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Determination Of Bacteriophages Ability That Isolated From Poultry Feces To Lysis Salmonella Typhimurium That Isolated From Stool Of Children Who Suffering From Diarrhea

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ABSTRACT

This study was conducted to isolate Salmonella from 350 stool samples taken from children under five years in age, suffering from diarrhea during the period from March 2019 to March 2020 in Tikrit city / Iraq. The results showed that isolate ten isolates of S. enterica subsp. enterica at infection rate of 2.875% of the total patients suffering from diarrhea, as well as the results showed that the age group of 49-60 months was more susceptible to salmonellosis. The results of the serotyping diagnosis also showed that six isolates belong to the serotype S. Typhimurium and the other belong to the serotype S. Enteritidis. The results of the isolates sensitivity test to antibiotics showed that all isolates were sensitive to the antibiotics Azithromycin, Cefotaxime, Ceftriaxone and Chloramphenicol, while the sensitivity of the isolates to other antibiotics was varied, as 40% of the isolates were resistant to the antibiotics Ampicillin, Ceftazidime and Nalidixic acid, while the isolates were resistant to the antibiotics Gentamicin and tetracycline by 30%, 40%, and 80%, respectively. The results of the study showed the possibility of isolating two virulent bacteriophages that have the ability to infect and kill S. Typhimurium isolates. The results also showed that SPh2 is more tolerant of heat and extreme conditions of pH than SPh1.

Keywords: Diarrhea, Salmonella Typhimurium, Bacteriophages

INTRODUCTION

Diarrhea is caused by a number of pathogens including viruses, bacteria, and parasites. Because of the similarity of symptoms, it is difficult to distinguish between viral, bacterial and parasitic agents, and many causes of diarrhea are not precisely identified, which may lead to inappropriate treatment and thus lead to the emergence of antibiotic-resistant strains (Schatten & Eisenstark, 2015).

Salmonella is a foodborne pathogen that causes three distinct clinical syndromes: typhoid fever, enteritis, and bacteremia. There are more than 2500 serotypes of Salmonella, which infect a variety of hosts. Non-typhoidal Salmonella (NTS) mainly causes gastroenteritis in humans, but it can cause acute

bacteremia in young children and immunocompromised patients. estimations indicate that around 3.4 million cases of bacteremia due to non-typhoidal Salmonella infection occur each year globally (Albert et al., 2019). Reports indicate that 99% of Salmonella infections in humans are associated with serotypes of Salmonella enterica subspecies enterica (Qamar et al., 2020). Mortality rates due to enteric salmonella are a worrying Source on a global scale. Salmonella antibiotic resistance is an important virulence factor that makes Salmonella more difficult to treat with antibiotics that used clinically, and therefore clinical isolates of MDR-Salmonella are a source of serious concern for public health. Salmonella employs several mechanisms of antibiotic resistance, including: (1) formation of biofilms (2) enzymatic destruction of antibacterial drugs (3) decreased drug permeability into bacterial cells (4) alteration of drug target (5) Efflux pumps is the main resistance mechanism that used by S. enterica to resistant antibiotics (Hackett, 2015). The high rates of antibiotic resistance of bacteria necessitated finding alternative solutions to treat the pathological cases due to infection with resistant strains. The most prominent of these solutions was a return to use phages in the treatment of such cases (Nieth et al., 2015).

Phages are the most abundant biological entities on the planet, and exhibit a high degree of host specificity so they are found in all environments where there is a suitable host. An estimated 1023 phage infections per second globally occur in marine environments (Switt et al., 2013). Phages are viruses that have adapted to infect and kill bacteria. Phages have been used for biological control against pathogens or used in food preservation instead of chemical agents commonly used in meat products and which may have some negative effects on human health (Petong et al., 2019). Virulent bacteriophages have been used as biological control agents in biological control against bacterial pathogens. This field of research aims to produce sustainable solutions to control these pathogens and solve the problems associated with multidrug resistance of bacteria (Hooton et al., 2011). Today Phages are used against foodborne pathogens to humans and animals as an alternative of antibiotics, or in combination with antibiotics. Recent studies report very promising results, but nevertheless, phages are sensitive to various environmental factors such as pH and temperature, which leads to breakdown and loss of their ability to infect bacteria, thus reducing their therapeutic efficiency. The effect of pH is more pronounced in oral treatment against enteric pathogens, because phages usually lack stability in the highly acidic environment of the stomach, and the duration of stay of phages in the intestine is very short (Azeredo, 2018).

MATERIAL and METHODS

Samples collection

In this study, 350 stool samples were collected from children that suffering from diarrhea who attended to Salah al-Din General Hospital in Tikrit. The samples were collected during the period from March 2019 to March 2020, as about 1-2 g of stool was taken and placed in sterilized screw vial cap, then wrote on the it the names of the patients and the date. Then all samples were cultured immediately after collection.

Salmonella isolation

Salmonella has been isolated according to (England public health, 2018). Stool samples were cultured in selenite broth at 37°C for 18-24 hours, after the incubation period a filled loop with bacterial suspension was cultured on XLD agar at 37°C for 18-24 hours. Colonies with red color with black center were selected to re-cultured on the same medium to obtain pure isolates. pure isolates were re-cultured on nutrient agar until their diagnosis were completed.

Salmonella identification

The isolates were diagnosed to the genus level by using Vitek 2 compact system, in addition a set of biochemical tests according to (Mikoleit, 2015) as shown in Tab. (1) were conducted to diagnose isolates to subspecies level, as well as serotyping kit (62984: Salmonella Sero-Quick ID kit) was used to diagnose isolates to serotype level.

Antibiotic Sensitivity test

This test was done using the modified Bauer-Kirby method approved by World Health Organization (Jan,2009).

Study of phages

Isolation

Phages were isolated by mixing 1 g of chicken feces with 10 ml of Tryptic soy broth (TSB) by using Vortex mixer at high speed for 5 minutes to release phages to the medium, Then the samples were centrifuged by using a refrigerated centrifuge at 10000 rpm for 10 minutes at 4° C to obtain a supernatant containing only phages. In order to increase the number of required phages, 5 ml of the filtrate was mixed with 5 ml of sterile Tryptic soy broth (TSB) in Erlenmeyer flask, then 100 µl of liquid culture of the test bacteria with a 24-hour age was added to the flask. The samples were incubated by using a vibrating incubator at 150 rpm at 37°C for 24 hours, then the samples were centrifuged at 5000 rpm for 10 minutes and the supernatant was filtered through a Millipore filter (0.22 µM) to obtain a filtrate containing phages only (Huang et al., 2018).

Purification

Double-Layer Agar technique (DLA) was used to purify phages. A series of decimal dilutions were made for the sample containing the phages, then 100 µl of each dilution was taken and placed in sterile Eppendorf tubes, then 100 µl of liquid culture of the test bacteria was added to each tube, then these contents were added to a tube containing 3 ml Semi-solid nutrient medium, then all contents were mixed gently and poured onto the surface of nutrient agar, then the dishes were incubated at 37°C for 24 hours, after that the dishes were examined to observe the plaque zones of different diameters. To obtain pure species, each plaque zone was drawn by a sterile pipette and placed in Eppendorf tube containing 200 µl of SM buffer, then mixed with the vortex mixer at high speed for 5 minutes in order to release the phages to the buffer, then the samples centrifuge at 10000 rpm for 10 minutes at 4° C remove the semi-solid medium and decomposing bacteria, then the supernatant was filtered through a Millipore filter (0.22 µM) to obtain a filtrate containing only one type of phage (Hungaro et al., 2008).

Heat tolerance test

To perform this test, 900 µl of Tryptic soy broth (TSB) was placed in sterile Eppendorf tubes, then 100 µl of the bacteriophage suspension was added to it, then the tubes were placed in a water bath at 30 °C for 60 minutes, this process was repeated at temperatures (40, 50, 60, 70) °C. After conducting thermal treatments, the number of remaining phages was verified by using the double-layer agar technique (DLA). The percentage of viable bacteriophages after treatment was calculated as in the equation below (Huang et al., 2018).

Viable bacteriophages (%) = (Phage no. after treatment / Phage no. before treatment) x100

pH tolerance test

To performed this test, Fifteen Eppendorf tubes were prepared, each one containing 900 µl of Tryptic soy broth (TSB), then the pH of each tube was adjusted, as the pH values ranged from 2 to 12, then 100 µl of the phage suspension was added to each tube, then the tubes were incubated for 24 hours at 37°C. The number of remaining phages was verified by using the double-layer agar technique. The percentage of viable bacteriophages after treatment was calculated as in the equation above (Huang et al., 2018).

	Diarrheal cases	
Gender	%	no.
Male	%58.57	205
Female	%41.43	145

Results and discussion

Study sample

The results of the current study showed that 58.57% of total cases suffering from diarrhea were males, while 41.43% total cases were females suffering from diarrhea was 145 cases as shown in the Tab. (1). current study results approximately agreed with the results of Gebrekidan and his group (2015), as they revealed that 50.5% of the cases who suffer from diarrhea are males, while the percentage of females is 49.5% of the total number of cases. Gebreegziabher and his group (2018) found that the percentage of males suffering from diarrhea was 55.8%, while the percentage of females with diarrhea amounted to 44.2% of the total cases of diarrhea, and these percentages are consistent with the results of the current study. The results of the study also agreed with Ismail (2006), who found that the percentage of males suffering from diarrhea was 62.26%, which is higher than the percentage of females, which was 37.74% of the total cases. The results of this study also agreed with Alrifai and his group (2008), as they found that the prevalence of diarrhea cases among males was 62%, while among females it was 38%.

Table (1) Percentage of the number of males and females who suffer from diarrhea.

Isolation

The results of this study showed the possibility of isolating 38 isolates from the 350 stool samples of children suffering from diarrhea, which are believed that belong to Salmonella genus. Bacterial isolates were determined on XLD agar medium as in the Image (1) according to the phenotypic shape, as rounded colonies that are 1-2 mm in diameter, red in color and have a black center in the middle, were selected (Mikoleit,2015).

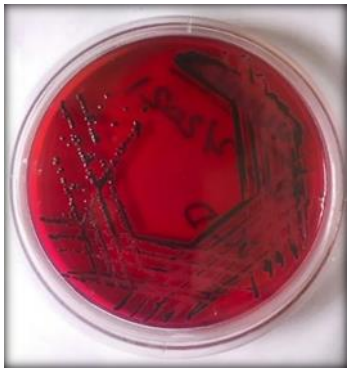


Image (1) Salmonella isolates Colonies on XLD agar

Identification

The results show wed that only 10 isolates belong to the genus Salmonella, as the isolates were diagnosed to the genus level by using Vitek 2 compact system, As well as The results of the tests as shown in Table (2). showed that all Salmonella isolates belong to Salmonella enterica subsp. enterica (Mikoleit, 2015). Diagnosis results to serotype level showed that isolates 1-6 belong to S. Typhimurium, while isolates 7-10 belong to the S. Enteritidis.

Table (2) Biochemical tests for the diagnosis of S. enterica subsp. enterica

	Tests	Results
Sugar fermentation	Arabinose	+
	Dulcitol	+
	Glucose	+
	Rhamnose	+
	Sorbitol	+
	Trehalose	+
	Xylose	+
	Maltose	+
	Lactose	-
	Inositol	-
Ornithine decarboxylation		+
Arginine decarboxylation		+
Lysine decarboxylation		+
Malonate test		-
Tartrate test		+

ONPG test	-

Incidence rate of Salmonellosis

The results of the current study showed that possibility to isolate 10 isolates, all of them belonging to S. enterica subsp. enterica, which was obtained from the culture of 350 stool samples of children suffering from diarrhea, meaning that the incidence of salmonellosis was 2.875% of the total number of patients. The results of the current study did not agree with the results of the study conducted by Al-Juboory and his group (2014) in Mosul city, as they stated that the rate of salmonellosis among patients suffering from diarrhea that they attended to Al-Razi Hospital was 0.994%, but the same researchers mentioned that the rate of salmonellosis among the patients visited Al-Khansa Hospital, it was 4.43%, which is close to the results of the current study. The results of this study were somewhat agreed with the results of the study conducted by Al-Kubaisy and his group (2015) in Baghdad, where they stated that the incidence of salmonellosis among children suffering from diarrhea was 4.7%. The results of this study are also somewhat agreed with the results of the study conducted by Hussain (2018) in the city of Nasiriyah, where it was stated that the incidence of salmonellosis among children under five years of age who suffer from diarrhea was 5%. The results of the current study did not agree with the results of the study conducted by Harb and his group (2017) in DhiQar Governorate, as they stated that the incidence of Non-typhoidal Salmonella was 10.3% of the total number of stool samples taken from children under five years of age who suffer from diarrhea. The results of the current study differed with the results of the study conducted by AL-Kaabi and AL-Yassari (2019) in Babylon, where the results of the study showed that the infection rate of salmonella was 18.66%.

Distribution of isolates according to age groups

The results of the current study showed that the age group 49-60 months was more susceptible to infection with salmonella, with an infection rate of 60% as shown in Table (4-6). As for patients suffering from diarrhea, and at the age of two years or less, they did not show any infection with Salmonella. According to a previous study by Prakash (2008) the rate of salmonella isolation was higher in children less than 5 years old. As the researcher Gebreegziabher and his group (2018) confirmed that the majority of patients infected with salmonella are children under the age of five, researchers attributed these results to the age difference, because children are less likely to wash their hands after defecation.

Table (3) Distribution of patients who infected with salmonellosis according to age group

Age group(month)	Patients	%
<12	0	%0
24-13	0	%0
36-25	1	%10
48-37	3	%30
60-49	6	%60
Total number	10	%100

Antibiotic susceptibility test

The results of the sensitivity test against Ampicillin showed that 40% of the isolates were resistant to this antibiotic. The results of the current study Approximately agreed with the results of Hussain and his group (2019), as their results showed that 45% of Salmonella isolates obtained from patients suffering from diarrhea were resistant to this antibiotic. the results of the current study Approximately agreed with results of Omar and his group (2015), which showed that salmonella isolates were resistant to this antibiotic by 46%. The results of the current study disagreed with the results of Harb and his group (2017), as they stated in their results that salmonella isolates were resistant to Ampicillin at percentage 12.1%. As for the sensitivity of the isolates to Augmentin, the results showed that 70% of the isolates were sensitive to this antibiotic. The results of the current study disagreed with the results of Almashhadany and his group (2019) in Erbil, which showed that all salmonella isolates were sensitive to this antibiotic. The results of this study also disagreed with the results of Harb and his group (2017), which showed that Salmonella isolates were resistant to this antibiotic at percentage of 6.1%. The results of this study showed that Salmonella isolates were resistant to Tetracycline with a resistance rate of 80% as shown in Table (4). The results of the current study approximately agreed with the results of Al-obaidy and his group (2014), as they stated in their results that Salmonella isolates were resistant to Tetracycline by 73.3%. The results of the current study disagreed with the results of Hussain and his group (2019) in Nasiriyah, as they mentioned that all Salmonella isolates were sensitive to this antibiotic. The results of this study also disagreed with the results of Njum and his group (2019) in Samawa, as their results showed that isolates were resistant to this antibiotic by 100%. The results of this study showed that all salmonella isolates were sensitive to Chloramphenicol at percentage 100%. This result agreed with results of Hussain (2018). The results of this study disagreed with the results of Tallal and Youssef (2010), which showed that all Salmonella isolates were resistant to this antibiotic, as well as the results of the current study also disagreed with the results of Al-obaidy and his group (2014), which showed that all Salmonella isolates were resistant to Chloramphenicol. The antibiotic Azithromycin showed a significant effect on salmonella isolates, as all isolates were sensitive to this antibiotic. The results of this study differed from

the results of Dibby and Shlash (2020) which showed that salmonella isolates were sensitive to this antibiotic at percentage 60%. The results of the sensitivity test of Salmonella isolates against the antibiotics of the cephalosporins group showed that all isolates were sensitive to Ceftaxime, Ceftriaxone, as for Ceftazidime, the study results showed that 60% of the salmonella isolates were moderately sensitive to this antibiotic. The results of this study almost agreed with the results of Aljanaby and Medhat (2017) in Najaf, where they stated that the salmonella isolates, they were sensitive to Cefotaxime by 97.44%. The results of the current study disagreed with the results of Hussain and his group (2019) in Nasiriyah, as they stated that all Salmonella isolates were sensitive to the two antibiotics Cefotaxime and Ceftriaxone by 35% and 50%, respectively, as well as the results of the current study also disagreed with the results of Harb and his group (2017) in Dhi Qar Governorate, as they stated that Salmonella isolates were sensitive to Cefotaxime and Ceftriaxone by 21.2% and 30.3%, respectively. The results of the sensitivity test of Salmonella isolates against Nalidixic acid showed that only 40% of the isolates were resistant to this antibiotic. The results of the current study approximately agreed with the results of Harb and his group (2017), as their results showed that the Salmonella

isolates were resistant to this antibiotic by 45.5%. As for the sensitivity of the isolates against Gentamicin, the results showed that only 20% of the isolates were resistant to this antibiotic. The results of the current study disagreed with the results of Salman and his group (2021), which showed that Salmonella isolates were resistant to this antibiotic at percentage 96.5%. The results of this study also disagreed with the results of Hassan and Alhatami (2017), which showed that 37.5% of Salmonella isolates were resistant to this antibiotic.

Table (4) of the sensitivity of salmonella isolates to antibiotics

Antibiotics	% Sensitive	% intermediate sensitivity	% resistance
Ampicillin	40	20	40
Azithromycin	100	0	0
Ceftazidime	0	60	40
Cefotaxime	100	0	0
Ceftriaxone	100	0	0
Chloramphenicol	70	30	0
Augmentin	50	20	30
Gentamicin	70	10	20
Nalidixic acid	30	30	40
Tetracycline	10	10	80

phages study

Primary isolation

During the study period, 65 stool samples were collected from local poultry to investigate about presence of bacteriophages that have the ability to analyze salmonella isolates that were isolated during the study period. The results of the initial isolation showed that only two samples contained phages that have the ability to analyze S. Typhimurium isolates. This result was inferred by observing the decomposition of the bacterial culture in the area treated with phage-containing filtrate as shown in Image (2). The results of the current study agreed with the results of Esmael and his group (2018) in Egypt, as they were able to isolate bacteriophages from chicken feces that have the ability to infect S. Typhimurium. Atterbury and his group (2007) were able to isolate salmonella bacteriophages from poultry faeces. Shin and his group (2012) were able to isolate salmonella bacteriophages from chicken feces. Huang and his group (2018) were able to isolate 5 phages that have the ability to infect Salmonella.

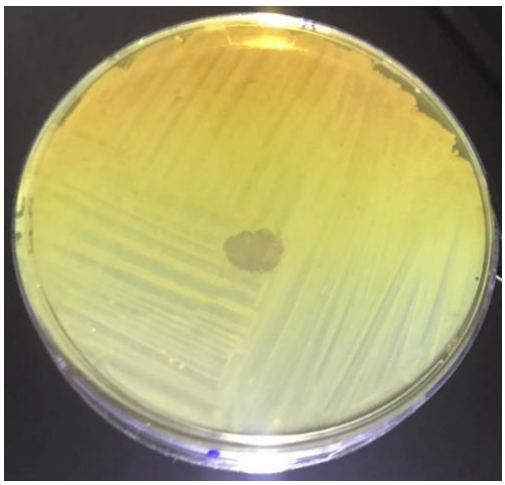


Image (2) decomposition of S. Typhimurium by phages

Phages purification

The Double-Layer Agar technique was used to isolate the phages obtained in the primary isolation from each other to obtain single pure phages. The results showed the presence of lysis zones of different diameters in the bacterial culture as in the Image (3). The difference in the diameters of the lysis zones indicates the difference in the virulence of the phages, as the more virulent phages degrade the test bacteria in less time compared to the less virulent phages. Depending on the diameter of the decomposition zone, the results showed the possibility of isolating two bacteriophages that have ability to decompose S. Typhimurium, these Phages are coded with SPh1 and SPh2. The results of this study agreed with the results of Esmael and his group (2018) in Egypt, as they were able to isolate three phages from chicken feces that have ability to infect S. Typhimurium. Also, Atterbury and his group (2007) were able to isolate 32 Salmonella bacteriophages from 26 poultry stool samples. Petsong and his group (2019) were able to isolate a phage of Salmonella from chicken farm samples.

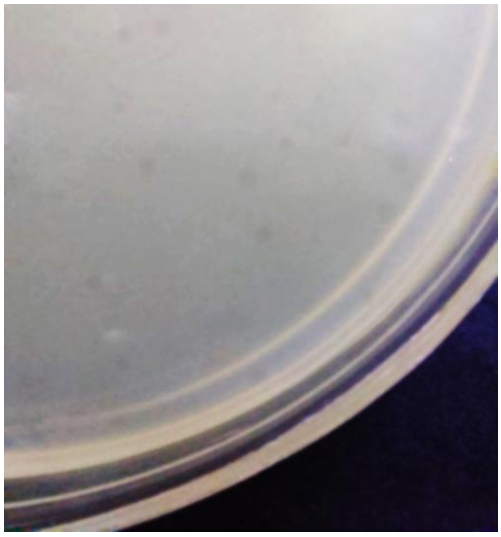


Image (3) Decomposition zones of different diameters due to different phages

Temperatures tolerate

The results of the current study showed that the bacteriophage SPh2 is somewhat more tolerant to high temperatures than SPh1 as shown in Figure (1). The results of heat treatment at 30 °C and 40 °C for 60 minutes showed that the samples containing the bacteriophage SPh1 as well as the samples containing the bacteriophage SPh2, that all phages were 100% viable. As for the samples containing the bacteriophage SPh1 that were treated at temperatures of 50°C and 60°C for 60 minutes, the phages had good temperature tolerance, as the percentage of viable phages after treatment was 80% and 55%, respectively, as well as for the samples containing the bacterium SPh2. The percentage of viable phages in the samples at the same temperatures was about 90% and 70%, respectively. The results also showed a significant decrease in the number of viable phages in samples containing SPh1 when treated at 70 °C, as the percentage of viable phages reached 20%, as for the samples containing the bacteriophage SPh2 when treated with the same temperature, the percentage of viable phages reached 40%. The results of this study are agreed with the results of the study conducted by Esmael and his group (2018), as they found that Salmonella bacteriophages that specifically infect the serotype S. Typhimurium retain their vitality when treated with heat at 30°C and 40°C. They also mentioned in their results that these phages do not It can withstand heat up to 80°C.

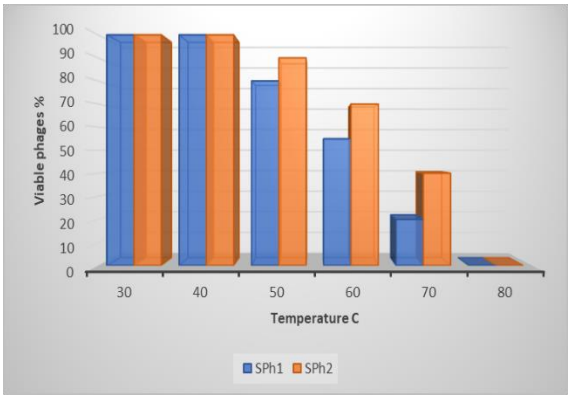
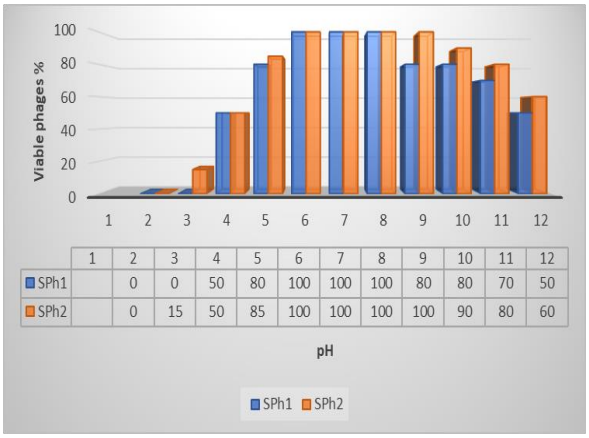


Figure (1) The ability of phages to tolerate different temperatures

pH tolerate

The results of the current study showed that the bacteriophage SPh2 is somewhat tolerant to the extreme acidic and alkaline conditions more than SPh1 as shown in Figure (2). The results also showed a significant decrease in the viable numbers of SPh2, reaching 15% when treated with Tryptic soy broth (TSB) broth, which has pH (3), as for the bacteriophage SPh1, the percentage of viable bacteriophages at this value was 0%. The results also showed good tolerance for both bacteriophages at pH ranging from 5 to 10. The results also showed that the bacteriophage SPh2 is more tolerant to alkaline conditions than SPh1. The results of this study almost agreed with the results of the study conducted by Esmael and his group (2018), as they found that Salmonella bacteriophages that specifically infect S. Typhimurium remain viable in a good percentage at pH ranging from 4 to 11.



	1	2	3	4	5	6	7	8	9	10	11	12
SPh1		0	0	50	80	100	100	100	80	80	70	50
SPh2		0	15	50	85	100	100	100	100	90	80	60

Figure (2) The ability of phages to tolerate different degrees of pH

CONCLUSION

The rate of diarrhea cases in males is more than females, and the age group of 49-60 months is more susceptible to salmonellosis. Salmonella enterica subspecies enterica is the most common cause of salmonellosis in children under five years of age. S. Typhimurium and S. Enteritidis, are the most prevalent serotypes. The phages that infect S. Typhimurium are more prevalent than those of S. Enteritidis.

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