DOI: 10.29409/ijcmg.v16i2.266

Genetics Research

Iraqi Journal of Cancer and Medical Genetics

Association of Typhoid Fever Severity with Polymorphisms in Nucleotide Oligomerization Binding Domain 2 (NOD2) and Natural Resistance Associated Macrophage Protein 1 (NRAMP1) Genes in a sample of Iraqi Patients

Bushra Jasim Mohammed[1], and Yasir Azem Hameed[1]

1 Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq.

Abstract

Background and aim: Typhoid fever is one of the major health problems facing humanity of all ages. This study was conducted to investigate the relative contribution of genetic factors to the severity of typhoid fever in a sample of the Iraqi population. Materials and Methods: Blood samples were collected from 52 patients suffering from typhoid fever that was previously diagnosed using conventional and genetic methods at Ibn Al-Baladi Hospital in Baghdad and 52 apparently healthy volunteers as a control group. DNA was extracted from peripheral blood and genotyped for NRAMP1 Intron 4 (469+14 G/C) / rs3731865 and NOD2 Exon 8 (2722 G/C) / rs2066845 single nucleotide polymorphism (SNP) using the Nested T-ARMS PCR method. Results: explained that patients with at least one copy of the NOD2 (C) allele, whether (homozygote or heterozygote) had a high risk of severity of Typhoid fever with highly significant ($P \leq 0.01$), as well as results found that there was no association between the occurrence of NRAMP1 rs3731865 Intron 4 (469+14 G/C) with severity of Typhoid fever. Conclusion: The incidence of NOD2 Exon 8 (2722 G/C) rs2066845 SNP plus the C allele could be related to the severity of Typhoid fever in this particular Iraqi population.

Keywords: *NOD2, NRAMP1, Typhoid fever, polymorphisms.*

Introduction

Typhoid fever is a prospectively, multisystemic illness that has been a public health problem, especially in the developing world (1). Globally, overall rates of typhoid fever are reported to be falling, however, there are between 11.9 and 26.9 million cases of typhoid fever each year with (129,000 – 135,900) deaths (2). Typhoid fever is endemic in Iraq, and hot weather and frequent interruptions in electricity and water supply during the summer months have resulted in an increased incidence (3). Numerous interventions were implemented to prevent and control outbreaks. In 2007, 2008, 2009 and 2010, a total of 36,208, 58,247, 49,113 and 49,139 suspected cases of typhoid fever were reported, respectively (4). Nucleotide oligomerization domain 2 (NOD2) is a family of intracellular bacterial sensors that are able to recognize highly conserved microbial motifs, play an antibacterial factor, limiting survival of intracellular invasive bacteria, and are cytosolic pattern recognition receptors (PRRs) that recognize particular motifs found in bacterial peptidoglycan. The NOD2 family is key bacteria-sensing receptors and is important in the early response against enteric pathogens, including S. typhi, mutations in NOD2 in humans have been associated with inflammatory disorders (5). The natural resistance associated macrophage protein 1 gene (NRAMP1) encodes a divalent cation transporter protein, includes Fe2+, which is a complementary factor to various enzymes ,bacterial proliferation, also necessary to produce hydroxyl radical, which reacts with nitric oxide to be biocidal peroxynitrite. Hence, it may influence the biocidal activity of macrophages, and NRAMP1 gene polymorphisms affect the development of some immune-related diseases (6). Studies on the relationship between genetic polymorphism and host typhoid fever susceptibility in Iraq were found to be rare and perhaps did not exist. That was what motivated us to conduct such a study, to examine the effect of genetic polymorphism of NOD2 and NRAMP1 genes of susceptibility to typhoid fever.

Corresponding Address:
Bushra Jasim Mohammed
Immunogenetic, Professor, Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq.
Email: dr.bushrajassim@ige.uobaghdad.edu.iq

Materials and Methods

Blood samples were obtained from 52 typhoid patients from Ibn Al-Baladi Hospital in Baghdad, under the approval of the Ethics Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad for using human samples of number (2553), patients with a range of age between (3-48 years), and 52 healthy controls of the same age and gender. Two ml of blood was taken from each subject, but in EDTA anticoagulant tubes and subjected to DNA extraction according to (7) and (8) using the genomic DNA purification kit (Geneaid /Taiwan), DNA concentration was estimated using the Nano drop spectrophotometer and measured in(ng\µl) as well as the purity was estimated according to (7) and (9), reading the absorbance at a wavelength between 260 and 280 nm. Genotyping for NOD2 Exon 8 (2722 G/C) / rs2066845 and NRAMP1 Intron 4 (469+14 G/C) / rs3731865 single nucleotide polymorphism (SNP) was carried out using the Nested T-ARMS PCR method. In brief, it consisted of Taq polymerase and four primers with two outer nested primers. Two pairs (instead of one pair) of PCR primers are used to amplify an 8 fragment(outer forward (OF), outer reverse (OR), inner forward (IF) and inner reverse (IR) primers. Primers of Nested T-ARMS PCR and their sequences are listed in tables 1and 2.

Table 1 Primers of NOD2gene Exon 8 (2722 G/C) SNP rs2066845

Target gene		Sequence (5'-3')	Tm (C°)	Product size	Reference
Nested	F	AAGTCTGTAATGTAAAGCCAC	56	bp 380	Designed in this study
	R	CCCAGCTCCTCCCTCTTC -'5			
Inner	F	ACTGCAGAGGGAGGAGGACT	56	bp 259	
	R	TTCAAAGACCTTCAGAACTGGC			
Allele G	FG	TTGGCCTTTTCAGATTCTCGG	56	bp 182	
Allele C	RC	TCGTCACCCACTCTGTTCCG	56	bp 117	

Table 2 Primers of NRAMP1gene Intron 4 (469+14 G/C) SNP rs3731865

Target gene		Sequence 5'-3'	Tm (C°)	Product size	Reference
Nested	F	GATTACAGGGTGAGCTACCAGC	56	bp 844	Designed in this study
	R	CTCTCATGTCCCTCTAGGCTATG			
Inner	F	CCGAGGAGTATGCTTGGTTAGA	56	bp 369	
	R	CGAGACTCCGACTGAAAAACAAA			
- Allele	FC	ATGGTTCTCCCTGTCCACGC	56	bp 245	
--Allele	RT	CTAAGGTGAGCTTGGGCGC	56	bp 161	

The ratio of the inner primer to the genotype-specific primer is 1:10 in a final reaction volume of 50 µl. The Nested T-ARMS PCR Cycling program DNA was used to detect NRAMP1 and NOD2 gene polymorphism, as illustrated in Table-3.

Table 3: Nested T-ARMS PCR Cycling Program for Human NRAMP1 and NOD2 genes.

Phase	Tm (C°)	Time	Cycles
Initial denaturation	94	5 min	1X
Denaturation	94	30 sec.	35X
Annealing	60	30 sec.	
Extension	72	1 min	
Final extension	72	5 min	1X

Agarose gel electrophoresis for Nested T-ARMS PCR product has been performed to visualize the PCR product size after finishing the PCR program using 2% agarose gel. The genotypes were differentiated by checking the amplicon sizes with reference to molecular size markers. The SAS (10) statistical analysis system program was used to detect the effect of difference factors in the study parameters. The least significant difference –LSD test (Analysis of Variation-ANOVA) was used to compare between means and the Chi-square test which was used to compare between percentage (0.01 probability) in this study.

Results

The results of the distribution of S. typhi according to age revealed that the highest number of typhoid fever infection was located in the age group (1-10) years that represented 20 patients with a percentage (38.46%) of the total number, followed by (11-20) years that showed 17 patients with a percentage (32.70%) , whereas the last three groups seemed almost equal in the age groups (21-30)years with 6 (11.60%), and (31-40) years that showed 5 (9.60%), lastly 4(7.70%) for the age group (41-50) years with a highly significant difference ($P \leq 0.01$), as shown in Table (4).

Table 4: Distribution of S. typhi according to age groups.

Age groups (year)	No.	Percentage (%)
1-10	20	38.46
11-20	17	32.70
21-30	6	11.54
31-40	5	9.60
41-50	4	7.70
Total	52	100%
Chi-Square (χ^2)	---	21.653 **
** ($P \leq 0.01$).		

The results of distribution of typhoid fever patients according to gender showed that males were 25 (48.08%) and females was 27 (51.92%), with no significant difference as shown in table (5).

Table 5: Distribution of patients with S. typhi according to gender

Gender groups	No.	Percentage (%)
Male	25	48.08%
Female	27	51.92%
Total	52	100%
Chi-Square (χ^2)	---	0.076 NS
NS: Non-Significant.		

The result of T-ARMS PCR for the human gene NOD2 rs2066845 SNP showed that the T-ARMS-PCR product was divided into four bands: 380 bp represented the nested outer PCR which served as a DNA template for the inner PCR of 259 bp and 182 bp for the allele (G) and 117 bp for the allele (C).The wild-type genotype (GG) had only three bands 380, 259 and 182 bp. The heterozygous mutant genotype (GC) would have four bands of 380, 259, 182 and 117 bp, and homogenotybe (CC) 'mutant' had three bands of 380, 259 and 117 bp as shown in figures (1) and (2).

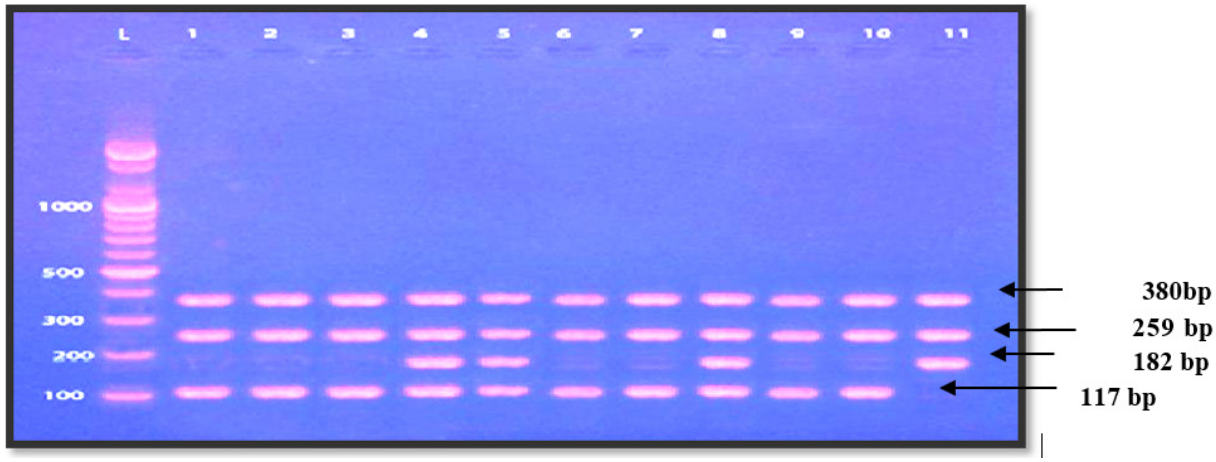

Figure1: T-ARMS-PCR bands of the human gene NOD2 rs2066845 SNP for the patient. (2%) agarose, DNA dye is RedSafe, V: 5/cm2, Time: 45 minutes, L: DNA ladder.

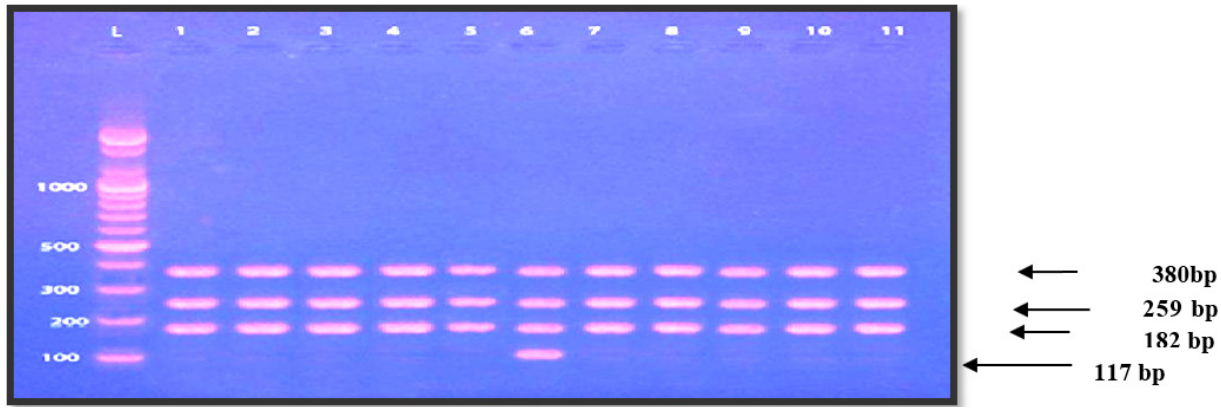

Figure2: T-ARMS-PCR bands of the human gene NOD2 rs2066845 SNP for control. 2% agarose, DNA dye is RedSafe, V: 5/cm2, Time: 45 minutes, L: DNA ladder.

The Genotype GG, which represented as wild type of the group of patients, was found in only 4 samples that represented a percentage of 7.69%, when compared to the control group 51 samples that represented a percentage of 98.08%, with highly significant (P≤ 0.01).The genotype GC, the heterozygote frequented 27 times in the patient group represented a percentage of 51.92% and one sample in the control group represented a percentage of 1.92%, with highly significant (P≤ 0.01), also the genotype CC, was repeated 21 times in the patient group represented a percentage 40.38%, and was zero for the control group, with highly significant (P≤ 0.01). The allele frequency of the G allele was 0.34 for the patient's group and 0.99 for the control group, the C allele frequented 0.66 in the patient's group, as well as for the control group with a highly significant (P≤ 0.01) as illustrated in Table (6).

Table 6 : Genotype and allele frequency of rs2066845 exon 8 (2722 G/C) - NOD2 gene

Genotype\Allele NOD2 gene	Control Group	Patient Group	P value
GG	(98.08%)51	(7.69%) 4	**0.0001
GC	(1.92%) 1	(51.92%) 27	**0.0001
CC	(0.00%) 0	(40.38%) 21	**0.0001
G	0.99	0.34	**0.0001
C	0.01	0.66	**0.0001

Furthermore, the prevalent genotype genotype of NRAMP1 rs3731865 G/C alleles in S. typhi patient was as fallowed; The T-ARMS PCR product of these was divided into four bands: 844 bp represents the nested outer PCR that served as a DNA template for the inner PCR of of 369 bp and 245 bp for allele G and 161 bp for allele C. The wild type of genotype GG had only three bands 844, 369 and 245 bp. The hetero-genotype GC had four bands of 844, 369, 245 and 161 bp. The rs3731865 SNP (CC), which was the mutant gene, had three bands 844, 369 and 161 bp. The results of T-ARMS- PCR for the patient and control samples are shown in figures (3) and (4).

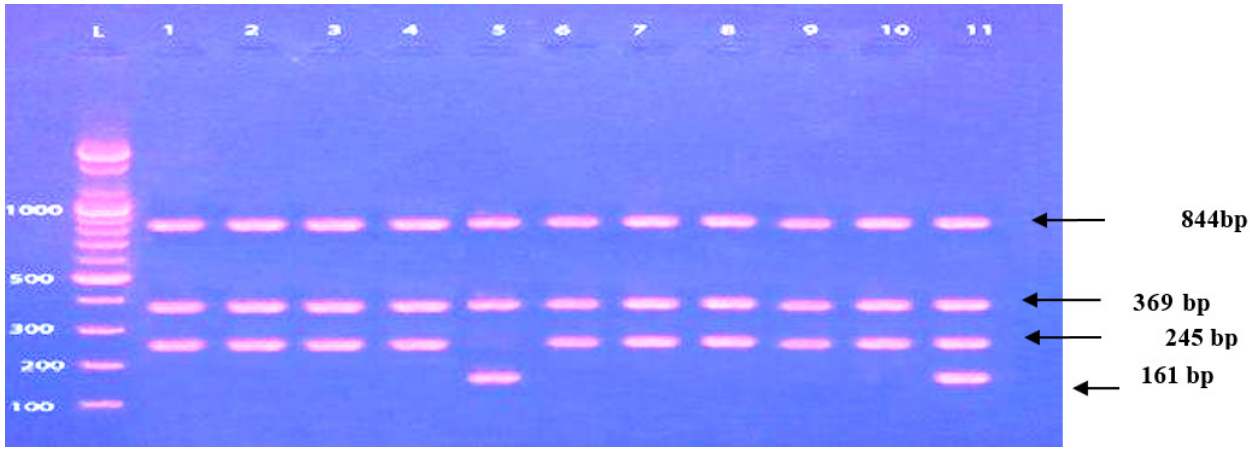

Figure 3 : T-ARMS-PCR bands of the human gene NRAMP1 rs3731865 SNP of the group of patients. (2%) agarose, DNA dye is RedSafe, V: 5/cm2, Time: 45 minutes, L: DNA ladder.

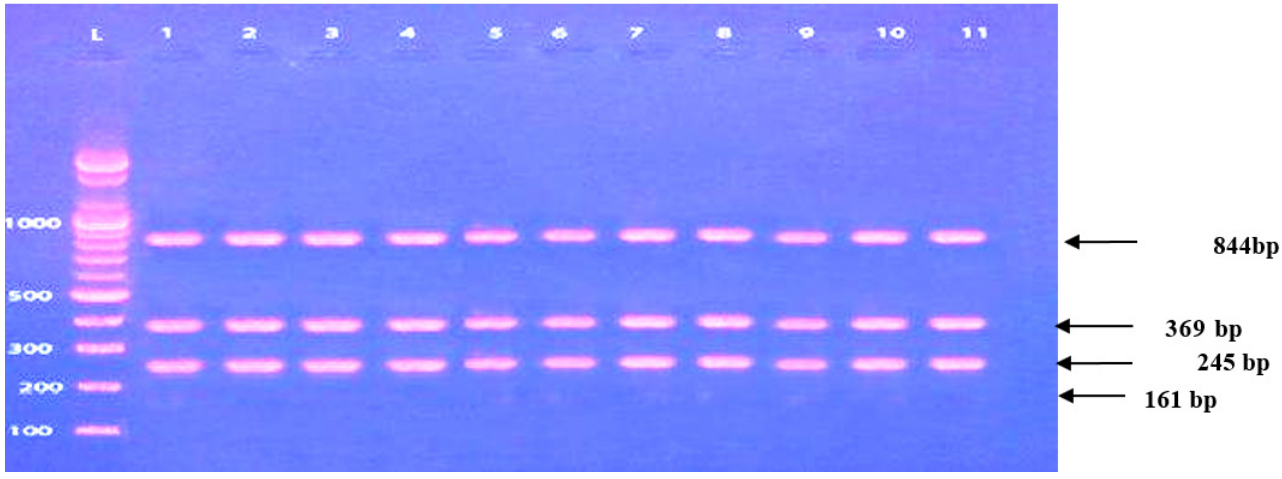

Figure4: T-ARMS-PCR bands of the human gene NRAMP1 rs3731865 SNP of the control group. The gel was 2% and the DNA dye is red Safe V: 5 /cm2, Time: 45 minutes. L: DNA ladder.

Genotype GG in the patient group was found in 50 out of 52 (96.15%) if compared to the control group 52 (100%),and the P value was 0.894 and the differences were not significant. The genotype GC, the heterozygote was 1 out of 52 in patient group represented 1.92% and one in the control group represented 1.92%, and P value was 0.844, also non-significant .The genotype CC, was repeated 1 out of 52 in the patient group represented 1.92% and the result was zero for the control group, P value was 0.844 with non-significant differences. The frequency of the allele was not significant for both alleles, G=0.97 and (C=0.03) for the patient group and G=1 and C=0.00 for the control group. From the above results, there were no significant differences between the patient and the control group, and also no association was concluded between the severity of typhoid fever and the SNP / rs3731865 SNP of the gene NRAMP1, as shown in Table (7).

Table 7: Genotype and allele frequency of the rs3731865 (469+14 G/C) - NRAMP1 gene.

Genotype\Allele NRAMP1 gene	Control Group	Patient Group	P value
GG	52 (100%)	50(96.15%)	0.894 NS
GC	0 (0.00%)	1 (1.92%)	0.844 NS
CC	0 (0.00%)	1 (1.92%)	0.844 NS
G	1	0.97	NS
C	0.00	0.03	NS

Discussion

Typhoid fever is related to the environment, behavior, situation, and sanitation. The disease could develop to a severe state in some patients. However, the molecular pathomechanisms of the severity of typhoid fever are not yet clear. The results of the distribution of S. typhi according to age groups revealed that the highest number of infections from typhoid fever was located in the age group (1-10) years, perhaps due to the lack of awareness of health among children and young people, in addition to the lack of hygiene conditions in schools and their health facilities, as well as the case for adolescents and youth, being the group most exposed to street food and unlicensed food sellers.

Previous studies agreed with current results such as the study by Britto et al. (4) ,when they suggested that typhoid and high disease burden among young children and adolescents, children were disproportionately affected by the increased incidence seen between 5 and 14 years of age. The Iraqi study carried out at Erbil Pediatric Hospital by Alchawishli et al .(11) was in agreement with the present study that showed that the age of the children was the most infected age category with S. typhi,also the study of Saha et al .(12) agreed with the current study in case typhoid fever was highly significant with children and the age of the youth. The results of the distribution of the patients with typhoid fever according to gender showed that the males were more than without significant differences, this findings was consistent with the study of Haque et al. (13) when they noticed that the frequency of S. typhi infections was less spread in male patients compared to female with no significant difference in the sample of children from Bangladesh ,also the study of Banik et al. (14) found that there was no significant difference between the gender groups when they studied the association between typhoid fever with age, sex ,blood phenotypes ABO and Rh among children in Dhaka, Bangladesh. Nucleotide Oligomerization Domain 2 plays an important role in the immune system, it recognizes bacterial molecules (peptidoglycans) and stimulates an immune reaction.

The NOD2 gene functions as an antibacterial factor that limits the survival of intracellular invasive bacteria and cytosolic pattern recognition receptors (PRRs) that recognize particular motifs found in the bacterial peptidoglycan. NOD2 are key bacteria-sensing receptors and are important in the early response against enteric pathogens, including S. typhi. Mutations in the NOD2 gene in humans have been associated with inflammatory disorders, such as typhoid fever. This may corroborate the significant correlation of the severity of typhoid fever with genetic mutations of the NOD2 gene, while the protective factor is allele G and the risk factor is allele C. These findings were considered statically significant. A previous study by Dwiyanti et al. (5) agreed with the current results, who found that there was an association between typhoid fever severity and mutations in the Indonesian sample of the NOD2 gene. Natural resistance-associated macrophage proteins, also called solute carrier family 11 member 1(SLC11A1) represent a family of proton/metal transporter proteins that are evolutionarily conserved across all species, from bacteria to humans. These facts suggest that NRAMP1 may inhibit intracellular pathogen replication by altering the phagolysosomal environment. Natural Resistance-Associated Macrophage Protein 1, which is encoded by the NRAMP-1, encodes a divalent cation transporter protein which is involved in the control of intraphagosomal microorganism replication and macrophage activation.

The NRAMP-1 can play an important role in the biocidal effects of macrophages.

Polymorphisms of the NRAMP-1 gene have been reported that are associated with susceptibility to diseases, such as tuberculosis, rheumatoid arthritis, sarcoidosis, and type I diabetes. Previous studies were in agreement with the current study such as Dunstan et al.(15) when they found that polymorphisms within the NRAMP1 gene were not expressed in correlation with typhoid fever when they studied typhoid fever and genetic polymorphisms in NRAMP1. Another study by Dwiyanti et al. (5) agreed with the current result, which found that there was no association between the severity of typhoid fever and polymorphism in the NRAMP1 gene in an Indonesian sample. Also, the study of Ma et al .(16) was

consistent with the present study that showed no association between typhoid fever severity and polymorphism in the NRAMP1 gene when they studied human genetic variation influences enteric fever progression in Malaysia. The change in the SNP from G to C will lead to a change in the type of amino acid produced and included in the protein structure of the NOD2 receptor, and it is discussed whether the new amino acid will change the structure of the receptor and its functional ability.

Conclusions

From the present results, it can be concluded that there was an association between the presence of the Nod2 (C) allele at the Exon 8 rs2066845 (2722 G/C) SNP locus with the severity of typhoid fever, while there was no association between the presence of NRAMP1 at the Intron 4 rs3731865 (469 + 14 G / C) SNP locus with the severity of typhoid fever.

Acknowledgments

The authors thank the staff of the Institute of Genetic Engineering and Biotechnology for post-graduate studies for their support. We also thank the referees for their thoughtful reading of the manuscript and their insightful remarks.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Bushra Jasim Mohammed and Yasir Azem Hameed carried out the experiment. Bushra Jasim Mohammed wrote the manuscript.

References:

1. Bhandari J, Thada P, Devos E. Typhoid Fever. Treasure Island (FL): Stat Pearls J. 2020;32(4):91-94.
2. Shubbar M, Zighair S. Incidence of Typhoid Fever and the Validity of the Widal Test in Some Districts in Iraq . Al-Anbar Medical Journal.2020;16(1):5-8.
3. Sadeq N, Jabar K . Knowledge, Attitude and Practice of Mothers towards Typhoid Fever Disease. Iraqi Journal of Medical Sciences . 2017;15(1): 71-77. 7.
4. Britto C, Pollard AJ, Voysey M, Blohmke CJ. An Appraisal of the Clinical Features of Pediatric Enteric Fever: Systematic Review and Meta-analysis of the Age-Stratified Disease Occurrence. Clin Infect Dis. 2017 Jun 1;64(11):1604-1611.
5. Dwiyanti R, Hatta M, Natzir R, Pratiwi S, Sabir M, Yasir Y. Association of typhoid fever severity with polymorphisms NOD2, VDR and NRAMP1 genes in endemic area, Indonesia. J Med Sci. 2017;17(3):133–139.
6. Hamed Y, Mohammed B, Tothli ,K.Conventional and Molecular Diagnosisof Salmonella typhi isolated from Iraqi Patients with Typhoid Fever.Indian Journal of Ecology. 2022; 49 (18): 493-499.
7. Sambrook J, Russell D.W. Molecular Cloning: A Laboratory Manual. 3rd Edition, Vol. 1, Cold Spring Harbor Laboratory Press, New York: 2001.32344p
8. Mohammed B. Association between TNF-α level and TNF-α gene polymorphisms in liver cirrhosis of Iraqi patients, BIOSCIENCE RESEARCH . 2018; 15(2): 1342-1349.
9. Mohammed B.A Search for the Effect of Gasoline Exposure and Viability, Apoptosis, Necrosis of Peripheral Blood Mononuclear Cells and Polymorphisms in Fas -FasL Genes in Blood of Gasoline Stations Iraqi Workers.Biomedicine J. 2017; 37 (3):375-381.
10. SAS. Statistical Analysis System, User's Guide. Statistical. Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. USA.2018.136p
11. Alchawishli K, Palpitany S, Smail B, Hassan S.Association Between Typhoid and Paratyphoid Fever with ABO Blood Group among Patients Attending Raparin Pediatric Hospital in Erbil. Journal. Diyala Journal of Medicine. 2019; 17(3): 12-15.
12. Saha S, Tanmoy AM, Andrews JR, Sajib MSI, Yu AT, Baker S .et al. Evaluating PCR-Based Detection of Salmonella Typhi and Paratyphi A in the Environment as an Enteric Fever Surveillance Tool. Am J Trop Med Hyg. 2019 Jan;100(1):43-46.
13. Haque M, Roy D, Rahman M. Risk Factors of Enteric Fever in Children: A Study in A Tertiary Care Hospital, Nilphamari, Bangladesh. IOSR Journal of Dental and Medical Sciences. 2019 ; 18(2): 8- 12.
14. Banik A ,Nurul Kabir A, Alam J. An association between typhoid fever and Age, Sex and Blood Phenotypes ABO and Rh among Children – a Study ina Tertiary Care Hospital, Dhaka, Bangladesh, IOSR-JDMS.2018; 17(9) :63-69.
15. Dunstan SJ, Ho VA, Duc CM, Lanh MN, Phuong CX, Luxemburger C, Wain J, Dudbridge F, Peacock CS, House D, Parry C, Hien TT, Dougan G, Farrar J, Blackwell JM. Typhoid fever and genetic polymorphisms at the natural resistance-associated macrophage protein 1. J Infect Dis. 2001 Apr 1;183(7):1156-60
16. Ma PY, Tan JE, Hee EW, Yong DWX, Heng YS, Low WX.et al. Human Genetic Variation Influences Enteric Fever Progression. Cells. 2021 Feb 6;10(2):345.