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ORIGINAL ARTICLE

TOLL Like Receptor Type Four Gene Polymorphism in Neonatal Sepsis in Zagazig University Children's Hospital

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ABSTRACT

Introduction: The immune response constitutes the first-line defense against microbial infections. Toll like receptors (TLRs) play a fundamental role in neonatal innate immunity, and one of them, toll like receptor 4 (*TLR4)* is considered a major pattern recognition receptor (PRR) in recognizing lipopolysaccharide (LPS) of gram-negative bacteria. Single nucleotide polymorphisms (SNPs) among various TLR genes have been identified and maybe related to susceptibility/resistance to certain infections and other inflammatory diseases. **Objective:** The purpose of this study was to find a relation between sepsis-related outcomes in neonates and TLR4 *Asp299Gly* gene polymorphism. **Method:** we carried out a case control study on 72 neonates. 36 neonates with culture proven gram-negative sepsis and 36 neonates as controls. Both groups were genotyped for TLR4 *Asp299Gly* gene polymorphism, by polymerase chain reaction (PCR), and restriction fragment length polymorphism analysis (RFLP) by using BccI enzyme. **Results:** No statistically significant association between TLR4 *Asp299Gly* gene polymorphism and severity of neonatal sepsis (*P*>.99).**Conclusion:** TLR4 *Asp299Gly* gene polymorphism is not associated with increased severity of gram-negative bacterial sepsis in neonates.

Keywords: Gram-negative sepsis; TLR4 *Asp299Gly* polymorphism; genotyping; neonatal intensive care unit (NICU).

INTRODUCTION

Despite improved neonatal care over the past decades, sepsis remains a major cause of morbidity and mortality in neonates admitted to the neonatal intensive care unit [1]**.**

Gram-negative bacteria are associated with more severe clinical manifestations, higher mortality, and increased risk of neonatal morbidity, potentially posing a significant disease burden [2].

Neonates must depend on their innate immunity for protection against invading organisms , given the limited exposure to antigens in utero and the relative immaturity of the adaptive immune system**[3].**

Susceptibility factors for newborn to sepsis include maternal and environmental exposures, immune status, and inflammatory responses. These interacting factors can be modified by variation among individuals in gene expression that may have significant clinical implications**[4].**

The innate immune response constitutes the first-line defense against microbial infections. It depends on pattern recognition receptors (PRRs) to alert the host to the presence of

invading pathogens (like bacteria, virus, fungus and protozoa), which are called pathogen-associated molecular patterns (PAMPs)**[5].**

Toll like receptors (TLRs) play a fundamental role in neonatal innate immunity, and one of them, toll like receptor 4 (TLR4) is considered a major pattern recognition receptor (PRR) in recognizing lipopolysaccharide (LPS) of Gram-negative bacteria **[6].**

Single nucleotide polymorphisms (SNPs) among various TLR genes have been identified and maybe related to susceptibility/resistance to certain infections. This 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 [7].

METHODS

Patients and study design:

We conducted this case control study in Neonatal Intensive Care Unit in Zagazig University Children's in the period from June 2018 to December 2018.

Written informed consent was obtained from all participants' parents 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.

We enlisted 72 neonates (36 as cases and 36 as healthy controls) in this study. The included neonatal cases were diagnosed with either early or late onset sepsis proven by gram-negative cultures. The mean gestational age was 38±1.2 weeks. Sex distribution included 37 males and 35 females, including both cases and controls.

Neonatal cases who had clinically suspected sepsis with negative cultures, or had sepsis from gram-positive organisms and those born prematurely were excluded from the study.

All newborns included in the study were subjected to thorough history taking including: prenatal history (premature labor, maternal peripartum infection, premature rupture of membrane, amniotic fluid problem, maternal bleeding, history of maternal disease), natal history (cord around neck, resuscitation at birth, invasive procedure and obstructed labor), postnatal history (gestational age, sex, birth weight, APGAR score, signs of neonatal sepsis).

Careful clinical examination was performed including: vital signs, general and local examination, signs of sepsis (temperature instability, respiratory compromise (respiratory distress, apnea, cyanosis), cardiovascular compromise (bradycardia, tachycardia, poor perfusion, hypotension), neurologic changes (hypotonia, lethargy, seizures), gastrointestinal compromise (feeding intolerance).

Cases were classified according to the severity of clinical presentation into the following:

1. Sepsis with manifestation of systemic inflammatory response and proven infection
2. Severe sepsis with manifestations of sepsis, organ dysfunction.
3. Septic shock
4. Multiorgan failure

all neonates including "control group + sepsis group" were subjected to routine laboratory investigations and sepsis screen (Complete Blood Count (CBC) with differential counts, C-reactive protein, procalcitonin, and cultures).

Genotyping for TLR4 *Asp299Gly* polymorphism was performed on the studied neonates through the following steps:

- Genomic DNA extraction was carried out from whole blood using the commercially available **InnuPREP Blood DNA Mini Kit (**Analytik Jena AG, Jena, Germany) as described in the user manual.
- PCR amplification of the TLR4 gene using specific primers. PCR reactions were performed using **2X PCR Master mix Solution** (iTaqTM, iNtRON, Korea) on Veriti 96 Well Thermal Cycler (Applied Biosystems, USA). We used the following primers:

the forward primer: 299TLR4F,5'GGCTTCATAAGCTGACTTT-3';

the reverse primer: 299TLR4R,5'-CATCCGAAATTATAAGAAAAG- 3'

- Restriction of the amplified product using BccI enzyme to differentiate between the wild type and mutated gene. We yielded two bands (222 and 94 bp) when asparagine was present at position 299 in the gene, and three bands (113, 109, 94 bp) when glycine was at position 299. Regarding the TLR4 gene, a loss of function SNP within the TLR4 gene results in substitution of aspartic acid to glycine at position 299 (*Asp299Gly).*

Statistical analysis:

Continuous variables were presented as the mean±SD if normally distributed or median(range) if not normally distributed. Normality was checked by Kolmogorov-Smirnov test. Homogeneity of variance was checked by Levene's test.

Categorical variables were presented by the count (percentage).

- **Fisher's Exact Test:** for (2X2) (RXC). It 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).
- **Threshold for significance**: *P*-value <.05 indicates a significant difference, *P*≤.01 indicates a highly significant difference, *P*≤.001 indicates a very highly significant difference while, *P*≥.05 indicates a non-significant difference.
- **Two-sided tests were used throughout.**

All statistical analyses were performed using IBM SPSS Statistics, version 24 (IBM; Armonk, New York, USA).

RESULTS

This study included 72 neonates. 36 neonates diagnosed with sepsis and 36 neonates as controls.

Regarding to the characteristics of the studied neonates, they were similar in neonates with sepsis and healthy neonates (*P*>.05) except for the type of feeding where a highly statistically significant difference was found (*P*<.001), as shown in Table 1.

In regards to the risk factors of the studied neonates, prenatal risk factors were positive in (21 out of 36, 58%) in the neonates with sepsis while natal risk factors were positive in only five (4%) patients, as shown in Table 2.

Regarding to the clinical presentation and findings of the studied neonates, respiratory distress was the commonest presenting symptoms (18 cases out of 36, 50%). Congenital anomalies were not detected in most neonates (22 out of 36; 61%), as shown in Table 3.

Concerning the vital signs of the studied neonates, neonates with sepsis had significant increases in heart rate (*P*=.008) and respiratory rate (*P*=.001) but a significant decrease in diastolic blood pressure (*P*<.001) compared to healthy neonates. No significant differences in systolic blood pressure (*P*=.18) and temperature (*P*=.065) were found between both groups.

In regards to the laboratory findings of the studied neonates, neonates with sepsis had very highly significant increases in levels of C-reactive protein (*P*<.001) and procalcitonin (*P*<.001) but very highly significant decreases in levels of hemoglobin (*P*<.001), platelets count (*P*<.001) and lymphocytic count (*P*<.001) compared to healthy neonates. No significant differences in total leukocytes count (*P*>.99) and granulocytic count (*P*=.14) were found between both groups, as shown in Table 4.

Regarding to the isolated organisms from cultures, *Escherichia coli* (44% of cases) and *klebsiella pneumoniae* (31% of cases) were the most prevalent gram-negative bacteria affected the studied neonates, as shown in Table 5.

In the matter of detecting TLR4 *Asp299Gly* polymorphism of the studied neonates, only two cases out of 36 showed heterozygous polymorphism of TLR4 gene with substitution

of A with G at position 896 in the coding gene *(Asp299Gly)*. No statistically significant association between TLR4 *Asp299Gly* polymorphism and severity of sepsis (*P*>.99).

Regarding the severity of sepsis among the studied neonates and TLR4 *Asp299Gly* polymorphism, six (17%) of cases had a clinical picture compatible with sepsis, seven (19%) with severe sepsis, four (11%) with septic shock, and 19 (53%) with multiorgan dysfunction syndrome (MODS), death was observed in 24 (66 %) patient, as shown in Table 6.

In terms of neonatal sepsis outcome, mortality was observed in 24 (66 %) of cases.

Table 1. Baseline characteristics of the studied neonates

Characteristics	**Controls**	**Cases**	**Test of significance**	***P*-value**
	***n*=36**	***n*=36**		
Gestational age (weeks)			Mann-Whitney *U* test=710.5	.47
mean±SD	38.2±1.4	38±1.2		
median(range)	38(35-40)	38(35-40)		
Sex, *n*(%)			χ^2=2.8	.25
Boys	17(47)	20(56)		
Girls	19(53)	16(44)		
Mode of delivery, *n*(%)			Fisher's exact test	.79
Normal vaginal delivery	10(28)	8(22)		
Cesarean section	26(72)	28(78)		
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)		
Onset of sepsis, *n*(%)			------	------
Early	------	18(50)		
Late	------	18(50)		
Feeding type, *n*(%)			χ^2=22.8	**<.001**
Exclusive breastfeeding	13(36)	2(5)		
Bottle feeding	5(14)	24(67)		
Mixed	18(50)	10(28)		
χ^2:Chi-squared test				

Table 2. Risk factors of the studied neonates with sepsis

Risk factors	***Total number=36***
Prenatal risk factors, *n*(%)	
No risk	15(42)
PROM	7(19)
Diabetes	2(6)
Hypertensive disorders of pregnancy	1(3)
UTI	1(3)
Anemia	1(3)
Placenta previa	1(3)
Polyhydramnios	2(6)
Previous abortion	6(17)
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. Clinical presentation of the studied neonates with sepsis

Vital signs	**Controls**	**Cases**	**Test of significance**	***P*-value**
	n=36	*n*=36		
Heart rate (bpm)			Mann-Whitney *U*=411.5	**.008**
mean±SD	126±35	143±35		
median(range)	127(121-141)	140(67-200)		
Systolic blood pressure (mmHg)			Mann-Whitney *U*=529	.18
mean±SD	72±8	67±21		
median(range)	70(62-90)	68(33-120)		
Diastolic blood pressure (mmHg)			Independent samples t-test=3.7	**<.001**
mean±SD	48±8	37±14		
median(range)	48(32-65)	38(12-70)		
‡Temperature (C^0)			Mann-Whitney *U*=485.5	.065
mean±SD	36.9±0.2	37.3±0.8		
median(range)	37(36.6-37.4)	37(35.5-39)		
Respiratory rate (cycle/min)			Independent samples t-test=3.6	**.001**
mean±SD	48±9	62±21		
median(range)	49(31-60)	65(20-97)		
Main presentation				
#Respiratory distress	------	18(50)		
$Cardiac illness	------	8(22)		
ǁ Neurologic illness	------	4(11)		
‡Abdominal illness	------	6(17)		

Table 4. Laboratory findings of the studied neonates

Laboratory findings	**Controls**	**Cases**	**Test of significance**	***P*-value**
	n=36	*n*=36		
C-reactive protein (mg/L)			Independent samples t-test=8	**<.001**
mean±SD	2.8±1.2	120±87.5		
median(range)	2.8(1.1-51)	99.5(10-353)		
Procalcitonin (ng/ml)			Mann-Whitney *U* test=0	**<.001**
mean±SD	0.3±0.1	36±33.7		
median(range)	0.3(0.1-0.5)	21(4-109)		
HB (gm/dL)			Independent samples t-test=18.1	**<.001**
mean±SD	18.3±0.5	11±2.3		
median(range)	18.4(17-19)	10.5(7-16.5)		
Platelets count (10^3/uL)			Independent samples t-test=8.5	**<.001**
mean±SD	265.5±54.9	107.2±97.3		
median(range)	269(154-346)	78(4-444)		
Total leukocytes count (10^3/uL)			Mann-Whitney *U* test=648	>.99
mean±SD	13.8±0.6	15.8±10.8		
median(range)	13.6(13.1-14.9)	13.9(2.3-37)		
Granulocytes (10^3/uL)			Mann-Whitney *U* test=517.5	.14
mean±SD	8.4±0.8	10.1±8.9		
median(range)	8.5(7.1-10)	6.6(0.4-29)		
Lymphocytes (10^3/uL)			Mann-Whitney *U* test=38.5	**<.001**
mean±SD	11.5±3.1	4.3±2.4		
median(range)	12(6.5-16.9)	3.8(1-8.1)		

Table 5. Culture results of the studied neonates with sepsis

Culture results	***Total number=36***
Urine culture, *n*	
E-coli	3/5
klebsiella pneumoniae	2/5
Blood culture, *n*	
E-coli	11/23
klebsiella pneumoniae	7/23
Acinetobacter baumannii	5/23
CSF, *n*	
Acinetobacter baumannii	4/5
E-coli	1/5
Sputum, *n*	
E-coli	½
klebsiella pneumoniae	½
Pus, *n*	
klebsiella pneumoniae	1/1

Table 6. Severity of sepsis among the studied neonates with sepsis and TLR4 *Asp299Gly* polymorphism

Sepsis severity, *n*(%)	**Wild type (AA)**	**Heterozygous allele (AG)**	**Homozygous allele (GG)**
Sepsis	5(14)	1(3)	----
Severe sepsis	6(17)	1(3)	----
Septic shock	4(11)	0(0)	----
MODS	19(53)	0(0)	----

Abbreviations; MODS, multi-organ dysfunction syndrome

DISCUSSION

Sepsis is still a critical challenge in the neonatal critical care medicine, in spite of continued advances in neonatal medicine. It is one of the most common causes of death in Neonatal Intensive Care Units [8,9]**.**

Identifying genetic variations in the genes involved in the pathogenesis of neonatal sepsis may predict neonatal susceptibility to, and outcomes of sepsis and help to identify neonates at higher risk of death or serious complications who require aggressive therapy [10].

Therefore, it's important to discover the roles of sepsis-related genes, which may provide theoretical basis to understand sepsis pathogenesis **[11] .**

The 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].**

Toll like receptors (TLRs) play an important role in innate immunity, and one of them, Toll like receptor type 4 (TLR4), the specific receptor for lipopolysaccharides (LPS) of gram-negative bacteria plays a significant role as a receptor recognizing PAMPs [13].

This study represents a case-control study to evaluate TLR4 *Asp299Gly* gene polymorphism

in relation to the severity, prognosis, and outcome of gram-negative bacterial sepsis in neonates. 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 [7].

The current study enrolled 36 neonates with sepsis (20 males and 16 females) who were admitted to NICU of Zagazig University children's Hospital. In addition to 36 apparently healthy neonates as a control group.

In this study, the mean gestational age of the studied neonates was 38 weeks and ranged from 35 to 40 weeks. This is matched with a study reported by **Özgur et al.** [14] on TLR4 *Asp299Gly* polymorphism and severity of gram negative sepsis in neonates.

In contrary, **Abu-Maziad and coworkers** [15]**,** studied the role of polymorphic variants on septic preterm infants with a birth weight ≤ 1500 gram (VLBW).

Concerning the incidence of early (≤ 3d) and late (>3d) onset sepsis, it was equally distributed in the studied neonates. Opposing that, **Sampath et al.** [16] demonstrated that early onset sepsis was detected in (3.4%) while late onset sepsis occurred in (19.8%) of infants in their study about TLR4 *Asp299Gly* polymorphism and gram negative sepsis.

Regarding to the mode of delivery and the incidence of sepsis, our study revealed higher incidence of sepsis in neonates born via caesarean section than in those born via vaginal delivery. An Egyptian study by **Shehab El-Din et al.** [17] showed similar findings.

In the matter of the clinical presentation, respiratory distress was the most presenting symptom in the current study (50%) of cases, followed by cardiovascular manifestations (22%), gastrointestinal manifestations (17%) and neurologic manifestations (11%).

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.** [17] and another study conducted by **Simonsen et al**. [18] in North America.

Concerning the isolated organisms from cultures, we reported that *Escherichia coli* (44% of cases) was the most prevalent gram-negative bacteria followed by *klebsiella pneumoniae* (31%), and *Acinetobacter baumannii* (25%). These findings were in agreement with the results reported by **Shah et al.** [19] in their systematic review about sepsis in NICU.

However, the predominance of *klebsiella pneumoniae* among the causative gram-negative pathogens was reported in two previous studies. One Egyptian study by **Fahmey**[20] and one Indian study by Muley et al. [21].

Regarding the severity of sepsis in the current study, six (17%) of cases had a clinical picture compatible with sepsis, seven (19%) with severe sepsis, four (11%) with septic shock, and 19 (53%) with multiorgan dysfunction syndrome (MODS).

Tsai et al.[22] obtained similar results in their study about the clinical outcomes of gram-negative neonatal sepsis in Taiwan.

In terms of neonatal sepsis outcome in the present study, we observed mortality in 24 (66 %) of cases. This implicates the greater mortality and morbidity associated with gram-negative bacterial infection in neonates [2].

With regard to TLR4 *Asp299Gly* polymorphism, we studied TLR4 *Asp299Gly gene* (*AG,GG, AA* genotype) polymorphism in relation to the severity, prognosis, and outcome of gram-negative bacterial sepsis in neonates. We focused on the association between *TLR4* SNPs and gram-negative infections as TLR4 recognizes mainly lipopolysaccharide of gram-negative bacteria.

The current study revealed lack of association between the analyzed TLR4 *Asp299Gly* polymorphism and severity of neonatal sepsis.

Only two cases out of 36 showed heterogenous allelic variation (*AG*) in the TLR4

Asp299Gly polymorphism. But no polymorphism was noticed in the control group.

This was nearly matched with **Özgur et al.** [14] who studied the relation between TLR4 *Asp299Gly* polymorphism and gram-negative neonatal infection. Although they did not find any polymorphism in the cases (16 cases), yet they observed four heterozygous TLR4 *Asp299Gly* polymorphisms *(AG)* in the control group.

On the contrary, **Sampath et al.** [16] found heterogenous allelic variation (*AG*) in 25% of cases in their study about TLR4 *Asp299Gly* polymorphisms in very low birth weight (VLBW)) infants.

In accordance with this research, a study conducted by **Feterowski et al.** [23] showed that the TLR4 *Asp299Gly* polymorphism is not associated with the development or the outcome of sepsis, yet this study was performed on septic patients after major visceral surgery in surgical ICU.

In line with this present study, **Nakada et al.** [24] conducted a study to evaluate the clinical outcome of TLR4 *Asp299Gly* gene polymorphisms in critically ill Japanese patients, but no Japanese participants carrying the TLR4 gene *Asp299Gly* polymorphism were detected.

In addition, **Jessen et al.**[25]observed no relation between severity of sepsis and TLR4 *Asp299Gly* gene polymorphism in their study on adult patients with gram-negative sepsis admitted to a Danish hospital. Despite being conducted in adult, **Jessen et al.** [25] supported the results of the current study.

In agreement with our study, **Özgur et al.** [16] showed no relation between the two polymorphisms: TLR4 *Asp299Gly* and *Thr399Ile* and gram-negative infection in neonates. They performed a case-control study on term neonates of Turkish ethnic background.

Ahmad-Nejad and coworkers[26] obtained matched results, they observed no relations between TLR4 gene *Asp299Gly* SNPs and the outcome of sepsis in critically ill patients admitted to surgical Intensive Care Unit (ICU).

The results of the present study are concordant with the results of **Zhu et al.** [27] who conducted a meta-analysis on 17 eligible articles to investigate the association between TLR4 *Asp299Gly* polymorphism and neonatal sepsis. In a subgroup analysis depending on the type of pathogens, they did not show an association of this TLR4 *Asp299Gly* polymorphism with gram-negative bacterial sepsis.

Liu and coworkers[11] designed a meta-analysis enrolled 28 published articles containing 51 independent case-control studies including 6,537 septic neonates and 8,832 controls for a more comprehensive conclusion on association between TLR4 *Asp299Gly* gene polymorphism and susceptibility to and severity of sepsis in neonates. The results of this meta-analysis suggested that TLR4 *Asp299Gly* polymorphism may have no statistically significant influence on sepsis susceptibility**.**

In the current study, two cases out of 36 cases had heterogeneous (*AG*) TLR4 *Asp299Gly* polymorphism. Similar results were reported by **Saleh et al.** [28] in their case-control study to investigate TLR4 *Asp299Gly* gene polymorphisms among neonatal sepsis in Egypt.

On the contrary, some prior epidemiological studies reported that TLR4 polymorphisms, namely *Asp299Gly* were associated with more severe course of sepsis [16,29].

Perhaps the discrepancy between the results of the previous studies may be attributed to the population characteristics. As **Sampath et al.** [16] enrolled VLBW infants while **Nachtigall et al.** [29] studied the TLR4 gene SNPs in critically ill adult patients.

CONCLUSIONS

Taken all together, there is no significant association between TLR4 *Asp299Gly* polymorphism and severity or prognosis of gram-negative bacterial sepsis in neonates.

Conflict of interest: Nothing to declare

Financial disclosure: Nothing to declare

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