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Evaluating the virulence factors of *Salmonella typhi* isolated from children suffering from severe diarrhea using molecular genetic techniques

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Abstract. *Jaber AS. 2023. Evaluating the virulence factors of* Salmonella typhi *isolated from children suffering from severe diarrhea using molecular genetic techniques. Biodiversitas 24: 3543-3549. Salmonella enterica subsp.* serotype Typhi is the bacteria that causes typhoid fever. Although its prevalence has significantly decreased in affluent nations, it continues to be a leading cause of illness and mortality in emerging nations. Because of changes in ecology, eating habits, and agricultural and food production techniques, *Salmonella* infections have increased in frequency in industrialized countries. This study aimed to use qualitative RT-PCR (based on the *ttr* gene) and conventional PCR to measure the frequency of *Salmonella typhi* in Iraqi children with chronic diarrhea (based on *PagN* and *TolC* genes). This study included 100 stool samples collected from children suffering from continuous diarrhea. Those samples were inoculated on CHROM-agar and incubated 37°C for 18-24 hours, then *Salmonella* was identified using a Vitek 2 compact system. DNA has been extracted, and qRT-PCR has been done to detect *Salmonella* sp. by the amplification of the ttr gene. Then PCR also done to amplify *TolC* and *PagN* genes. The amplified products then sequenced to reveal mutations within the amplified regions. The results of *PagN* gene showed 41 samples were positive, and 40 samples were positive for *TolC*. Results of qRT-PCR according to the *ttr* gene amplification showed 38 samples positive. The blood group O+ showed a higher number of positive samples (13), and males showed higher positive samples than females (23 and 15, respectively). Sequencing revealed various number of mutations within the amplified regions of both *PagN* and *TolC* in the samples which isolated from children with the longest period of illness.

Keywords: *pagN* gene, qRT-PCR, *Salmonella typhi*, Transition, Transversion, *ttr* gene

INTRODUCTION

The stomach is an important site of entry for infections. Understanding the infection process requires identification of the relevant protective barriers (Rooks and Garrett 2016). Unexpectedly, human beings vary from one another in terms of our ability to combat gut colonization by enteropathogens (Wotzka et al. 2017). Epidemiologic statistics show that most people who are exposed to *Salmonella* (94-99.8%) either have very mild symptoms of the acute illness or stay well (Dróżdż et al. 2021). It will be interesting to see whether some traits may distinguish between susceptible and hardy individuals based on barrier functionality, microbiota composition, or other variables (Porras et al. 2021).

More than 1300 children die from diarrheal diseases per day, or around 480,000 children globally, under the age of five, according to the World Health Organization in 2016 (Diarrhoea - unicef data 2021). In many Eastern Mediterranean Region (EMR) nations, infectious diarrhea is a major cause of morbidity and mortality, especially in young children under the age of five (Harb et al. 2019), making diarrheal sickness one of the most challenging health issues (Mokdad et al. 2018). Pediatric diarrhea cases have increased over time in other EMR countries including Iran and Iraq, where they increased from 10.3% to 19.6% between 2008 and 2010 and 14.9% to 21.3% between 1997 and 2000, respectively. Salmonellosis is one of the most harmful zoonotic diseases in the world (Dróżdż et al. 2021). Most of the time, its etiologic agents, *Salmonella* sp., are harmful to humans and may especially harm young children (under 5 years old), elderly adults (over 64 years old), people with impaired immune systems, and pregnant women (Lowden et al. 2015). *Salmonella* sp. are thought to be opportunistic infections or maybe a part of certain animals' regular gut microbiota. For instance, without exhibiting any symptoms, up to 80-90% of reptiles are *Salmonella* sp. carriers (Ross et al. 2019).

TolC is a classic example of an OEP (outer membrane efflux protein). Gram-negative bacteria use these proteins to move microscopic substances and toxins through their outer membrane. The genomes of some bacteria, notably Pseudomonas species, include several copies of OEPs (Dijun et al. 2014). Contrarily, enterobacteria possess a single *tolC* gene and its progeny, which collaborate with a number of transporters. *TolC* inactivation has a profound influence on the physiology and pathogenicity of enterobacteria. *TolC* seems to have a very broad and important role in enterobacteria's physiological adaptation to challenging environments, according to recent studies (Raspoet et al. 2019). *TolC* has been recognized to have a crucial role in the structure and function of the *E. coli* outer membrane for at least 30 years. Because *tolC* mutants are hypersensitive to several dyes, medications, and detergents while tolerating colicin E1, early studies hypothesized that *tolC* mutants had abnormalities in the cell membrane. Later experiments revealed that *TolC* and its homologs in other

Gram-negative bacteria facilitate the transport of several toxic substances through the outer membrane (Liu 2021).

Additionally, the *Salmonella* invasion *PagN* (phoP-activated gene) outer membrane protein has been discovered (Lambert and Smith 2008). Through the use of a TnphoA random-insertion screening intended to find PhoP-activated genes, it was originally discovered in *S. Typhimurium* (Lambert and Smith 2009). The majority of the investigated serotypes include the *pagN* gene, which is located on the distinctive genomic island of centisome 7 (Nanjoo et al. 2022). The PhoP/PhoQ transcriptional regulatory system controls the transcription of *pagN*. PhoQ is auto-phosphorylated and transfers its phosphate to the cytoplasmic DNA-binding protein PhoP in response to an acidic environment, a low Mg2+ concentration, or the presence of antimicrobial peptides (Su et al. 2021). Following that, PhoP either activates or inhibits the transcription of certain *Salmonella* genes. The first study was Lambert and Smith (2009) were the first to demonstrate that pagN deletion in *S. typhimurium* led to a reduction in *Salmonella* invasion of enterocytes two methods have been demonstrated, namely qualitative RT-PCR (based on the *ttr* gene) and conventional PCR-were used to determine the frequency of *Salmonella typhi* in Iraqi children with persistent diarrhea (based on *PagN* and *TolC* genes). Compare the sensitivity of the two molecular methods as well. On the other hand, this research also intended to identify alterations in the amplified forms of two *Salmonella typhi* genes (*TolC* and *PagN*). Detect the bacterium with a high sensitivity and specificity by targeting the ttr gene. Mutations within both *PagN* and *TolC* genes increased the severity of the infection. Paying attention to blood groups has an important role in infection, especially blood group O+, especially in children, this is what makes it different from some previous studies.

MATERIALS AND METHODS

Culturing on Chrom agar and diagnosis of *Salmonella* sp. by Vitek2

This study included 100 stool samples collected from children (1-8 years) who were accompanied with their parents visiting department of pediatrics at Bint Alhuda Hospital (Dhi- Qar, Iraq) and were suffering from continuing diarrhea, abdominal cramps, tenderness and fever. The fresh stool samples is refresh on tetrathionate broth medium and then inoculated on CHROM agar *Salmonella* selective culture medium at 37°C for 18 to 24 h, to detect the presence of *Salmonella* sp. Then the positive isolates have been cultured on *Salmonella Shigella* Agar (S.S agar), and incubated for 24 h at 37°C. After the completion of the incubation period a test tube has added standard sterile saline (3.0 mL). An application stick or sterile swab was used to transfer sufficient pure-crop colonies and suspend the isolated colonies in the normal saline. The turbidity (0.5-0.63) of McFarland was adjusted and Densi Chek TM was used. Then the prepared bacterial suspension is transferred to the Gram Negative card (GN card), which have sealed and placed in the VITEK system (BioMe'rieux) to detect the positivity of the samples for *Salmonella typhi*.

Use VITEK-2 system to confirmed identification, data were analyzed using Vitek-2 database, which allows organism identification in a kinetic mode beginning 180 min after the start of incubation, identification card is based on established biochemical methods, there are 43 biochemical tests measuring carbon source utilization, enzymatic activities and resistance. Final identification results are available in approximately eight hours or less. After being identified by the Vitek 2 system, the samples were diluted in normal saline and 1 ml of each dilution was cultured on MacConkey agar and S.S agar.

DNA extraction

DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO, USA). The eluted DNA was stored in the deep freezer until the day of the PCR. The reagent is designed to isolate high-quality total DNA generated from cells. The DNA is preserved because the reagent efficiently suppresses DNase activity while disrupting cells and dissolving cell components during sample homogenization (Kit leaflet). The Rapid-DNA MiniPrep provides a quick method for directly extracting up to 50 g (per prep) of high-quality DNA from samples.

Sample DNA concentration and purity were evaluated using the QuantusTM Fluorometer and Promega QuantiFluor® Dye Systems from Promega (USA). The 1X TE buffer was made by diluting the 20X Tris EDTA (TE) Buffer (pH 7.5) to 1X using nuclease-free water. The QuantiFluor® Dye working solution was diluted to 1:400 using 1X TE buffer. The blank sample was produced by filling a 0.5 ml PCR tube with 200 µL the QuantiFluor® Dye working solution (without DNA sample or standard). Two hundred microliter of the QuantiFluor® ONE DNA Dye were mixed with one liter of the QuantiFluor® ONE DNA System standard. The liquid was combined three times using gentle pipetting. Tubes were centrifuged at 2,000 g for 5-10 sec to remove any air bubbles and collect the liquid at the bottom of the tube. Tubes were incubated at room temperature for five minutes without being exposed to light. The prepared blank and standard tubes are then read using the Quantus apparatus to calculate the standard to blank ratio. The samples were then created by mixing 1 µL of sample with 200 µL of water and placed in the calibrated Quantus apparatus to be read.

Gene amplification for *TOLc* and *PAGn*

A portion of the *Salmonella* genome has been amplified by using particular primers. The primer sequences for each pair are listed in Table 1. Prior to being dissolved in free ddH2O, the primers were lyophilized to provide a stock solution with a final concentration of 100 pmol/L. As a suspended work primer, they were then held at -20 to establish a concentration of 10 pmol/L. To form a final volume of 100, 10 µL of the stock solution was combined with 90 µL of free ddH2O water. At room temperature, the Pre-mix Master Mix (Cat#25025) was defrosted and thawed. The Taq PCR Pre Mix, 1 µL of each forward and reverse primer, 3 µL of eluted DNA, and 7.5 µL of

nuclease-free water were combined to create the reaction mix for each sample. The tubes were then put in a tiny centrifuge for optimal mixing. The tubes were then put into a thermal cycler that had been configured to go through 35 cycles of denaturation at 95°C or 25 seconds, annealing at 52°C for 25 sec, and extension at 72°C. A Basic Local Alignment Search Tool Bio ID program from the NCBI was used by sequence and looked up in the nucleotide databases to identify the sample. It was then submitted to GenBank (ID). Related sequences of a sample or samples were obtained from the nucleotide database of the NCBI (www.ncbi.nlm.gov/nucleotide). qPCR detection The Real-time PCR reaction mixture was prepared by adding 10 µL of KAPA SYBR® FAST master mix, 3 µL of DNA, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 6 µL of nuclease-free water to a fresh, sterile PCR tube. The tubes were then attached to the thermal cycler device, which was made to do denaturation at 94°C for 5 minutes, 35 cycles at 94°C for 35 sec, 1 min at 60°C, and lastly 7 min at 72°C. For the purpose of amplifying detection, FAM channel was chosen.

Statistical analysis

Microsoft Excel and SPSS version 24.2 were both employed in the research's statistical analysis. Two categorical parameters have been analyzed using the chi-square approach. P-value has a 0.05 likelihood, according to research.

RESULTS AND DISCUSSION

Results

The first diagnosis of *Salmonella typhi* was done by amplifying the *tolc* gene and the *PagN* gene. Then the amplified products were run through agarose gel electrophoresis, gel electrophoresis for *TolC* gene (Figure 1.A) and for *PagN* gene (Figure 1.B), both ran with 100bp DNA ladder. The second diagnosis of *Salmonella* was targeted by q-PCR using the *ttr* gene (Figure 2). The results shown a multiple logarithmic curves, each curve represent the amplification of a single sample.

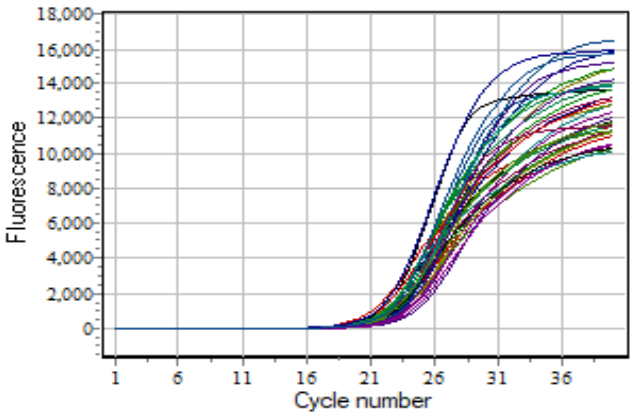

Figure 2. qPCR amplification results of the *ttr* gene

Table 1. Nucleotides sequences of primers set used in this study

Gene	Forward primer sequence	Reverse primer sequence	Reference
TR	5-CTCACCAGGAGATTACAACATGG-3	5-AGCTCAGACCAAAAGTGACCATC-3	Nairet et al. (2019)
PagN	5-TTCCAGCTTCCAGTACGTTTAG-3	5-GCCTTTGTGTCTGCATCATAA-3	Yue et al. (2019)
TolC	5-GCAGACGCTGATCCTCAATAC-3	5-TTGCGCCGACGAAGTTATAC-3	Roozbehani and Brown (2021)

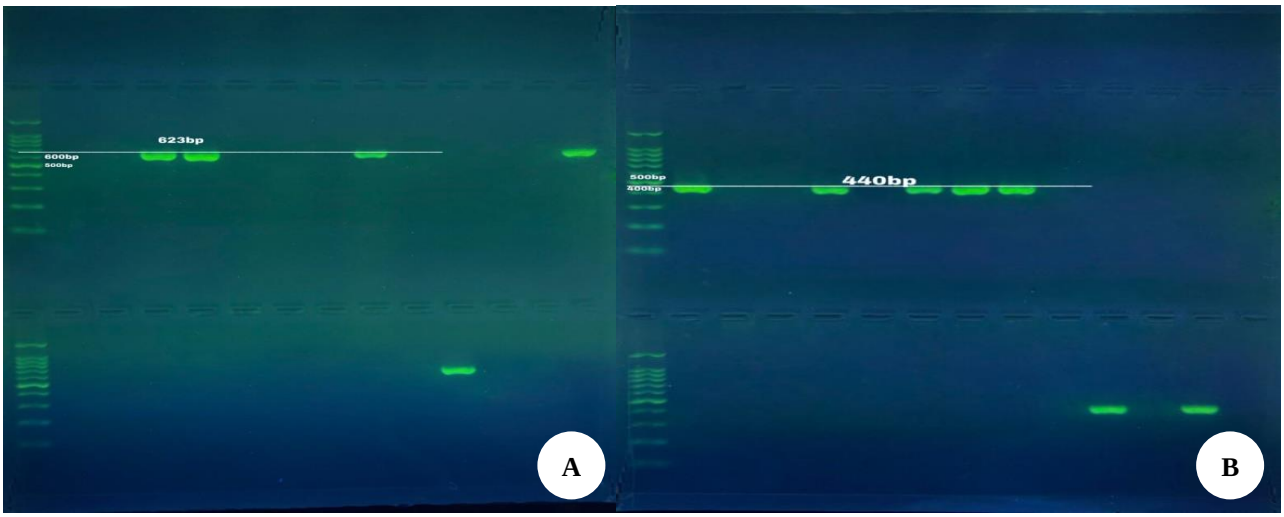

Figure 1. A. *Tolc* gene PCR output showing a 623 bp band. The final result was achieved by electrophoresis on 2 percent agarose at 5 volts/cm2. With a 1-hour TBE buffer. B. *PagN* gene PCR output showing a 440 bp band. The final result was achieved by electrophoresis on 2 percent agarose at 5 volts/cm2. With a 1-hour TBE buffer

The results of *PagN* gene showed 41 samples were positive and 59 were negative. The *TolC* differ with one sample (40 positive and 60 negative). The number has reduced to 38 by the qPCR. This differences among the results of tests was significant (chi-square 3.21, P-value= 0.031) (Table 2).

The distribution of samples on the demographic parameters, Male showed higher number of positive samples than female (23 and 15, respectively). The blood group O+ showed the higher number of positive samples (13) and the lower number of positive samples are shown by AB- and O- blood group which both showed only one sample positive. The middle age group 3-4 years showed the higher number of positive samples (14). The infection time showed one sample has spent 7 days suffering from the symptoms and the period of 4 days showed the higher number of positive sample (15) (Table 3).

The results of PCR showed that 40 samples were positive for the *TolC* gene, and 41 samples were positive for the *PagN* gene. Three samples of each gene, representing the infection phase that lasted the longest, were chosen for sequencing.

A chromatogram sometimes referred to as an electropherogram, and a visual representation of a DNA sequence made up of nucleotide sequences and peaks are produced by automated DNA sequencing. as a computer output. A good sequence usually starts at base 20 and is characterized by towering, distinct peaks with little overlap. The results showed clear distinguishable peaks that represent good sequencing results.

According to alignment to NCBI reference sequence, there are various mutations on this stretch. The three samples under study each had a distinct mutation frequency. The number of these mutations and the accompanying shifting of amino acids are detailed in Table (4). It is clear that sample 1, which had 5 mutations, has the greatest mutation number. This quantity of mutation included one gap at nucleotide 407bp and 2 transition (shifting between purins and pyrmidins at nucleotide 489bp and 573bp) and 2transversion at nucleotides 493bp and 583bp. Both samples 2 and 3 showed only one gap mutation at the same position 441bp.

After aligning *PagN* gene sequences for the studied samples, different variations appeared among the samples, the highest number of mutations have appeared in the second sample (5 mutations) those mutations include 3 transversion in the positions (28bp, 265bp, and 301bp), a single gap (346bp) and a single transition (311bp). Both first and third samples showed 3 mutations but at different positions, the first sample showed one gap at (7bp), one transversion (165bp), and one transition (205bp). The third sample showed two gaps at (19bp and 23bp) and one transversion (218bp). as shown in the (table 5) which summarize the mutations of each sample.

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Table 2. Comparison of positive and negative results among the used tests

Test	Positive	Negative	Chi-square	P-Value
Conventional PCR-*TOLc* gene	40	60	3.21	0.031
Conventional PCR-*PAGn* gene	41	59		
qPCR- *ttr* gene	38	62		

Table 3. Distribution of positive and negative samples among the demographic parameters

Parameter		Results		chi-square	P-Value
		Positive	Negative		
Gender	Male	23	28	1.580	0.147
	Female	15	31		
Blood	A+	3	13	9.080	0.246
	A-	4	2		
	B+	5	4		
	B-	2	3		
	AB+	9	9		
	AB-	1	7		
	O+	13	20		
	O-	1	1		
Age	1-2	11	16	4.205	0.240
	3-4	14	13		
	5-6	8	23		
	7-8	5	7		
Infection time (days)	1.00	1	6	57.42	0.001
	2.00	1	30		
	3.00	12	21		
	4.00	15	1		
	5.00	4	0		
	6.00	5	0		
	7.00	1	0		

Table 4. Summary information of sequence alignment for *TolC* gene

Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
Gap	407	A	ID: CP053702.1	*Salmonella enterica* subsp. Entericaserovar Typhi strain CMCST_CEPR_1 chromosome, complete genome	96%
Transition	489	G/A			
Transversion	493	C/A			
Transition	573	G/A			
Transversion	583	A/T			
Gap	441	T	ID: CP053702.1	*Salmonella enterica* subsp. Entericaserovar Typhi strain CMCST_CEPR_1 chromosome, complete genome sequence	97%
Gap	441	T	D: CP051218.1	*Salmonella* sp. SCFS4 chromosome, complete genome	97%

Table 5. Summary information of sequence alignment for *Pagn* gene

Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
Gap	7	C	ID: CP053702.1	*Salmonella enterica* subsp. entericaserovar Typhi strain CMCST_CEPR_1 chromosome, complete genome	98%
Transversion	165	A/T			
Transition	205	C/G			
Transversion	28	A/T	ID: CP034712.1	*Salmonella enterica* subsp. entericaserovar 43:a:1,7 strain RSE18 chromosome, complete genome	95%
Transversion	265	C/G			
Transversion	301	C/G			
Transition	311	C/T			
Gap	346	T			
Gap	19	C	ID: CP034712.1	*Salmonella enterica* subsp. entericaserovar 43:a:1,7 strain RSE18 chromosome, complete genome	98%
Gap	23	T			
Transversion	218	A/T			

Discussion

Antibiotics may both promote and repress the production of a number of efflux pump proteins, efflux processes have a significant effect on antimicrobial resistance (AMR), (Roozbehani and Brown 2021). One of the efflux pumps, AcrAB-*TolC*, is composed of the periplasmic adaptor protein AcrA, the inner membrane transporter AcrB, and the outer membrane protein *TolC*. As a fundamental mechanism of multidrug resistance in gram-negative bacteria, the AcrAB-*TolC* efflux pump is over expressed (Chowdhury et al. 2019).

In Gram-negatives such as *E. coli* (AcrAB-TolC), *Salmonella enterica* (AcrAB-TolC), *Klebsiella pneumoniae* (AcrAB-TolC), single Resistance Nodulation cell Division tripartite systems (single RND-tri systems) appear to be dominant (Hernando-Amado et al. 2016).

The value of RND efflux pumps as molecular targets is underscored by their propensity to support the acquisition of additional resistance mechanisms (Nolivos et al. 2019), their over expression in dormant bacteria and their role in cell-to-cell communication, biofilm formation, pathogenicity, and virulence. Strategies against multidrug efflux pumps have been targeting their (i) expression (Jesin et al. 2020), (ii) complex assembly (Abdali et al. 2017), or (iii) the fully assembled active transporter.

Investigation explain, two detection approaches were used (conventional PCR and real time- PCR) The findings confirmed a previous study's finding that authorized PCR might raise the detection level of *Salmonella* spp. by increasing the sensitivity of the diagnosis in comparison to PCR (Kasturi and Drgon 2017). Further concurred with Kuppuswamy's previously reported outcomes (Siala et al. 2017). AUC of 0.90 was obtained from the ROC analysis of the PCR technique and the culture method, with a 95% confidence range between 0.76 and 1.0. confirming the validity of the PCR method (Chirambo et al. 2020). The lack of sensitivity and specificity of Widal test with the necessary to repeat the test make it unsuitable for rapid diagnosis of enteric fever (Mengist and Tilahun 2017). in study (Noha et al. 2023). Explain that combined Widal and blood culture increased their sensitivity, specificity and accuracy to 89.2%, both tests are used in combination for diagnosing enteric fever in less developed regions as cheap, easy and available options. In our current study, we use Nucleic acid amplification techniques for diagnosis of *S. enterica* isolated from fecal samples, qPCR was used for detecting ttr gene from *S. enterica* isolated from fecal samples. Test is rapid than culture, *ttr* gene was the highest sensitivity, specificity, agree with the study (Noha et al. 2023; Chirambo et al. 2021). Noha study was the highest sensitivity, specificity and accuracy was for qPCR samples (99%, 99.01% respectively) and Chirambo, reported the sensitivity of qPCR for these genes ranged from 90.3% to 99.53% and the specificity ranged from 90.31% to 99.3% according to the used genes.

Tetrathionate reductase is encoded by the *ttr* gene, which was employed in this work for q-PCR detection. Typhoidal *Salmonella* is recognized by the presence of gene combinations specific to the *Salmonella enterica*, *serovars*, *typhi* and *paratyphi*. By mediating interactions with mammalian cells, the *PagN* outer membrane protein may encourage the adhesion and invasion of epithelial cells. The *pagN* gene is raised in both human cells and laboratory animals, and *pagN* mutants are less competitive in vivo, according to earlier studies (Lambert and Smith 2009). Numerous mutations were found in the amplified products after the *PagN* and *TolC* genes were sequenced; these alterations may enhance the severity of *Salmonella* sp. This finding differed with a prior research that demonstrated that *pagN* mutation did not totally abolish adhesion and invasion the size of the impact (Lambert and Smith 2008), since the isolates were obtained from the patients who had the longest length of infection. PagN is an outer membrane protein that may contribute to the virulence of *S. typhimurium.* On the other hand, the *Tolc* study's findings were consistent with earlier research that demonstrated that mutations led to higher levels of nitroxoline resistance because they increased the the *tolC* gene's expression. By over expressing *tolC* from an inducible plasmid in a mutant with little resistance, this was verified (Liu et al. 2021). Mutations in different genes causing increased expression of the EmrAB-TolC pump lead to an increased resistance to nitroxoline. The structurally similar antibiotics nitroxoline and nitrofurantoin appear to have different modes of action and resistance mechanisms (Balint et al. 2020).

The findings indicated that O+ blood type had a greater incidence of infection. The findings of this investigation

contradicted a prior study conducted in Iraq and shown that those with the B+ blood type had a greater probability of contracting *Salmonella typhi* infection (42.59%), followed by those with the O+ blood group (31.48%) (Al-Zubaidy 2022). Our study was inconsistent with the study of (Abegaz 2021), who explained that Early etiological studies indicated that blood type O has a connection with increased incidence of cholera, plague, tuberculosis infections, and mumps, whereas blood type B is also associated with increased incidence of gonorrhea, tuberculosis, *E. coli*, and salmonella infections; and blood type AB is associated with increased incidence of smallpox and salmonella infections. It fit with the ideas and expectations of the researchers who had investigated human ABO blood groups and associated diseases as well as health benefits to combat circulatory diseases, infectious disease, malaria, metabolic diseases, and so on (Abegaz 2021). The antigenic individuality in the individuals present on the surface of the red blood cells plays a very powerful role in development of certain specific and systemic diseases and infections (Sridevi 2021).

Test methods demonstrated high concordance, although stool culture and multiplexed ttr primers had superior specificity and sensitivity, molecular detection methods used here could be used of *Salmonella* exposure (Chirambo et al. 2021). The *ttr* gene has also been employed in a prior research that demonstrated detection based on multiplex-PCR, where ttr specificity and sensitivity were (90.30%, 99.30%) and (99.53%, 95.46%), respectively. This study has succeeded in employing the *ttr* gene to boost the sensitivity of the diagnostic.

In conclusion, we can conclude that children with blood group O+ are with a higher risk of infection with *Salmonella* sp. agree with many studies have proven the association between ABO blood types and diseases, others did not confirm it because uncertainty by inconsistent results. It is clear that ABO blood types are not the exact cause of diseases, but they can be susceptible and surrender to disease and health problems. qRT-PCR detect the bacterium with a high sensitivity and specificity by targeting the *ttr* gene. Mutations within both *PagN* and *TolC* genes increased the severity of the infection. *PagN* is contribute to the virulence of *S. typhimurium*, *pagN* mutants have been shown to be less competitive in vivo and thus this may be due to their lessened ability to interact with mammalian cells. Preference is given to methods of confirmatory molecular diagnosis in the future and observation of the sequence to detect mutations occurring within the genes of these pathogenic strains because they infect children, and thus the rapid detection of the pathogen and its virulence against the host is done. Numerous and intensive studies should be done on the genes of virulence and resistance to strains of *Salmonella enterica subsp* isolated from children, and focus attention on modern molecular methods that are important in diagnosis because of their great impact on child health.

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