Genotyping Polymorphism of the *CD14* Gene and the Risk of Sepsis in Iraqi Neonates

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Abstract

Background: Neonatal sepsis (NS) is a clinical syndrome of systemic illness accompanied by bacteremia occurring in the first month of life. It remains one of the main causes of mortality and morbidity despite the progress in hygiene, introduction of new and potent antimicrobial agents for treatment, and advanced measures for diagnosis. **Aim:** The present study was conducted to evaluate the role of the genotypic and allelic frequency of *CD14* gene polymorphism at position –C159T for the susceptibility to sepsis in neonates. **Methods:** This study was conducted on 75 neonates who were admitted to Maternity and Childhood Teaching Hospital at Neonatal Intensive Care Unit (NICU) at AL-Diwaniyah city, Iraq, and 75 healthy neonates as a (control group). RFLP-PCR technique was performed for the detection the genotype and alleles in *CD14 (C-159t)* gene in neonatal sepsis patients samples as well as in healthy control groups. The statistical significance of the measured OR is assessed by a special χ^2 (Chi-square) formula. **Results:** the risk of having proven sepsis, compared to healthy controls was significantly increased by 13.1 times for neonates with TT genotype. Conversely, the presence of C allele significantly reduced the risk of having culture positive neonatal sepsis by 13.1 times. **Conclusion**: There is significantly higher prevalence of detection of SNP of *CD14 (C-159*t) gene polymorphism among patients with proven septic and probable septic neonates as compared to control group. This provides strong evidence that *CD14 (C-159*t) gene may play a major role in the susbtiplity for sepsis.

Key words: Neonatal sepsis, *CD14* gene, Genotype**,** RFLP

Introduction:

Neonatal sepsis (NS) is systemic illness accompanied by bacteremia occurring in the first month of life **(1).** So it is responsible for 30- 50% of the total neonatal deaths in developing countries. It is estimated that up to 20% of the neonates develop sepsis and approximately 1% die of sepsis related causes **(2).**

World Health Organization (WHO) estimates that out of the four million neonatal deaths all over the world every year, over 35% are due to infection in the neonatal period **(3)**. The prognosis and outcome of neonatal sepsis depend on early diagnosis and efficient antibiotic therapy **(4).** The diagnosis of sepsis is difficult because of non-specificity of clinical signs and symptoms and overlapping of symptoms with other noninfectious causes of systemic inflammation **(5)**.

The variation in the ability to recognize pathogens may influence the risk of infection. One of the primary molecules that functions in recognition of pathogen is CD14 **(6)**. CD14 is a pattern recognition receptor that plays a central role in innate immunity through recognition of bacterial lipoglycans, primarily lipopolysaccharide (LPS). CD14 has a unique ability to discriminate non-self lipoglycans of infectious pathogens first and for most LPS from non - infectious self **(7).** In Iraq the distribution of under-five death by age-groups was 55% of all neonatal death in age of 0-28 days of life, and neonatal sepsis is responsible for about 16% of the total of neonatal deaths **(8).**

Materials and Methods

***Subject* :** This study was conducted on 75 neonates who were admitted to Maternity and Childhood Teaching Hospital at NICU at AL-Diwaniya city, Iraq, and 75 healthy neonates as a (control group) in the period from April to December 2015. They were evaluated for neonatal sepsis with sepsis screen tests, Blood culture, and subjected to broad-rang PCR amplification for *CD14* gene polymorphism. Informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study.

***Genotyping*:** The genotypes of *CD14* gene polymorphism was determined by PCR–restriction fragment length polymorphism (RFLP),with special thermo cycling condition , and CD14 (*C-159t*) Polymorphism primers were provided from (Bioneer company, Korea) **, Table (1)** ,**Table (2) and Table (3).**Genomic DNA was extracted from frozen blood of neonatal sepsis patients and healthy control samples by using AccuPrep®Genomic DNA extraction kit (Bioneer. Korea). The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm). RFLP-PCR technique was performed for detection the genotype in *CD14 (C-159t)* gene in neonatal sepsis patients samples as well as in healthy control group. REFLP PCR

master mix was prepared for CD14 gene by using AVAII restriction enzyme .Then the PCR products were visualized in an ethidium bromide-stained 1.5% agarose gel using a UV Transilluminator.

Statistical analysis: Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using IBMSPSS version 23 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2016. The statistical significance of the measured OR is assessed by a special χ^2 (Chi-square) formula.

Result

The results presented in our study were based on the analysis of 2 study groups of newly born infants. The cases group were 75 infants with a clinical diagnosis of neonatal sepsis. This group was further classified into 2 groups based on the results of blood culture. A positive bacterial culture was identified in 30 cases and these were labeled as "Proven sepsis" group. The culture negative cases were 45 in number and were thus labeled as "Probable sepsis" group. In addition, a random sample of 75 healthy control neonates was group matched to the cases group on gender, birth weight and gestational age. As shown in ,**Table (4),** females constituted around a third of all the 3 study groups. No obvious or statistically significant differences in gender composition was observed between the 3 study groups. In addition, no statistically significant differences were observed between the 3 study groups in proportion of low birth weight and preterm.

The products of successful binding between the extracted DNA and specific primers for *CD14* gene were detected by gel electrophoresis analysis using DNA marker (100 bp DNA ladder) and the products size was 497 bp for both patients and control groups. The distribution of *CD14*- gene (C-159t codon) polymorphism was detected by PCR-RFLP technique, at this locus there're three genotype; homozygote (TT) at 353bp, heterozygous (CT) at 497bp, 353bp and 144bp, and wild type (CC) which still undigested, the genotype distribution had no deviation from Hardy-Weinberg equilibrium in all study groups**, Figure (1)**.

The risk of having proven sepsis, compared to healthy control was significantly increased by 13.1 times for neonates with TT genotype. Conversely having a CC genotype significantly decreases the risk of proven sepsis by 5.1 times. The CT genotype marginally decreased the risk of sepsis by 50%. This last effect was too weak to be statistically significant**, Table (5)**.Also the risk of having probable sepsis compared to healthy controls was significantly increased by 14.4 times for neonates with TT genotype. Conversely having a CC genotype significantly decreases the risk of proven sepsis by 5.9 times**, Table (6).**

Discussion

Neonatal sepsis with its high mortality rate still remains a diagnostic and treatment challenge for the neonatal health care providers. An early diagnosis of neonatal septicemia helps the clinician in instituting antibiotic therapy at the earliest, thereby reducing the mortality rates in the neonates. In present study, sex distribution among neonates with proven and probable sepsis was 47/75(62.7%) males and 28/75(37.3%) females, **Table (4).**These results confirm other studies which have shown that males have been reported to be 2-6 times more likely than females to develop sepsis **(9).** Nearly 3:2 ratio in this study is constant with this data. The male preponderance has been attributed to the deficiency of an X linked immuno-regulatory gene. A predominance of male infant is apparent in almost all studies of sepsis in newborn **(10).**

The current study conducted that the polymorphism in the promoter region of the *CD14* gene, and the T allele shows increased transcriptional activity when assayed in reporter assays (**11**). As would be predicted, individuals homozygous for the-159T allele have increased levels of CD14 **(12).** A number of studies have been performed to examine whether the-159 polymorphic site is associated with infection or sepsis, with conflicting results **(13).**

Genotype analysis revealed 3 genotypes TT, CC, TC in the C-159T polymorphism in septic neonate, TT genotype was the most frequent in both proven and probable sepsis groups (53.3% and 55.6%) respectively. On the other hand, CC genotype was the predominant in control group (56%).There was a statistical significant higher rate of TT and lower CC genotypes in proven septic neonates as compared to control group ($p<0.001$), OR = 13.14 and 95% CI 4.37 – 39.48) ,**Table (5).**

Also there was a statistical significant higher rate of TT and lower CC genotypes in probable septic neonates as compared to control group ($p<0.001$), OR = 14.38 and 95% CI 5.18 – 39.9), **Table (6).**As there was a significant result with TT genotypes in relation to sepsis, it was verified that TT genotype represents a risk factor for sepsis.The homozygous mutant genotype (TT) is uncommon in the control population (8.0%) but has an increased frequency in sepsis neonates (53.3%). This genotype confers an odds ratio (OR) of (13.1). While the heterozygous genotype (CT) is found in (36%) of the control subjects and (26.7%) of the patients and confers an OR of (0.65), in contrast the wild-type homozygous genotype (CC) had a higher frequency in the control subjects (56%) compared with sepsis neonates (20%) (OR) of (0.2), Moreover, the TT genotype has obviously suggests as an etiological factore for sepsis, as it has etiologic fraction (EF) of (0.493), In contrast, the CC genotype had rather preventive role as it had Protective Fraction (PF) of (0.450), **Table (5)**.

The T allele has higher frequency (66.7%) in proven septic neonates than C allele (33.3%) but in healthy neonates T allele (26 %), C allele (74 %), the T allele has obviously suggests as an etiological factor for sepsis, as it has an etiologic fraction (EF) of (0.574), In contrast, the C allele has rather preventive role as it had protective fraction (PF) of (0.82), **Table (5)**.

Similarly the T allele with higher frequency (68.9%) in probable septic neonates than C allele (31.1%) but in healthy neonates T allele (26 %), C allele (74 %), the T allele has etiologic fraction (EF) of (0.11), In contrast, the C allele has rather preventive role as it had Protective Fraction (PF) of (0.62), **Table (6)**.

The distribution of genotypes (TT, CC and CT) in patients and controls is in agreement with result of **Sharaf *et al*.,(14),** It was found that TT genotype was 47.5% among septic neonates, while TT genotype was 25% in control group .

Also, **Martin *et al.,(*15),** revealed that the homozygotic-159 T mutation of CD14 gene (CD14-159T) is associated a high mortality rate in sepsis due to reduced response of the innate immune system in carriers of these mutation and might be associated with a higher rate of bacterial infections. Other study **An-qiang *et al*., (16),** has reported that the CD14-159C/T to be associated with sepsis with inconsistent results. **De Faria *et al*., (17),** reported that the CD14 C- 159T gene polymorphism has been associated with increased in vitro CD14 expression in the serum of children with altered IgE serum levels and positive allergic test in different population. These data provide insight into-the pathogenic role of the CD14 C-159T polymorphism in pathogenesis of sepsis as 159 TT may favor increased risk of acquiring infection. Larger population studies may clarify the actual role of genetic polymorphisms in a susceptibility to infection and may provide a clue to open the possibility for a new therapeutic intervention.

Conclusions:

There is significantly higher prevalence of detection of SNP of *CD14 (C-159*t) gene polymorphism among patients with proven septic and probable septic neonates as compared to control group. This provides strong evidence that *CD14 (C-159*t) gene may play a major role in the susbtiplity for sepsis. With the evidence that genetic factors play a role in the susbtiplity for sepsis as confirmed by other studies ,the evaluation of other genetic polymorphisms properly addresses the need for a more rigorous and objective studies.

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Table (1): The Restriction Enzymes were Used in RFLP-PCR Assay with their Company and Country of Origin

Restriction enzymes	Target gene	Company/Country
AVAII	**CD14**	**Biolabs/ U.K**

Table (2): The Multiplex PCR Primers with their Sequence and Amplicon Size

Primer	Sequence		Amplicon
P1 primer	**F**	GTGCCAACAGATGAGGTTCAC	497bp
	R	CCTCTGTGAACCCTGATCAC	
P2 primer	**F**	CCTGAAACATCCTTCATTGC	442bp
	R	CGCAGCGGAAATCTTCATC	

Table (3): PCR Thermocycler Conditions Used in a Study

PCR step	Temp.	Time	repeat
Initial denaturation	96°C	3min.	1
Denaturation	96°C	40 sec.	38cycle
Annealing	56°C-P1 58°C-P2	40 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	10 min	1
Hold	4°C	Forever	-

Table (4): Description of the 3 Study Groups by Gender, Birth Weight and Gestational Age.

	Study group						
	Proven sepsis (Culture positive)		**Probable sepsis (culture negative)**		**Healthy controls**		
	No	**%**	**No**	**%**	**No**	**%**	**P**
Gender							0.98[NS]
Male	19	63.3	28	62.2	46	61.3	
Female	11	36.7	17	37.8	29	38.7	
Total	30	100.0	45	100.0	75	100.0	
Birth weight categories (gm)							0.26[NS]
Acceptable birth weight (=>2500 gm)	7	23.3	8	17.8	25	33.3	
LBW (1500-2490 gm)	11	36.7	23	51.1	31	41.3	
Extremely LBW (<1500gm)	12	40.0	14	31.1	19	25.3	
Total	30	100.0	45	100.0	75	100.0	
Gestational age categories (weeks)							0.72[NS]
At term (37-41)	8	26.7	14	31.1	26	34.7	
Preterm (Gestational age <37 weeks)	22	73.3	31	68.9	49	65.3	
Total	30	100.0	45	100.0	75	100.0	

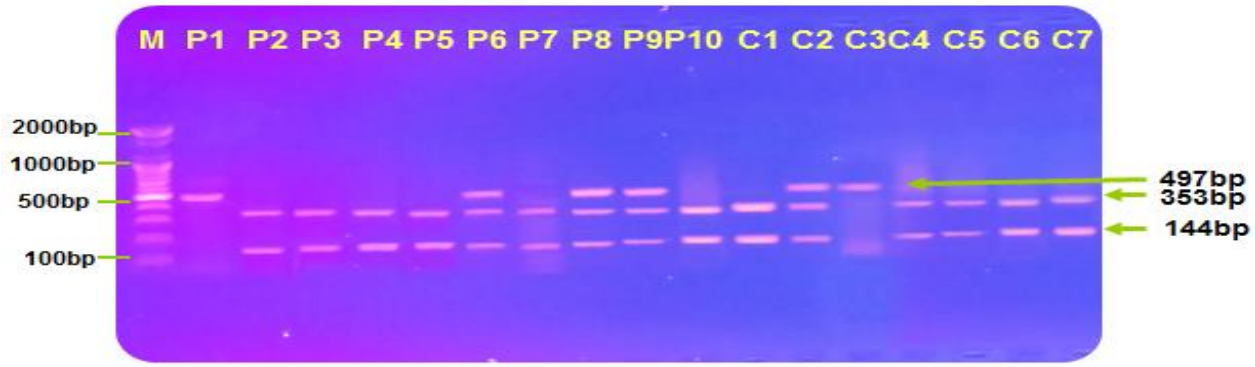

Figure (1): Agarose Gel Electrophoresis Image that Shown the RFLP-PCR Product Analysis of 497bp PCR Product *CD14-P1* Gene *(C-159t Codon)* in neonatal sepsis patient and healthy control blood sample that digestion by (*AVAII*) restriction endonuclease where M: Marker (2000-100bp), Lane (P1and C3) Patient and Control Sample Show (CC) Homozygote as Undigested 497bp Product Size, Lane (P2, P3,P4, P5, P7,P10, C1,C4,C5,C6, and C7)Patient and Control Sample Show (TT) Homozygote as 353bp and 144bp Product Size, Lane (P6, P8, P9, and C2) Patient and Control Sample Show (CT) Heterozygous as 497bp, 353bp and 144bp Product Size.

Table (5): The Risk of Proven Sepsis (Culture Positive) Compared to Healthy Controls by CD14 (C-159t) Gene Polymorphism.

	Healthy controls		*Proven sepsis (Culture positive)*			*Inverse OR*				
	N	**%**	**N**	**%**	**OR**		**95% CI OR**	**P**	**EF**	**PF**
CD14 (C-159t) polymorphism										
TT	6	8.0	16	53.3	13.1	**	(4.37 - 39.48)	<0.001	0.493	**
CC	42	56.0	6	20.0	0.2	5.1	(0.07 - 0.54)	0.001	**	0.450
CT	27	36.0	8	26.7	0.65	1.5	(0.25 - 1.65)	NS	**	0.127
Total	75	100.0	30	100.0						
CD14 (C-159t) alleles frequency										
T allele	**39**	26.0	40	66.7	7.18	**	2.74 - 18.79	0.0001	0.574	**
C allele	111	74.0	20	33.3	0.068	6.2	0.02 - 0.19	< 0.0001	**	0.82
Total	150		60							

Table (6): The Risk of Probable Sepsis (Culture Negative) Compared to Healthy Control Group by CD14 (C-159t) Gene Polymorphism.

	Healthy Controls		*Probable Sepsis (Culture Negative)*			*Inverse OR*				
	N	**%**	**N**	**%**	**OR**		**95% CI OR**	**P**	**EF**	**PF**
***CD14 (C-159t)* Genotype polymorphism**										
TT	6	8.0	25	55.6	14.4	**	(5.18 - 39.9)	<0.001	0.517	**
CC	42	56.0	8	17.8	0.17	5.9	(0.07 - 0.41)	<0.001	**	0.465
CT	27	36.0	12	26.7	0.65	1.5	(0.29 - 1.45)	NS	**	0.127
Total	75	100.0	45	100.0						
***CD14 (C-159t)* alleles frequency**										
T	39	26.0	62	68.9	1.19	**	0.38 - 3.698	NS	0.11	**
C	111	74.0	28	31.1	0.16	5.01	0.09 - 0.282	< 0.001	**	0.62
Total	150		90							

*** EF= etiologic fraction, PF= protective fraction, P=P-Value, OR=Odds Ratio, NS=Non significant, CI= confidence interval**

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