

Isolation and Identification of *Shigella Sonnei* from Children with Diarrhea and Detection of the Most Important Virulence Factors

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Abstract

Some reports showed that the sensitivity of *Shigella* species to antibiotics changed dramatically over time due to the indiscriminate use of drugs. The *Shigella* strains became gradually resistant to most antimicrobials, which led to failure of treatment and increased mortality. This study was conducted in Tikrit city and included the isolated of *Shigella sonnei* from 530 stool samples were collected from children suffering from diarrhea during the period from March 2018 to March 2019. Stool samples taken from children aged 1-60 month. The study showed that all the fifteen isolates obtained were *Shigella sonnei*, meaning that the infection rate was 2.83% of the total number of patients. The age group from 49-60 months is the group most likely to be affected by *Shigella*. The results of determining some virulence factors indicated that all *Shigella sonnei* isolates possess the ability to form biofilms, and 93.3% of these isolates produce beta-lactamase. As for the ability of isolates to cause keratoconjunctivitis, the results showed that 80% of the isolates were positive for the sereny test, that is, it has the ability to cause the disease. The results of the study showed that all isolates were resistant to antibiotics Nalidixic acid and Co-trimoxazole, and 93.3% of the isolates were resistant to Penicillin. Antibiotics Chloramphenicol and Azithromycin showed a significant effect on *Shigella* isolates, as 93.3% of isolates were sensitive to these antibiotics. The antibiotic Ciprofloxacin showed an asymptotic inhibition to the antibiotics Chloramphenicol and Azithromycin, as the results showed that 86.6 isolates were sensitive to this antibiotic. Antibiotics of the third generation cephalosporins Cefotaxime and Ceftriaxone, showed convergent effects in their ability to inhibit *Shigella sonnei* isolates, as the results showed that the isolates were sensitive to the antibiotics at a rate of 73.4% and 80% respectively. The percentage of isolates sensitive to the antibiotics Amikacin and Tetracycline were 40% and 46.7% respectively.

Keywords: Diarrhea, *Shigella sonnei*; virulence factors; Sensitivity to antibiotics

Introduction

Shigella belongs to Enterobacteriaceae family, class of gamma proteobacteria in the phylum of proteobacteria. The genus of shigella includes four species *S. sonnei*, *S. dysenteriae*, *S. flexneri* and *S. boydii*. The natural habitat of these species is in the intestine of humans, apes, and monkeys^[1]. All *Shigella* species are Slender rod-shaped bacteria and are found in coccobacillary forms in young culture^[2]. *Shigella* species are non-spore forming, non-motile, gram negative, oxidase negative, facultative anaerobic bacteria. Their colonies are convex and round and have intact edges. The colonies have a diameter of about 2mm within 24 hours^[2]. *Shigella* divides into four

serogroups depending on the O-antigen structure (A, B, C and D) The serogroup A, known as *S. dysenteriae*, contains 16 serotypes. The serogroup B known as *S. flexneri* contains 19 serotypes. The serogroup C known as *S. boydii* contains 20 serotypes and the serogroup D known as *S. sonnei* has one serotype. With the exception of the serogroup D which can be distinguished on the basis of chemical metabolism assays, the serogroups A, B, C are physiologically similar^[3]. The infectious dose of 10-100 germ cells is sufficient to cause shigellosis disease in adults^[4]. The infection usually occurs in young children who do not care about hygiene after the toilet. Shigellosis is more prevalent in day care centers where children meet from different areas. Transmission is also through food

and water^[5]. Shigella disease outbreaks through water is associated with *S. flexneri*, while cases of foodborne illness are caused by infection with *S. sonnei* bacteria. The disease caused by *S. dysenteriae* is particularly severe^[6]. Shigellosis appear suddenly after an incubation period of 12 hours to approximately two days and include high fever, anorexia, nausea, abdominal pain and diarrhea^[7]. The toxins of Shigella, enterotoxin 1 (ShET1) and ShET2 produced by some strains of Shigella induce fluid secretion in the intestine, which explains the aqueous phase of diarrhea^[8]. This study aimed to isolate *Sonnei Shigella* and determine some of its virulence factors and its sensitivity to some antibiotics

Materials and Methods

1. Sample collection

Stool samples were collected from children suffering from diarrhea during the period from March 2018 to March 2019. Samples taken from children aged 1-60 month. All samples collected from Patients attending to the Salah Al-Din Hospital and some health centers in Tikrit.

2. Shigella Isolation

Stool samples were cultured by taking a full loop of feces and culturing them into sterile test tubes containing 5 ml of Shigella broth. Then the tubes incubated at 37°C for 18-24 hours. After the incubation period and observation of growth, a ring filled with the bacterial suspension was cultured on the XLD agar. The dishes incubated at 37°C for 18-24 hours. The red-colored colonies were recultured on the same medium to obtain pure isolates. Pure isolates were cultured on SS agar and incubated at 37°C for 18-24 hours. After this period, the suspicious Shigella colonies were cultured onto the solid nutrient medium to complete the diagnosis^[9].

Virulence Factors Tests

1. Biofilm Formation Assay

Glass tubes containing 5mL of brain heart infusion broth were inoculated with pure bacterial colonies. The tubes incubated at 37°C for 48 hours, then the contents of the tubes poured and all tubes stained with crystal violet dye solution at a concentration of 0.1% for 15 minutes. Then it was washed with distilled deionized water and

left to dry at room temperature. The positive result was observed through the presence of a layer of dyed material sticking to the inner wall of the tubes^[10].

2. β -lactamase Production

The β -lactase enzyme was investigated using the rapid iodometric method. A pure colony was added to an Eppendorf tube containing 100 μ l of penicillin G (1000 U/ml) and then incubated at 37°C for 30 minutes. 50 μ l of the starch solution (1% w/v) was added and mixed well with the contents. Then 20 μ l of iodide solution was added to the tube and mixed for one minute. The result is positive when the color is changed from blue to white^[11].

3. Sereny test

A bacterial suspension was prepared, its turbidity equal to the turbidity of McFarland Standard No.1. One conjunctiva of each guinea pig was inoculated with 20-25 μ L bacterial suspensions of the test strain. The development of keratoconjunctivitis was observed in the guinea pig after 1-3 days^[12].

Antibiotic Sensitivity test

This test was done using the modified Bauer-Kirby method approved by World Health Organization^[13].

Results and Discussion

Study sample

530 fecal samples were collected from children suffering from diarrhea for Isolated *Shigella spp.* Patients ages ranged between a week and 5 years.

All the samples taken from the patients were cultured into Shigella broth, Then the growing bacteria in Shigella broth were streaked on Xylose Lysine Deoxycholate Agar (XLD), The results showed that 21 samples (3.96%) did not show any bacterial growth as in Table(1). The remaining 509 stool samples showed bacterial growth when grown on selective medium (XLD) Agar. The samples taken from males were 298 samples (56.23%), and the number of samples taken from females was 211 samples (39.81%), as in Table(1). This percentage agreed with a previous study conducted by Gebrekidan et al^[14] in which they revealed that 50.5% of patients with diarrhea are males and 49.5% of patients with diarrhea are females. In a similar study conducted

by Ismail^[15], who found that the percentage of diarrhea among males was 62.26%, which while among female patients were 37.74%. Al-Rifai^[16] also found that the prevalence of nosocomial diarrhea in hospitals among males 62% was more than females 38%.

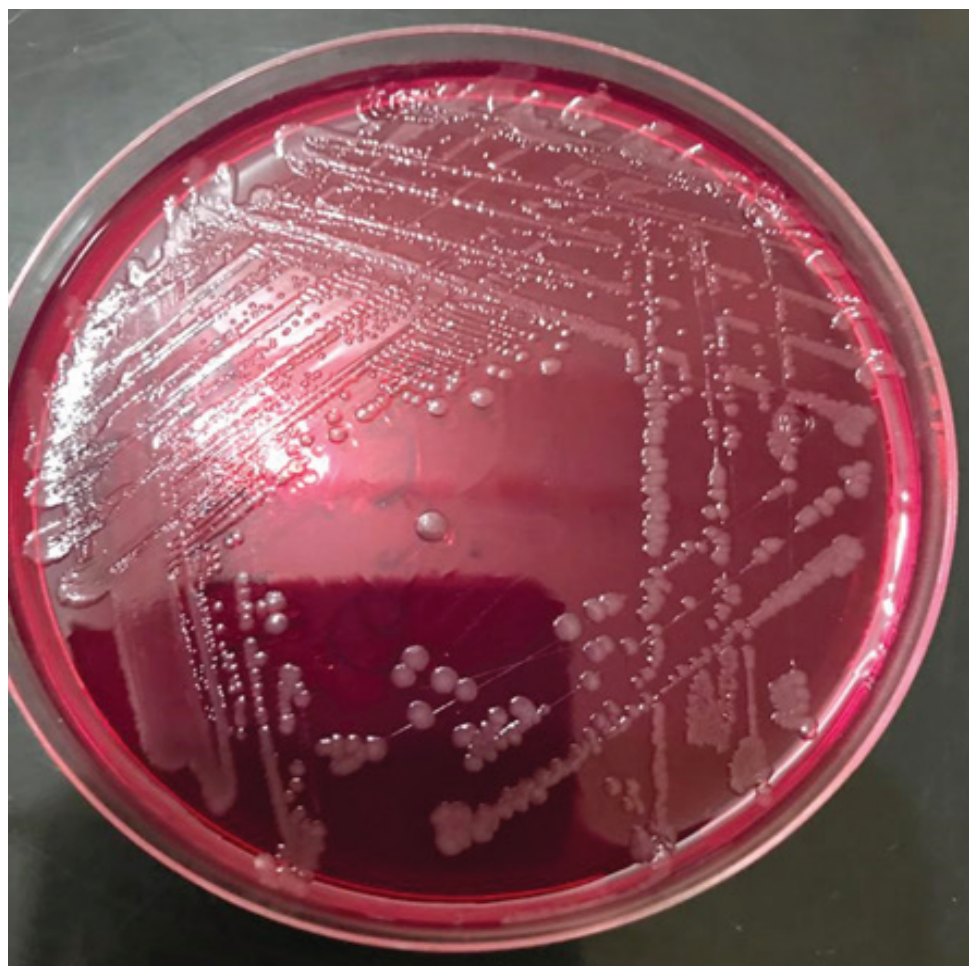
Table(1): Results of stool samples culture.

Gender	Male	Female
Results	No. %	No. %
No growth	17 3.21	4 0.75
Growth	298 56.23	211 39.81

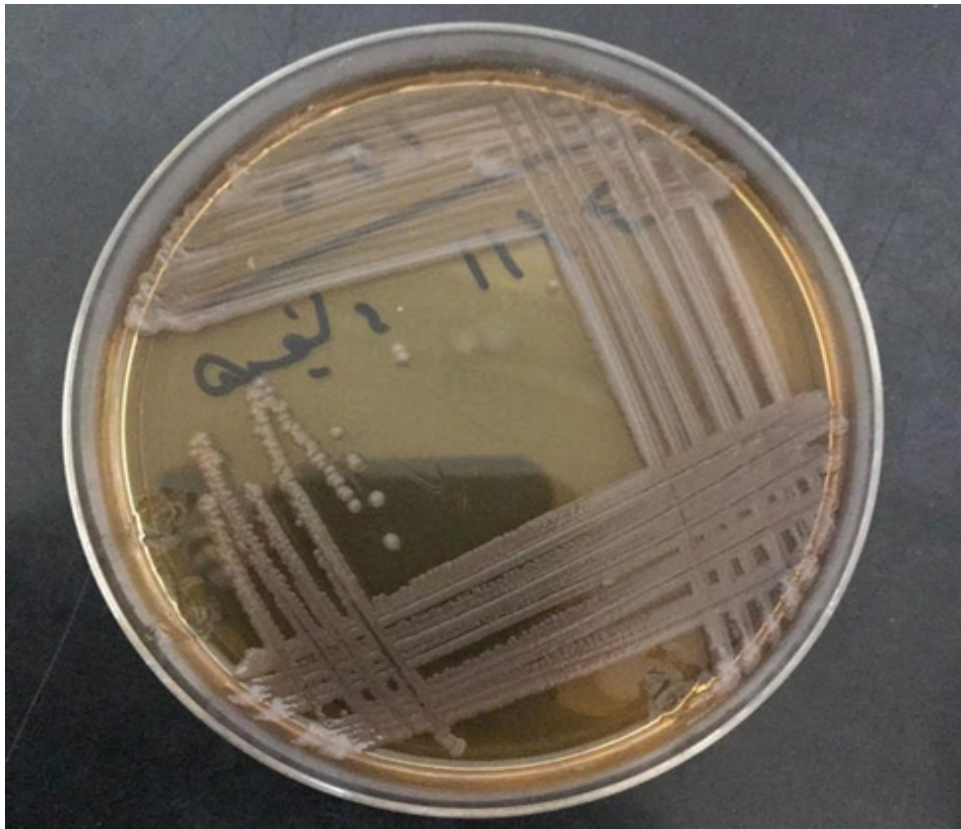
Isolation

In this study Shigella broth was used to increase the number of Shigella, in order to increase the possibility of isolating it from stool samples^[17]. Bacterial isolates were identified on the primary isolation media according

to the morphology and color of the colonies. Three selective media were used to isolate Shigella, which are XLD agar, SS agar and HE agar. 530 fecal samples were cultured on XLD Agar. 103 isolates were identified, possessing the same morphological traits as Shigella species on this medium. Then isolates cultured on the same medium to obtain pure cultures of the isolates as in the figure(1). All pure isolates were cultured again on (SS) agar. Shigella species colonies are transparent and do not contain a black center^[18]. Some strains of Shigella, such as *S. sonnei*, *S. dysenteriae* serovar 1, may ferment lactose relatively slowly when incubated for two days or more^[18], therefore after 24 hours of isolates cultivation on this medium, the transparent colonies were selected as in the figure(2). 96 isolates were obtained, that possess colonies morphological characteristics similar to Shigella colonies on SS agar medium.



Figure(1): Isolates colonies on XLD agar.



Figure(2) Isolates colonies on SS agar.

Isolates Identification

VITEK2 system was used to identification isolates.15 isolates of Shigella were identified, all belong to the *S.sonnei* which recorded a high probability ranging between 95% and98%.

Occurrence of *Shigella sonnei*.

The results of the current study revealed that *Shigella sonnei* infection rate was 2.83% of the total samples.The current study agrees with the previous study conducted in Erbil city.The infection with Shigella among children attending Erbil Hospital is 3.4%^[19].Also,a previous study was conducted in Tikrit Teaching Hospital to determine the prevalence of infectious diarrhea caused by Shigella among children under five years of age,the study showed that neglecting infection was 2.5% ^[20] .

The percentage of children infected with Shigellosis

Depending on age,the results revealed that *Shigella*

sonnei was isolated in a high percentage from patients with diarrhea, specifically from the age group(49-60) months,with an infection rate of 46.68%, as shown in the table(2).This result may be due to age,because children wash their hands after a defecation less than adults, and are more likely to play in soil contaminated with feces.

Table(2): The percentages of patients infected with Shigella for each age group.

Age	No.	%
<12 M.	0	0%
13-24 M.	2	13.3%
25-36 M.	2	13.3%
37-48 M.	4	26.66%
49-60 M.	7	46.68%
Total	15	100%

Virulence factors

1. β -lactamase production

The production of lactamase enzyme was detected by the Rapid iodometric method figure(3).The results showed that 14 isolates produced this enzyme at a rate of 93.33%.A negative test result does not mean that the bacteria are not producing β -lactamase enzyme,as there may be other mechanisms that cause this result, such as reduced permeability or decreased enzyme affinity for penicillin-binding proteins (PBPs)^[21].Another reason may be production low amounts of the enzyme in periplasmic space, which makes detection more difficult [22].

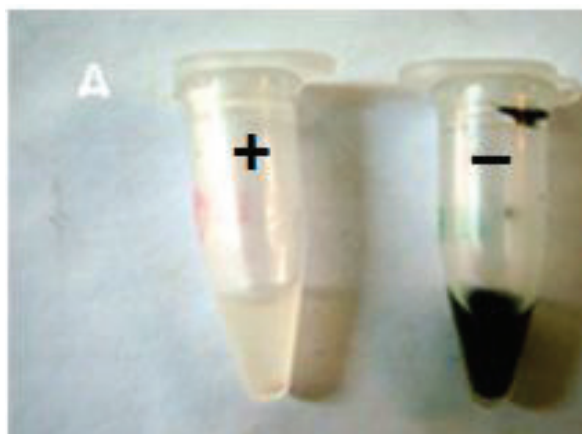


Figure (3) β -lactamase test

2. Biofilm Formation Assay

The ability of *Shigella* isolated from patient's feces to form biofilms was assessed using qualitative and quantitative tests.The tube method (TM) was used in the qualitative test of biofilm formation^[10].The results showed that all isolates are able to form biofilms.This result was inferred through a thick membrane visible on inner wall of tube and the bottom of the tube figure (4).

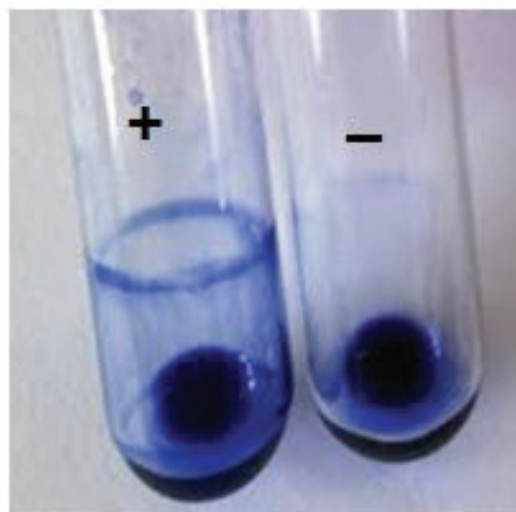


Figure (4) Biofilm Formation Assay

3. Sereny test

The Sereny test was performed to determine the ability of the isolates to invade cells. In this test, the guinea pig's eye was inoculated with bacterial suspension, its turbidity equal to turbidity of McFarland's No.1.This test is positive if keratoconjunctivitis occurs. The test results showed that 12 isolates were positive for the sereny test,at a rate of 80%.In a previous study,the results showed that 15 isolates out of 18 *Shigella* isolates from the feces of children suffering from acute diarrhea have the potential to induce keratoconjunctivitis in eyes of experimental animals on sereny test^[23].

Antibiotic susceptibility test

An antibiotic susceptibility test was performed on all bacterial isolates by the disc diffusion method.10 antibiotics were used in this study as in table (3).The results showed that all *Shigella sonnei* isolates were resistant to Nalidixic acid and Co-trimoxazole at a rate of 100%.The current study does not agree with the study of Abdel-Rahman et al,^[24] in Baghdad, as the results showed that 54.23% of *Shigella* isolates are resistant to nalidixic acid.

Table(3) *Shigella sonnei* sensitivity to some antibiotics.(R.resistant,I. intermediate,S. sensitive).

No. Antibiotics	R. No.	%R	I. No.	% of I	S. No.	% of S
1 Ampicillin	14	93.3%	1	6.7%	0	0
2 Nalidixic acid	15	100%	0	0	0	0
3 Co-trimoxazole	15	100%	0	0	0	0
3 Amikacin	5	33.3%	4	26.6%	6	40%
4 Chloramphenicol	0	0	1	6.7%	14	93.3%
5 Azithromycin	0	0	1	6.7%	14	93.3%
5 Cefotaxime	2	13.3%	2	13.3%	11	73.4%
6 Ceftriaxone	2	13.3%	1	6.7%	12	80%
7 Cefixime	4	26.6%	4	26.6%	7	46.7%
8 Ciprofloxacin	1	6.7%	1	6.7%	13	86.6%
9 Gentamicin	6	40%	0	0	9	60%
10 Tetracycline	5	33.3%	3	20%	7	46.7%

The results also showed that 93.3% of *Shigella sonnei* isolates were resistant to Ampicillin, this result agreed with the results of Subhash et al,^[25]. While 93.3% of isolates were sensitive to chloramphenicol This result agreed with the findings of Siraj^[26] as all *Shigella sonnei* isolates were resistant to this antibiotic. Azithromycin also showed a significant effect on *Shigella sonnei* isolates, as 93.3% of isolates were sensitive to this antibiotic. These results agreed with the results of the study conducted by Jomezadeh et al,^[27] in Iran, as they indicated that all *S. sonnei* isolates are resistant to this antibiotic. Cefotaxime, Ceftriaxone, which belongs to cephalosporins group, showed convergent effects in their ability to inhibit *S. sonnei* isolates, as the results showed that 73.4% of isolates were sensitive to Cefotaxime and 80% of isolates were sensitive to Ceftriaxone. As for the other antibiotic Cefixime which belongs to the same group, it showed a lesser effect, as only 46.7% of isolates were sensitive to this antibiotic. In a previous study, the results showed that the resistance to

the antibiotic Ceftriaxone was 9%^[28]. Jomezadeh et al,^[27] stated in their study that all *Shigella* isolates were resistant to this antibiotic. The antibiotic Ciprofloxacin showed a similar inhibitory effect for Chloramphenicol and Azithromycin, as the results showed that 86.6% of isolates were sensitive to this antibiotic. The results of Madhavan and her group^[26] agree with current study results, which showed that 90% of isolates were resistant to this antibiotic. As for Gentamicin, 40% of *Shigella sonnei* isolates were resistant 60% of isolates were sensitive. A previous study^[27] reported that 35% of *Shigella* isolates are resistant to Gentamicin, these results are close to the results of the current study. The percentage of isolates sensitive to antibiotics Amikacin and Tetracycline were 40% and 46.7% respectively while the percentage of resistant isolates was 33.3% for both antibiotics. This findings of da Cruz et al do not agree with the results of the current study, as they showed that 80% of the isolates were resistant to Tetracycline.

Conclusion

Shigella sonnei is one of the pathologic agents that cause diarrhea because it has virulence factors that enable it to do so, the most important of which is its ability to adhere to the host's cells and then invade them. In addition, it is resistant to many antibiotics.

Conflict of Interest: None

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Ethical Clearance: Not required

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