been used in biomedical systems due to its low production cost, safe to use and can be easily prepared[9]. It has been reported in literature that nanoparticles can attack bacteria through six main mechanisms: (i) Destruction of cell wall (ii) Release of toxic ions (iii) Destruction of protons efflux pumps and modification of membrane charges (iv) Formation of reactive oxygen species (ROS) (v) DNA, RNA and proteins degradation by ROS, (vi) Drop ATP production [10]. Antibacterial activity of zinc oxide nanoparticles ZnO-NPs focuses on formation of ROS, including hydrogen peroxide H₂O₂, OH⁻ (hydroxyl radicals) and O₂⁻² (peroxide). ROS possess several mechanisms to effect cell wall, including its destruction due to ZnO-localized interaction, increased membrane permeability, internalization of NPs due to loss of proton motive force and uptake of toxic dissolved zinc ions. All of these lead to weak mitochondria, intracellular outflow and release gene expression of oxidative stress which causes eventual cell growth inhibition and then death [11].

**Materials and Methods**

*Isolation and Identification of Shigella spp.*

This study was conducted during the period from March 2020 to March 2021 in Tikrit city, Iraq. *Shigella* were isolated from 150 stool samples taken from children under 5 years of age, suffering from bloody diarrhea by using *Shigella* broth and SS agar. Colorless colonies that grew on SS agar were isolated [12]. Isolates were identified at the species level by using the compact Vitek 2 system. As well as the isolates were identified at the strain level by *stx* gene sequencing.

**DNA Extraction**

Purification Kit of Genomic DNA was used to extract DNA. This kit was manufactured by Promega in USA [13].

*Preparation of Primer*

*stx*₁ primer in Table 1 was synthesized by Alpha Corporation in Canada as a lyophilized product and was dissolved in sterile deionized water, according to the manufacturer's instructions to get final concentration of *stx*₁ primer stock solution (100 μM). The working solution was prepared by adding 10 μl of the stock solution to 90 μl of deionized water.

**Table 1:** *stx*₁ primer used in this study

<table>
<thead>
<tr>
<th>Sequence 5’-3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: CAGTTAATGTGGTTGCGAAG</td>
<td>895</td>
</tr>
<tr>
<td>R: CTGCTAATAGTTCTGCGCATC</td>
<td></td>
</tr>
</tbody>
</table>

**PCR Reaction**

PCR reactions were achieved at volumes of 25μl in Eppendorf tubes. All reaction ingredients were frozen separately and used at optimum concentration. Table 2 shows the ingredients of the PCR reaction. PCR reaction for the gene was used by this study which was achieved according to the reaction conditions (Table3) [14].
Table 2: PCR reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Master Mix (2X)</td>
<td>12.5</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>8.5</td>
</tr>
<tr>
<td>Template of DNA</td>
<td>2</td>
</tr>
<tr>
<td>F. Primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>R. Primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 3: PCR conditions [14] to detect stx1 gene.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Cycles</th>
<th>Temperature (°C), Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>InitialDenaturation</td>
<td>1</td>
<td>95 °C, 3min.</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95 °C, 60sec.</td>
</tr>
<tr>
<td>Annealing</td>
<td>35</td>
<td>57 °C, 50sec.</td>
</tr>
<tr>
<td>FinalExtension</td>
<td></td>
<td>72 °C, 120sec.</td>
</tr>
<tr>
<td>Extension</td>
<td>1</td>
<td>72 °C, 7min.</td>
</tr>
</tbody>
</table>

Electrophoresis

PCR products electrophoresis was performed 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 of PCR products and 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 50 minutes [15].

Antibiotic Sensitivity Test

This test was done by using Bauer-Kirby method which is approved by World Health Organization [16].

ZnO-NPs Preparation

Zinc nanoparticles (ZnO-NPs) manufactured by a Sky Spring Nanomaterials Inc., USA were used in this study. Stock suspension of ZnO-NPs was prepared by suspending 10000 µg of it in 10 ml of deionized distilled water to get a concentration of 1000 µg/ml. The stock suspension was dispersed by sonication at 100W and 40 kHz for 40 minutes to form homogeneous suspension. The stock suspension was kept in dark vial at room temperature until use [17, 18]. The concentrations of 200, 150, 100, 50, 25 µg/ml used in the study, were prepared by diluting the stock suspension to an appropriate concentration by using distilled water. A magnetic stirrer was used to avoid aggregation and deposition of particles during dilution. Zinc oxide nanoparticles were spherical in shape and their size ranged between 10-30 nanometers.

Results and Discussion

Isolation and Identification of Shigella spp.

Culturing of 150 stool samples on SS agar led to appear 11 isolates with colorless colonies with a diameter of 2-3 mm and a convex surface (Figure 1). These phenotypic characteristics were similar to the phenotypic characteristics of Shigella colonies on this medium [19].
The results of identification, using the Vitek 2 compact system, showed that only 6 isolates belonged to *S. sonnei*, at a ratio of 4% of the total samples. Current study results agreed with the results of the study conducted by Rabatti and Rasheed [20] in Erbil city, where they isolated *Shigella* at a ratio of 3.6% from patients attending Erbil Children’s Hospital. In addition, the results of this study somewhat agree with the study results obtained by Tawfik *et al.* [21], where they found the number of children infected with *S. sonnei* representing 2.58% of the total number of all cases. The results of this study disagree with the results of study conducted by Ali [22] in Hawija city where he isolated *Shigella* from children aged under five years, at an infection rate of 9.3%. The results of our study are very far from the results of the study of Abdulrahman *et al.* [23] where they isolated *Shigella* at 14% ratio of the total stool samples collected in Baghdad.

**Detection of stx1 gene**

The detection results of *stx1* gene produced only two isolates that possessed the *stx* gene, and this gene appeared on the agarose gel as a band of size 895 bp (Figure 2). The appearance ratio of *stx1* gene was 33.33% of the total *S. sonnei* isolates. Sváb [24] stated in his results that gain of Stx phages can occur in different milieus as independent events, and that various horizontal transfers in charge of emergence of Stx phages in strains of *S. sonnei*.

**Figure 2:** Electrophoresis of *stx1* gene on agarose gel (2% concentration at 5 volt/cm for 50 min.), The bands in the lanes 2, 5 at a size of 895 bp is a typical band of the *stx1* gene of *Shigella sonnei* strains. M: DNA ladder (100-1500bp)

**Nucleotide Sequencing Results**

The *stx1* gene sequence was used to study the two isolates of *S. sonnei*. The searches for identical sequences were carried out using BLAST software available at the National Center for Biotechnology Information (NCBI). Sequence analysis results of the *stx1* gene for the two
isolates showed match ratio of 99% with the *stxl* gene sequences of *S. sonnei* in the gene bank under the sequence ID: CP055292.1 (Table 4). Isolate No. 2 showed the presence of three types of genetic variants, two of which C/T and G/A were transitional variations at sites 2842188 and 2842252, respectively, and the third C/G was a transversional variant at site 2842165. Isolate No. 6 showed transitional variant T/C at site 2842219 and transversional variant A/C at site 2842301.

**Table 4: Matching ratio of *Shigella sonnei* local isolates with standard strains in NCBI**

<table>
<thead>
<tr>
<th>No. Of Sample</th>
<th>Type of Substitution</th>
<th>Location</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Predicted Effect</th>
<th>Sequence ID with Compare</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Transversion</td>
<td>2842165</td>
<td>C/G</td>
<td>AGC\AGG</td>
<td>Missense</td>
<td>ID: CP055292.1</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Transition</td>
<td>2842188</td>
<td>CT</td>
<td>GCG\GTC</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Transition</td>
<td>2842219</td>
<td>TC</td>
<td>ACT\ACC</td>
<td>Silent</td>
<td>ID: CP055292.1</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Transversion</td>
<td>2842301</td>
<td>AC</td>
<td>ACA\ACCA</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Recording of *Shigella sonnei* Local Isolates in NCBI**

The two isolates of *S. sonnei* were registered at the National Center for Biotechnology Information using *stxl* gene sequencing. The isolates obtained accession number and became a reference to Iraq, The Middle East and the world with the registration numbers: OK127759.1, OK127760.1

**Phylogenetic Tree**

The Iraqi strains registered in NCBI showed a global agreement of 99-100% with Malaysia (CP060117.1), China (CP000038.1), United Kingdom (CP066810.1), Hungary (CP019689.1), Somalia (CP023645.1), Spain (CP022672.1), Italy (CP035008.1), India (CP041322.1), Australia (CP045932.1), Nigeria (CP046286.1), Switzerland (CP049183.1), USA (CP053751.1) and South Korea (CP055292.1), as shown in Figure 3 of the phylogenetic tree of *S. sonnei*.

**Figure 3: Phylogenetic tree of *Shigella sonnei***

**Antibiotic susceptibility test**

*Shigella* strains sensitivity against 10 antibiotics was tested by using the disc diffusion method (Figure 4). Inhibition zones diameters were measured and compared with the standard diameters to determine the sensitive and resistant strains for each antibiotic as in the Table 5.
Sensitivity test results for *Shigella* strains showed that the strain OK127759.1 was resistant to antibiotics azithromycin, ceftazidime, cefotaxime, ceftriaxone, augmentin and tetracycline. While the results showed that this strain was sensitive to gentamicin and intermediate sensitive to antibiotics ampicillin, chloramphenicol and nalidixic acid. These results differed from results of the study by Dhattalet al. where they mentioned that all *shigella* isolates were sensitive to antibiotics nalidixic acid [25]. As for the strain OK127760.1, the results showed that it was resistant to all the antibiotics mentioned in the Table 5. However, it was intermediate sensitive to gentamicin. These results differed from results of the study by Dhitalet al, where they mentioned that *Shigella* isolates were sensitive to antibiotics azithromycin, ceftazidime, cefotaxime and ceftriaxone [26].

**Figure 4**: Sensitivity test of *Shigella sonnei* strains to antibiotics

**Table 5**: *Shigella* strains resistant to antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Symbol</th>
<th>Con. μg/disk</th>
<th><em>Shigella sonnei</em> Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OK127759.1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>AM</td>
<td>25</td>
<td>I</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>AZM</td>
<td>15</td>
<td>R</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>CAZ</td>
<td>30</td>
<td>R</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30</td>
<td>R</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>CRO</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>10</td>
<td>I</td>
</tr>
<tr>
<td>Augmentin</td>
<td>AMC</td>
<td>30</td>
<td>R</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>CN</td>
<td>10</td>
<td>S</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>NA</td>
<td>30</td>
<td>I</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TE</td>
<td>10</td>
<td>R</td>
</tr>
</tbody>
</table>

**Effectiveness Inhibition of ZnO-NPs**

The inhibition effectiveness of ZnO nanoparticles was tested by using agar well diffusion method [27]. In this test duplicates plates were used for each isolate. The results showed that the concentration of 200 μg/ml ZnO-NPs had the highest inhibition activity against the strain
OK127759.1 and for the strain OK127760.1 with inhibition diameter of 22 mm and 21 mm, respectively (Figure 5). And as for the other concentrations, the inhibition diameters ranged from 9 -20 mm (Table 6).

**Figure 5:** Agar well diffusion method to test Inhibition effectiveness of ZnO-NP Against *Shigella sonnei* strains.

<table>
<thead>
<tr>
<th>Concentrations (µg/ml)</th>
<th><em>Shigella sonnei</em> Strains</th>
<th>Inhibition diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OK127759.1</td>
<td>OK127760.1</td>
</tr>
<tr>
<td>25</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>50</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>100</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>200</td>
<td>22</td>
<td>21</td>
</tr>
</tbody>
</table>

**Table 6:** Diameters of inhibition zones

**Conclusion**

Bloody diarrhea caused by infection with *S. sonnei* producing shiga toxin is considered one of the most dangerous symptoms that threaten the lives of infected children. Thus, the present study would suggest the possible utilization of ZnO NPs to prevent the fatal diseases caused by *S. sonnei* strains that produce shiga toxin and are resistant to many antibiotics.

**Conflicts of Interest**

The authors declare that they have no conflict of interest. **Ethics Approval**
The research was agreed by the local Ethical Committee in Tikrit University.

**Authors Contribution**

H.R. Alwan designed the research and collected the samples and performed all assays that were related to the identification of isolations, DNA extraction and investigation of virulence gene. M.N. Maaroor and Sh.N. Dahham analyzed the data and interpreted the results. A.H. Dhayea drafted the manuscript and reviewed it and recorded isolations in NCBI. All authors read and agreed to manuscript version.
References


