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ORIGINAL ARTICLE

# Polymorphism of Toll-Like Receptors Type 2 Gene in Neonatal Sepsis in NICU of Zagazig University Children Hospital

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**ABSTRACT**

**Background:** Neonatal sepsis is a major cause of morbidity and mortality among newborn infants. Sepsis is a complex syndrome that is initiated by infection and is characterized by a systemic inflammatory response. The inflammatory cascade is a complicated process that involves humoral and cellular responses, complement, and cytokine cascades. Recently, genetic variation in crucial genes in the inflammatory response and coagulation pathways has been the focus for innovative research aiming to elucidate the mechanisms behind sepsis, in particular, identification of genetic variation in TLRs. This study aimed to investigate the association between the prescence of certain genetic polymorphisms of toll-like receptor type 2 genotype and risk, severity, prognosis and outcome of gram-positive bacterial sepsis in neonates in NICU at Zagazig University Childern Hospital. **Methods:** This is a case–control study , 72 subjects were included in the present study divided into two groups; group I : 36 healthy newborns as control group, group II: 36 bacteremic cases with early or late onset neonatal sepsis. For Detection of TLR2 [Arg753Gln] polymorphisms, blood specimens were collected in vacutainer tubes containing EDTA then DNA extraction was performed and determination of the TLR 2 gene polymorphisms was accomplished by PCR followed by RFLP. **Results:** TLR2 [Arg753Gln] polymorphism was detected in 3 subjects [8.3%] in the sepsis group, while it was not detected in the control group. **Conclusions:** There is no significant association between TLR2 Arg753Glu polymorphism and severity or prognosis of gram-positive bacterial sepsis in neonates.

**Key words:** Sepsis, Toll-like receptor 2, Single nucleotide polymorphism, Genetic susceptibility, Newborn infant

## INTRODUCTION

Despite significant advances in supportive care and in understanding the molecular basis of sepsis and its associated immune response, sepsis remains a major cause of neonatal morbidity and mortality, especially among very low-birth- weight [VLBW] infants. [1] .

Neonatal sepsis is an invasive infection, usually bacterial, occurring during the neonatal period. Signs are multiple, nonspecific, and include diminished spontaneous activity, less vigorous suckling, apnea, bradycardia, temperature instability, respiratory distress, vomiting, diarrhea, abdominal distention, jitteriness, seizures, and jaundice. Diagnosis is clinical and based on culture results. [2] .

The susceptibility of neonates to sepsis includes maternal and environmental factors, and host's immune, inflammatory, and coagulation responses, resulting in important interindividual differences with significant clinical implications. [3] .

There is recently a great deal of interest in linking genetic and phenotypic variation in the form of severity of, and susceptibility to, common multifactorial diseases such as sepsis. [4] .

The genetic background has recently been recognized as an important element in the host response to infection, contributing to the variability in the clinical outcome of critically ill neonates. [5] .

Polymorphisms in genes coding for proteins involved in the recognition of bacterial pathogens [Toll-like receptor 2 , CD14] and the response to bacterial pathogens [tumor necrosis factor [TNF]-α, inter- leukin [IL]-1, interleukin-1 receptor antagonist [IL-1RA], IL-6, IL-10] can influence the amount or function of the protein produced in response to bacterial stimuli. [6] .

Combining population genomics, bio-informatics, and clinical data may lead to the discovery of the variations that exist among the genes involved in determining susceptibility to sepsis in neonates. [7] .

## METHODS

A case–control study was carried out in the Neonatal Intensive Care Unit [NICU] at Zagazig University Children Hospital, Zagazig University, Egypt, in the period spanning from May 2018 to November 2018.

A total of 72 neonates were enrolled in the study. Thirty six term neonates with culture proven gram positive sepsis who were diagnosed within the first month after birth were enrolled as cases. Thirty six healthy sex-matched and age-matched neonates were enrolled as controls. Control volunteers were recruited from our governmental child health care centers that regularly monitor the growth and development of the infant from the first day of life. Neonatal cases who had clinically suspected sepsis with negative blood cultures, sepsis due to gram –ve bacteria and those born prematurely were excluded from the study.

Written informed consent was obtained from all participants 'patients' and the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The work has been carried out in accordance with The Code of Ethics of the World Medical Association [Declaration of Helsinki] for studies involving humans.

Both cases and controls were subjected to thorough history taking including gestational age, birth weight, the order in family and mode of delivery were carried out. Detailed clinical examination was performed for both case and control groups. Neonates with sepsis were subjected to routine laboratory investigations and sepsis screen [complete blood count [CBC] with differential counts, C-reactive protein, procalcitonin, and blood culture].

Cases were strictly followed-up during the period of NICU stay. Clinical course, the timing of control of the infection by sepsis markers, recommended antibiotics, duration of hospital stay, need for positive airway pressure and disease outcome, either discharged or expired, were recorded. Both cases and controls, were genotyped for TLR2 gene [Arg753Gln] polymorphisms.

**Lab Methods**

A 2-ml sample of peripheral venous blood was collected from all studied groups into ethylenediaminetetra-acetic acid tubes which were stored in a central freezer at −20°C and transported once a week to a central laboratory for processing. Genomic DNA was extracted from the peripheral venous blood with a purification kit, **InnuPREP blood DNA Mini Kit [Analytik Jena AG,Jena, Germany]**.

The identification of the gene polymorphism was carried out using polymerase chain reaction [PCR]. PCR primers **[operon, Invitrogeon],** were designed to allow a distinction of wild-type and mutant TLR2 alleles based on the prescence of restriction enzyme recognition sites. For TLR2 gene, the forward primer was

753TLR2F, 5`-TTGACTCCATTGAAAAGAGC-3`; and the

reverse primer was 753TLR2R, 5`TAAATATGGGAACCTAGAC-3`.

Primers were purchased as lypophilized agents from [operon, Invitrogen],reconstituted with sterile deionized water to make stock.Then,dilutions were made from this stock to reach 10 µM concentration. Both outer and inner PCR reactions were performed using **2X PCR Master mix Solution [iTaqTM, iNtRON, Korea],** with a concentration of 1 µM of the respective sense and antisctrense primer, and 5 µM of genomic DNA and water to complete volume up to 25 µl.

PCR was performed using the recommended thermal cycling conditions.PCR reactions were run at 95ºC for 4 min. followed by 30 cycles at 95ºC for 30 s, 55º C for 30 s, 72ºC for 30 s, and afinal cycle at 72ºC FOR 5 min.

Electrophoresis through agarose gel is the standard method to separate , identify and purify DNA fragments.The location of DNA within the gel can be determined directly by staining with low concentration of the fluorescent intercalating dye,ethidium bromide.

For identifying of Arg753Glu polymorphism, each PCR product was digested by Pst1 restriction endonuclease enzyme. 10 µl of the PCR reaction were digested with 1 U Pst1 restriction endonuclease in 1 x NEBbuffer 1 at 37ºC overnight.The final volumes of the reactions were 20 ml.

The digestion of the TLR2 gene PCR product resulted in a single band of 300 bp when arginine was present at position 753 in the gene and two bands [190 and 110 bp] when glutamine was at position 753.Regarding the TLR2 gene, a loss of function single nucleotide polymorphism [SNP] within the TLR2 gene results in substitution of arginine to glutamine at position 753 [*Arg753Gln]*.

Genotypes were assigned and confirmed by independent laboratory investigators who were unaware of the patients' phenotypes. Patients were then divided into two groups according to the assigned polymorphism: Arg753Gln heterozygous carriers [TLR2 group], and wild type carriers [wild type group].

**Statistical Analysis**

The obtained data were collected and statistically analyzed using IBM SPSS Statistics,version 24 [IBM;Armonk, New York, USA]. Continuous variables were presented as the mean±SD if normally distributed or median[range] if not normally distributed.Normality checked by Kolmogorov-Smirnov test.Homogenecity of variance was checked by Levene's test.Categorical data were presented by the count and percentage. Fisher's Exact test for [2X2] [RXC] is an alternative to chi-squared test to discover if there is a relationship between two categorical variables when the expected cell count is less than five. Independent-samples t-test is used to determine if a difference exists between the means of two independent groups on a continuous dependent variable.Mann-Whitney U test [nonparametric alternative to independent-samples t-test]. *P*-value <.05 indicates a significant difference, *P*-value ≤.01 indicates a highly significant difference, *P*-value≤.001 indicates a very highly significant difference while *P*-value ≥.05 indicates a non-significant difference.

## RESULTS

The current study enrolled 36 neonates with sepsis [18 males and 18 females] who were admitted to NICU at Zagazig University children's Hospital, in addition to 36 apparently healthy neonates as a control group **[table 1]**. There was no statistically significant difference between the studied groups regarding gestational age. The mean of gestational age was [38.22±1.05] and [38.44±0.97] for case and control group respectively **[table 1]**. There was no statistically significant difference regarding mode of delivery in both groups, CS is the commonest in both groups [61.1%] and [58.8%] in case and control group respectively **[table 1]**. The most common prenatal risk factors included maternal PROM [63.8%] followed by diabetes [11.2%], while natal risk factors were positive in only five [14%] patients **[table 2]**. The most common cause of admission in NICU of the studied

cases was respiratory manifestations[15 out of 36]. Also, The largest percentage of studied cases had no congenital anomalies[27 out of 36] **[table 3].**

In the current study 24 subjects had EOS and 12 subjects had LOS..The most common organisms detected in this study was coagulase negative staph [55.6%] followed by staphylococcus aureus [33.3%], listeria monocytogens [6.7%], streptococcus aglactacia [2.8%] and Enterococcus faecalis [2.8%] **[table 4]**. In the current study,only three cases out of 36 cases had heterogeneous [GA] TLR2 Arg753Glu polymorphism.Also there was no significant difference in allelic frequencies of the SNPs between the two groups [P>0.05] **[table 5].** The digestion of the TLR2 gene PCR product resulted in a single band of 300 bp when arginine was present at position 753 in the gene and two bands [190 and 110 bp] when glutamine was at position 753 **Figure [1]**. Regarding the TLR2 gene, a loss of function single nucleotide polymorphism [SNP] within the TLR2 gene results in substitution of arginine to glutamine at position 753 [*Arg753Gln]*.

**Table 1.** Comparison between the studied groups regarding demographic characteristics:

| **Characteristics** | **Controls** | **Cases** | **Test of significance** | ***P*-value** |
|---|---|---|---|---|
| | ***n*=36** | ***n*=36** | | |
| Gestational age [weeks] | | | Mann-Whitney *U* test=710.5 | 0.353 |
| mean±SD | 38.44±0.97 | 38.22±1.05 | | |
| median[range] | 38.5[37-40] | 38[37-40] | | |
| Sex, *n*[%] | | | $\chi^2$=2.057 | 0.151 |
| males | 12[33.3] | 18[50] | | |
| females | 24[66.7] | 18[50] | | |
| Mode of delivery, *n*[%] | | | Fisher's exact test | 0.475 |
| Normal vaginal delivery | 17[47.2] | 14[38.9] | | |
| Cesarean section | 19[52.8] | 22[61.1] | | |
| Consanguinity, *n*[%] | | | Fisher's exact test | >.99 |
| Negative | 29[81] | 30[83] | | |
| Positive | 7[19] | 6[17] | | |
| Sibling death, *n*[%] | | | Fisher's exact test | .11 |
| Negative | 36[100] | 32[89] | | |
| Positive | 0[0] | 4[11] | | |

**Table 2.** Distribution of studied case group according to risk factors:

| Risk factors | *Total number=36* |
|---|---|
| Prenatal risk factors, *n*[%] | |
| No risk | 5[13.8] |
| PROM | 23[63.8] |
| Diabetes | 4[11.2] |
| UTI | 3[8.5] |
| chorioamnionitis | 1[3] |
| Natal risk factors, *n*[%] | |
| No risk | 31[86] |
| Breech delivery | 1[3] |
| Cord prolapse | 1[3] |
| Accidental hemorrhage | 2[6] |
| True knot of cord | 1[3] |
| Abbreviation; PROM, premature rupture of membrane; UTI, urinary tract infection | |

**Table 3.** Distribution of studied case group according to clinical presentation:

| Variables | *Total number=36* |
|---|---|
| Main presentation, *n*[%] | |
| #Respiratory manifestations | 15[41.7] |
| $Cardiac illness | 11[30.6] |
| ‖ Neurologic illness | 7[19.3] |
| ‡Abdominal illness | 2[5.6] |
| Temprature instability | 1[2.8] |
| Associated congenital anomalies, *n*[%] | |
| No | 27[75] |
| Cardiac anomalies | 7[19.4] |
| Hydrocephalus | 1[2.8] |
| Cleft palate | 1[2.8] |
| # includes respiratory distress, apnea<br>$ includes tachycardia, bradycardia, poor perfusion, hypotension<br>‖ includes lethargy, poor activity, poor tone, irritability, seizures<br>‡ includes vomiting, abdominal distension, feeding intolerance | |

**Table 4.** Distribution of studied case group according to severity of sepsis:

| | N=36 | 100 % |
|---|---|---|
| Severity of sepsis: | | |
| Sepsis | 14 | 38.9 |
| Severe sepsis | 11 | 30.6 |
| Septic shock | 6 | 16.7 |
| MOF [multi organ failure] | 5 | 13.8 |

**Table 5.** Types of organisms detected in blood cultures of the studied case group:

| | N=36 | 100 % |
|---|---|---|
| **Types of organisms:** | | |
| **Staphylococcus aureus** | **12** | **33.3** |
| **Coagulase negative staph** | **20** | **55.6** |
| **Listeria monocytogen** | **2** | **6.7** |
| **Streptococcus aglactacia** | **1** | **2.8** |
| **Enterococcus faecalis** | **1** | **2.8** |

**Table 6.** Comparison between Toll-like receptors type 2 Polymorphism genotype and allele distribution among the studied groups:

| | Case | Control group | Test of significance | *P*-value |
|---|---|---|---|---|
| | **N=36[%]** | **N=36 [%]** | | |
| Polymorphism :<br>Homozygous wild[G/G]<br>Homozygous mutant [A/A]<br>Heterozygous mutant[G/A] | <br>33 [91.7]<br>0 [0]<br>3 [8.3] | <br>36 [100]<br>0 [0]<br>0 [0] | <br>Fisher's exact test | 0.209 |
| Alleles:<br>G allele<br>A allele | <br>69 [95.8]<br>3 [4.2] | <br>72 [100]<br>0 [0] | <br>Fisher's exact test | 0.118 |

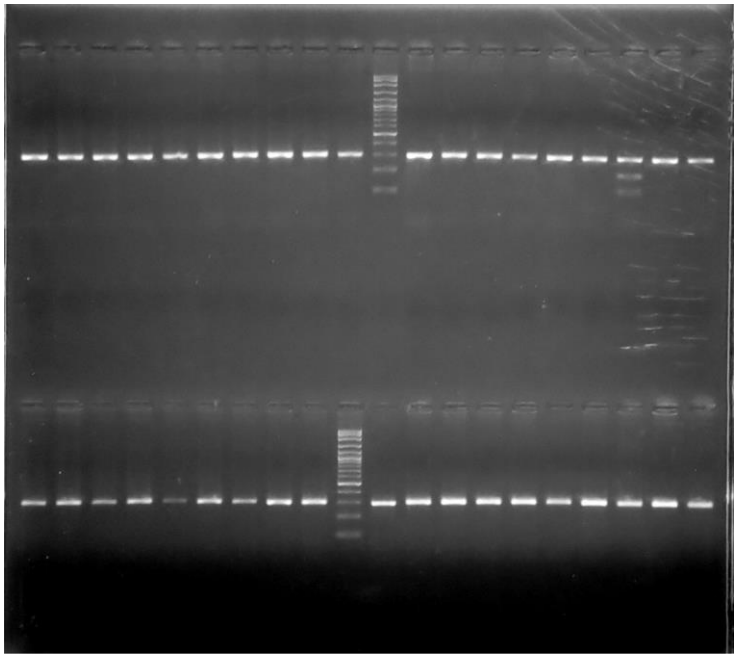

**Figure 1.** Gel after restriction by Pst1 restriction endonuclease enzyme

## DISCUSSION

Neonatal infections, including bacterial infection, are considered a major health care problem worldwide, with an annual mortality of more than one million deaths. The susceptibility of neonates to sepsis includes maternal and environmental factors, and host's immune, inflammatory, and coagulation responses, resulting in important interindividual differences with significant clinical implications. [8] .; [9] .

The increased frequency and severity of

systemic neonatal infections can be attributed to the immaturity of the defense immune mechanisms [especially in preterm] and the interaction between the pathogen and the host. [10] .

Recently, advanced researches are directed towards successful modulation of the neonatal immune system in order to reduce the incidence of sepsis, sepsis-related morbidity and mortality.

The genetic background has recently been recognized as an important element in the host response to infection, contributing to the variability in the clinical outcome of critically ill neonates. [11] .

Innate immune system depends on pattern recognition receptors [PRRs] to detect conserved structures of pathogens [like bacteria, virus, fungus and protozoa], which are called pathogen-associated molecular patterns [PAMPs] [12] .

TLRs constitute a family of transmembrane proteins that recognize PAMPs. They play a fundamental role in innate immune responses. [13] .

This study represents a case-control study to evaluate TLR2 Arg753Glu gene polymorphism in relation to the severity, prognosis, and outcome of gram-positive bacterial sepsis in neonates in an attempt to elucidate the role of these polymorphisms in the pathogenesis or susceptibility to neonatal sepsis.

As identification of genetic variations in the TLRs may lead to the development of TLR based gene therapy as well as the development of certain molecules to target these TLRs responsible for disease pathogenesis [14] .

The current study enrolled 36 neonates with sepsis [18 males and 18 females] who were admitted to NICU at Zagazig University children's Hospital, in addition to 36 apparently healthy neonates as a control group. This is matched with Susanna Esposito et al. [2014] study who included 50 % of cases with culture proven sepsis as males and 50% as females.

In this study, the mean gestational age of the studied neonates was 38 weeks and ranged from 37 to 40 weeks.

This was in contrary with [12] .who studied the role of polymorphic variants of TLRs on septic preterm infants with a birth weight ≤ 1500 gram [VLBW].

Concerning the incidence of early [≤ 3d] and late [>3d] onset sepsis, The largest percentage [66.7] of the studied cases had early onset sepsis. This was consistent with Betty Chacko et al. who found that The incidence of EOS was 20.7 per 1000 live births and it constituted 55.4% of overall sepsis.

In contrary, [15] .demonstrated that early onset sepsis was detected in [3.4%] while late onset sepsis occurred in [19.8%] of infants in their study about Toll-like receptor genetic variants and it's association with sepsis in VLBW infants.

Regarding to the mode of delivery and the incidence of sepsis, the present study revealed higher incidence of sepsis in neonates born via cesarean section than in those born via vaginal delivery. An Egyptian study by [16] .showed similar findings.

study who found that premature rupture of membranes, and presence of clinical chorioamnionitis were significantly associated with sepsis.Also found that neonates with biochemical evidence of sepsis showed a statistically significant incidence of PROM lasting 18 hours or more, labour room stay more than 9 hours and 3 or more vaginal examinations.

In the matter of the clinical presentation, respiratory distress was the most common presenting symptom in the current study [41.7%] of cases, followed by cardiovascular manifestations [30.6%], neurologic manifestations [19.3%] and gastrointestinal manifestations [5.6%].

Similar results were obtained in previous studies about sepsis in term and preterm neonates in different countries. One Egyptian study by [Shehab El-Din et al., 2015] and another study conducted by [17] .in North America.

Concerning the isolated organisms from cultures, Coagulase negative staph [55.6% of cases] was the most prevalent gram-positive bacteria in the current study followed by staphylococcus aureus [33.3%], and Listeria

monocytogen [6.7%].

These findings were in agreement with the results reported by [13] . who reported that Coagulase-negative Staphylococcus [CONS] [50%] was the most commonly isolated organism from blood cultures.

The current study revealed lack of association between the analyzed TLR2 Arg753Glu polymorphism and severity of neonatal sepsis.

Only three cases out of 36 showed heterogenous allelic variation [GA] in the TLR2 Arg753Glu polymorphism. But no polymorphism was noticed in the control group.

These results were consistent with [18] .study who found that the genotype on TLR2 was G/G and no mutation was found in both groups,Also there was no significant difference in allelic frequencies of the SNPs between the two groups [P>0.05].

Also revealed lack of association between TLR2 Arg753Glu polymorphism and susceptibility to sepsis, The genotype frequencies for the disease subgroups were identical with 106 G/G genotypes [95%] and 6 G/A genotypes [5%] in the community acquired-disease group and with 294 G/G genotypes [95%] and 14 G/A genotypes [5%] in the hospital-acquired-disease group.

Also [19] .studied 299 premature babies in Milan, Italy and found no association between TLR2 gene rs3804099 polymorphism and sepsis.

Also [13] .study on 535 prematue infants at lowa Children'S Hospital found that TLR2 gene rs3804099 polymorphism increase susceptibility to Gram positive septicemia in premature infants.

In the current study, three cases out of 36 cases had heterogeneous [GA] TLR2 Arg753Glu polymorphism. Similar results were reported by [20] .in their case-control study to investigate TLR2 Arg753Glu gene polymorphisms among neonatal sepsis in Egypt.

## CONCLUSIONS

There is no significant association between TLR2 Arg753Glu polymorphism and severity or prognosis of gram-positive bacterial sepsis in neonates.

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