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

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ABSTRACT

Shigella infection strains producing shiga toxin results in bloody diarrheais 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, ceftazidime, cefotaxime, ceftriaxone, augmentin and tetracycline. And it was sensitive to gentamicin and intermediately sensitive to ampicillin, chloramphenicol and nalidixic acid. As for the strain OK127760.1, the results showed that this strain was intermediately 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.

عزل وتشخيص*Shigella Sonnei* المنتجةلسم الشيگا من الأطفال الذين يعانون من الإسهال الدموي وتقييم الفعالية التثبيطيةلأوكسيد الزنك النانوي

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الخلاصة

تؤدي الإصابة بسلالات الشيگلا المنتجة لسم الشيگا إلى إسهال دموي يمثل أعراضًا خطيرة لدى الأطفال دون سن الخامسة ويمكن أن يتسبب في الوفاة. لذلك أجريت هذه الدراسة لعزل سلالات *Shigella sonnei* المنتجة لسم الشيگا من 150 عينة مأخوذة من براز دموي لأطفال دون سن الخامسة يعانون من الإسهال خلال الفترة من آذار 2021 إلى آذار 2022 في مدينة تكريت / العراق.أظهرت النتائج إمكانية عزل ست عزلات من *S. sonnei*بنسبة 4% من مجموع العينات. تم التعرف على العزلات المنتجة لسم الشيگا باستعمال الجين *stx*. أظهرت النتائج أن اثنين من عزلات *S. sonnei* تمتلكان الجين *stx* وبنسبة 33.33% من مجموع سلالات *S. sonnei*. تم تسجيل عزلات *S. sonnei* المنتجة لسم الشيگا في المركز الوطني لمعلومات التكنولوجيا الحيويةNCBI،وتم قبولها على أنها سلالات عراقية في NCBI تحت أرقام التسجيل OK127759.1وOK127760.1.أظهرت السلالات العراقية المسجلة في NCBIتوافقاً مع سلالات عالمية بنسبة 99–100%، التي سجلت بأرقام تسجيل ماليزيا (CP060117.1) والصين (CP000038.1) والمملكة المتحدة (CP066810.1) والمجر (CP019689.1)والصومال (CP023645.1)وإسبانيا (CP022672.1)وايطاليا (CP035008.1)والهند (CP041322.1)وأستراليا (CP045932.1)ونيجيريا (CP046286.1)وسويسرا (CP049183.1)والولايات المتحدة الأمريكية (CP053751.1) وكوريا الجنوبية (CP055292.1).أما بالنسبة لاختبار الحساسية، فقد أظهرت النتائج أن السلالة (OK127759.1) كانت مقاومة للمضادات الحيويةazithromycin، ceftazidime، cefotaxime، ceftriaxone، augmentin وtetracycline. كما كانت حساسة للمضاد الحيوي Gentamicin ومتوسطة الحساسية للمضادات ampicillin وchloramphenicol. أما بالنسبة للسلالة (OK127760.1) فقد أوضحت النتائج أن هذه السلالة كانت متوسطة الحساسة للمضاد الحيوي gentamicin ومقاومة لبقية المضادات الحيوية المستخدمة في الاختبار .أظهر أوكسيد الزنك النانوي ZnO–NPs بتركيز 200 ميكروغرام / مل أعلى فعالية تثبيطية تجاه السلالة OK127759.1والسلالة OK127760.1 بقطر تثبيط 22 ملم، 21 ملم على التوالي.

INTRODUCTION

Shigella dysenteriae serotype 1 is considered a unique species among *Shigella* species in producing a potent toxin which is known 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 threat 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 processknown 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 H_2O_2, OH^- (hydroxyl radicals) and O_2^{-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

stx1 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 *stx1* 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:*stx1* primer used in this study

Sequence 5´-3´	Product size (bp)
F: CAGTTAATGTGGTTGCGAAG	895
R: CTGCTAATAGTTCTGCGCATC	

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

Component	Volume (µl)
Green Master Mix (2X)	12.5
Nuclease Free Water	8.5
Template of DNA	2
F. Primer (10 µM)	1
R. Primer (10 µM)	1
Total volume	25

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

Stages	Cycles	Temperature (°C), Time
InitialDenaturation	1	95 °C ,3min.
Denaturation	35	95 °C,60sec.
Annealing		57 °C,50sec.
FinalExtension		72 °C,120sec.
Extension	1	72 °C,7min.

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 1000µg/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].

Figure 1: *Shigella* colonies on SS agar

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 e 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.*

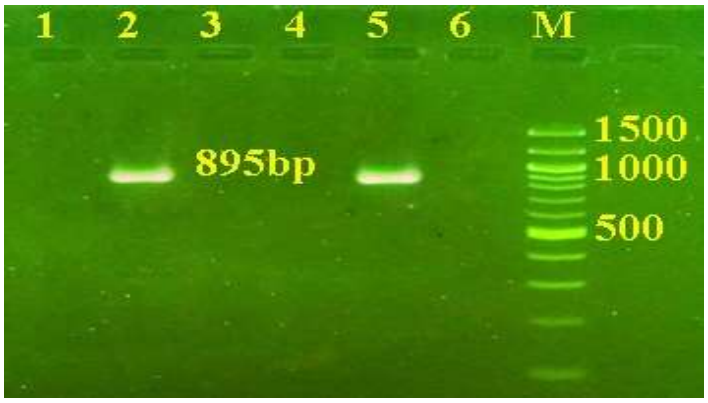

Figure 2: Electrophoresis of *stx1* gene on agarose gel (2%concentration at 5 volt/cm for 50 min.), The bands in the lanes 2,5 at a size of 895 bp is a typical bandof the *stx1* gene of *Shigella sonnei*strains. M: DNA ladder (100-1500bp)

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 *stx1* 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. IsolateNo. 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

Source: *Shigella sonnei*								
No. Of Sample	Type of Substitution	Location	Nucleotide	Nucleotide Change	Amino Acid Change	Predicted Effect	Sequence ID with Compare	Identities
2	Transversion	2842165	C\G	AGC\AGG	Serine\ Arginine	Missense	ID: CP055292.1	99%
	Transition	2842188	C\T	GCC\GTC	Alanine\ Valine	Missense		
	Transition	2842252	G\A	CAG\CAA	Glutamine\ Glutamine	Silent		
6	Transition	2842219	T\C	ACT\ACC	Threonine\ Threonine	Silent	ID: CP055292.1	99%
	Transvertion	2842301	A\C	ACA\CCA	Threonine\ Proline	Missense		

Recording of Shigella sonnei Local Isolates in NCBI

The two isolates of *S. sonnei* were registered at the National Center for Biotechnology Information using *stx1* 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*.

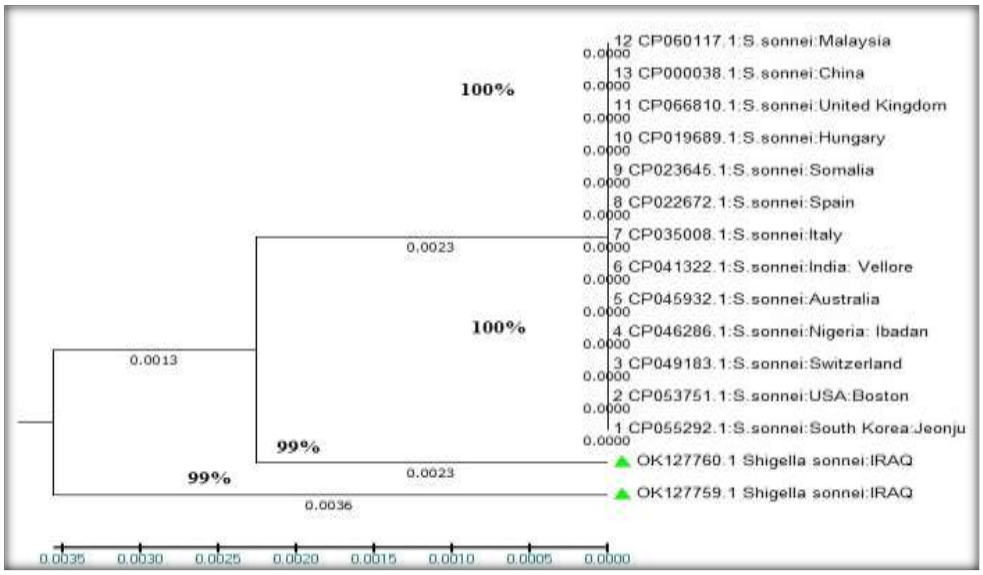

Figure 3: Phylogenetic tree of *Shigella sonnei*

Antibiotic susceptibility test

Shigella strains sensitivity against10 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 Table5.

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 intermediately sensitive to antibiotics ampicillin, chloramphenicol and nalidixic acid. These results differed from results of the study by Dhital*et 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 intermediately sensitive to gentamicin. These results differed from results of the study by Dhital*et al,* where they mentioned that *Shigella* isolates were sensitive to antibiotics azithromycin, ceftazidime, cefotaxime and ceftriaxone [26].

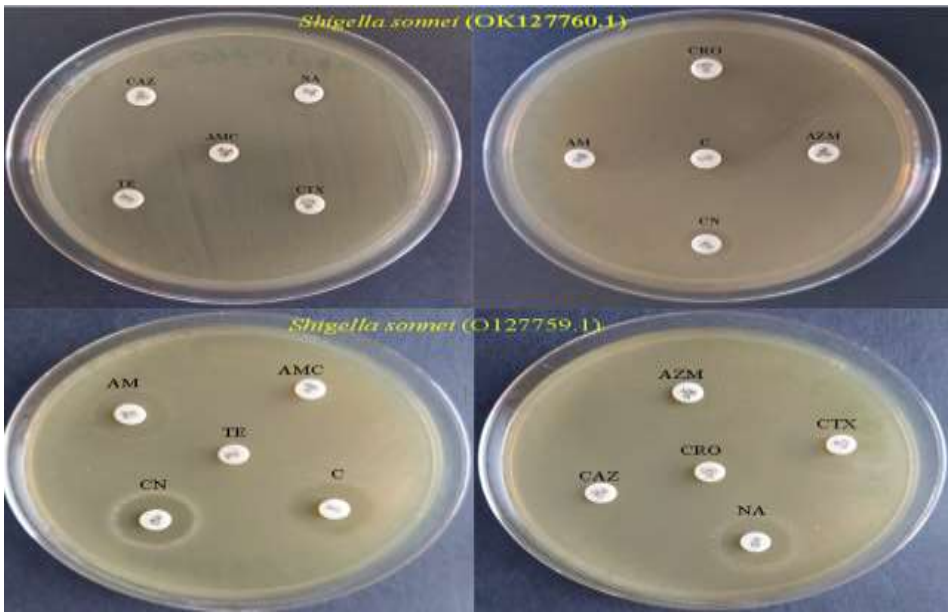

Figure 4: Sensitivity test of *Shigella sonnei* strains to antibiotics

Table 5: *Shigella* strains resistant to antibiotics

Antibiotics	Symbol µg/disk	Con. µg/disk	*Shigella sonnei* Strains	
			OK127759.1	OK127760.1
Ampicillin	AM	25	I	R
Azithromycin	AZM	15	R	R
Ceftazidime	CAZ	30	R	R
Cefotaxime	CTX	30	R	R
Ceftriaxone	CRO	10	R	R
Chloramphenicol	C	10	I	R
Augmentin	AMC	30	R	R
Gentamicin	CN	10	S	I
Nalidixic acid	NA	30	I	R
Tetracycline	TE	10	R	R

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 6: Diameters of inhibition zones

Concentrations (µg\ml)	*Shigella sonnei*Strains		
	OK127759.1	OK127760.1	
25	12	9	Inhibition diameter (mm)
50	15	12	
100	17	16	
150	20	18	
200	22	21	

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. Maaroof 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

[1] E.N. Biernbaum and I.T. Kudva," AB_5 Enterotoxin-Mediated Pathogenesis: Perspectives Gleaned from Shiga Toxins," *Toxins,*Vol.14, No.1, p. 62, 2022.doi.org/10.3390/toxins14010062

[2] S. Domonkos, B. Balázs, V. Bálint, M. Gergely andT. István, "Comparative Genomic and Phylogenetic Analysis of a Shiga Toxin Producing *Shigella sonnei* (STSS) Strain,"*Frontiers in Cellular and Infection Microbiology*, Vol. 7, No. 229, pp.10, 2017. doi: 10.3389/fcimb. 2017. 00229

[3] M. Watanabe-Takahashi, M. Tamada and M. Senda, "Identification of a peptide motif that potently inhibits two functionally distinct subunits of Shiga toxin," *Communications biology*, Vol. 4, No. 538, pp 1-7, 2021.doi.org/10.1038/s42003-021-02068-3

[4] M.F.K. KhanandS.S. Rashid,"Molecular Characterization of Plasmid-Mediated Non-O157 Verotoxigenic *Escherichia coli* Isolated from Infants and Children with Diarrhea,"*Baghdad Science Journal*, Vol. 17, No. 3, 2020. doi.org/10.21123/bsj.2020.17.3.0710

[5] I.I Generalov, "Medical Bacteriology & Medical Virology – Lecture Course for students of medical universities," *in Medical Microbiology, Virology and Immunology*.VSMU Press, 2017,p. 392, ISBN 978-985-466-743-0. Available from: https://core.ac.uk/download/pdf/80151473.pdf

[6] M.S. Lee, J.W. Yoon JW and V.L Tesh, "Editorial: Recent Advances in Understanding the Pathogenesis of Shiga Toxin-Producing *Shigella* and *Escherichia coli*," *Frontiers in Cellular and Infection Microbiology*, Vol. 10, 2020.doi.org/10.3389/fcimb.2020.620703

[7] M. Fogolari,C. Mavian, S. Angeletti, M. Salemi, K.A. Lampel and A.T. Maurelli, "Distribution and characterization of Shiga toxin converting temperate phages carried by *Shigella flexneri* in Hispaniola, "*Infection, Genetics and Evolution*, Vol. 65, pp. 321-328, 2018. doi: 10.1016/j.meegid.2018.07.038. Epub 2018 Jul 31

[8] C. Adams, A. Vose, M.B. Edmond andL. Lyckholm, "*Shigella sonnei* and hemolytic uremic syndrome: A case report and literature review. ID Cases," Vol.10, No.8, pp.6-8, 2017. doi: 10.1016/j.idcr.2017.02.003

[9] A. Alwash,"The Green Synthesize of Zinc Oxide Catalyst Using Pomegranate Peels Extract for the Photocatalytic Degradation of Methylene Blue Dye," *Baghdad Science Journal*, Vol.17, No.3, p.787, 2020.doi.org/10.21123/bsj.2020.17.3.0787

[10] V.T. Nguyen, V.T Vu, T.H. Nguyen, T.A. Nguyen, V.K. Tran andP. Nguyen-Tri," Antibacterial Activity of TiO_2 and ZnO-Decorated with Silver Nanoparticles," *Journal of Composites Science*,Vol.3, No.2, p. 61, 2019. doi.org/10.3390/jcs3020061

[11] A. Sirelkhatim, S. Mahmud, A. Seeni, N.H.M. Kaus, L.C. Ann, S.K.M. Bakhori,*et al*.,"Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism,"*Nano-Micro Letters*, Vol.7, No.3, 2015. doi: 10.1007/s40820-015-0040-x

[12] P.M. Tille, "Bailey and Scott's Diagnostic Microbiology," 15th Edition, Elsevier. 2021; 842-855, ISBN: 978-0-323-68105-6

[13] S.A. Miller, D.D. Dykes DD andH.F. Polesky," A simple salting out procedure for extracting DNA from human nucleated cells,"*Nucleic Acids Research*,Vol.16, No.3, 1988.doi: 10.1093/nar/16.3.1215

[14] S. Yaghoubi, R. Ranjbar, M.M.S. Dallal, S.Y. Fard, M.H. Shirazi and M. Mahmoudi, "Profiling of Virulence-associated Factors in *Shigella* Species Isolated from Acute Pediatric Diarrheal Samples in Tehran, Iran. *Osong Public Health and Research Perspectives*,Vol.8, No.3, pp.220-226, 2017. doi: 10.24171/j.phrp.2017.8.3.09

[15] P.Y. Lee, J. Costumbrado, C.Y. Hsu and Y.H. Kim, "Agarose gel electrophoresis for the separation of DNA fragments.*Journal of Visualized Experiments*. Vol.62, No.3923 pp.1-5.2012.doi: 10.3791/3923.

[16] H. Jan,"Kirby-Bauer Disk Diffusion Susceptibility Test Protocol," ASM. Washington, D.C. 2009, https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf

[17] H.S. Abdulkrim, "Evaluation of Zinc Oxide Nanoparticles and *Bifidobacterium bifidum* Products In Treatment Diarrhea Caused by *Clostridium difficile* in the Rats. PhD [dissertation]. Tikrit University; 2020.

[18] M.A. Gharby and B.N. Al-Qadhi, "The Effect of Zinc oxide Nanoparticles (ZnO NPs) on the Viability of Leishmania tropic In Vitro," *Iraqi Journal of Science*, Vol. 58, No.2A, pp.600-610 2022. Retrieved from https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/6079

[19] PHE, "UK Standards for Microbiology Investigations Identification of Shigella species, Issued by the Standards Unit, Microbiology Services," PHE Bacteriology – Identification 2021, | ID 20 | Issue no: 3,pp. 1-22

[20] A.A. Rabatti and N.E. Rasheed, "Etiology of Bloody Diarrhea among Children Admitted to Maternity and Children's Hospital-Erbil," *AL-Kindy College Medical Journal* Vol.4, No.2, pp.19-24, 2008. https://jkmc.uobaghdad.edu.iq/index.php/MEDICAL/article/view/737

[21] S.M. Tawfeeq, M.N. Maaroof and I. Al-Ogaidi, "Synergistic effect of biosynthesized silver nanoparticles with antibiotics against multi-drug resistance bacteria isolated from children with diarrhoea under five years," *Iraqi Journal of Science*, Vol.58, No.1A, pp,14-52, 2022. Retrieved from https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/6189

[22] M.J. Ali, "Studying the types of bacteria isolated from children with diarrhea and their sensitivity to antibiotics. *Diyala journal for Pure Science*, Vol.8, No.1, 2012.

[23] T.R. Abdulrahman, Q.W. Jamal, W.A. Kadhim and S.A. Belal, "Evaluation of Plasmid-Mediated Quinolone Resistance associated with the *Qnr* Genes in Clinical Isolates of *Shigella spp*. in *Baghdad. Iraqi Journal of Medical Sciences*, Vol.13, No.1, pp.32-39, 2015.doi: iraqijms.net/upload/pdf/esite56d6bef046357.pdf

[24] D. Sváb, B. Bálint, B. Vásárhelyi, G. MarótiandI. Tóth,"Comparative Genomic and Phylogenetic Analysis of a Shiga Toxin Producing *Shigella sonnei* (STSS) Strain," *Frontiers in Cellular and Infection Microbiology*, Vol.7, No.299, 2017.doi: 10.3389/fcimb.2017.00229

[25] A. Madhavan, S. Balakrishnan and J.Vasudevapanicker, "Antibiotic susceptibility pattern of *Shigella* isolates in a tertiary healthcare center," *Journal of Laboratory Physicians*. Vol.10, No.2, pp.140-144, 2018. doi: 10.4103/JLP.JLP_93_17.

[26] S. Dhital, J.B. Sherchand, B.M. Pokharel, K. Parajuli, S.K. Mishra, S. Sharma, H.P. Kattel, S. Khadka, S. Khatiwada and B. Rijal, "Antimicrobial susceptibility pattern of Shigella spp. isolated from children under 5 years of age attending tertiary care hospitals, Nepal along with first finding of ESBL-production,"*BMC Research Notes*. Vol.5, No.10, 2017.doi: 10.1186/s13104-017-2512-1.

[27] A.A. Shareef, Z.A. Hassan, M.A. Kadhim and A.A. Al-Mussawi, "Antibacterial Activity of Silver Nanoparticles Synthesized by Aqueous Extract of Carthamus oxycantha M.Bieb. Against Antibiotics Resistant Bacteria. *Baghdad Science Journal*, Vol.19, No. 3, 2022.doi.org /10.21123/bsj.2022.19.3.0460