Open Access Maced J Med Sci **electronic publication ahead of print,**
published on August 31, 2019 as https://doi.org/10.3889/oamjms.2019.740

ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences.
https://doi.org/10.3889/oamjms.2019.740
eISSN: 1857-9655
Basic Science

Association of Gene Polymorphism of *Bactericidal Permeability Increasing Protein* Rs4358188, *Cluster of Differentiation 14* Rs2569190, *Interleukin 1β* Rs1143643 and *Matrix Metalloproteinase-16* Rs2664349 with Neonatal Sepsis

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Citation: Mustarim M, Yanwirasti Y, Jamsari J, Rukmono R, Nindrea RD. Association of Gene Polymorphism of *Bactericidal Permeability Increasing Protein* Rs4358188, *Cluster of Differentiation 14* Rs2569190, *Interleukin 1β* Rs1143643 and *Matrix Metalloproteinase-16* Rs2664349 with Neonatal Sepsis. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2019.740

Keywords: Neonatal sepsis; Single nucleotide polymorphism; BPI; CD14; IL1β; MMP16; Polymorphism

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Received: 08-Jun-2019; **Revised:** 25-Jul-2019; **Accepted:** 26-Jul-2019; **Online first:** 31-Aug-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Neonatal sepsis is a health problem because it causes serious morbidity and mortality in neonate intensive care units. The susceptibility of neonates occurs due to the immaturity of immune system development as well as due to maternal and environmental risk factors that can cause infection. Identification of genetic variation in genes involved in the inflammatory process can help clarify the pathophysiology of sepsis in high-risk patients, useful for the development of new diagnostic tools, and specific management plans for more accurate predictions of patient's prognosis.

AIM: This study aims to determine the association between gene polymorphism of *BPI* rs4358188, *CD14* rs2569190, *IL1β* rs1143643 or *MMP16* rs2664349 and the incidence of neonatal sepsis.

METHODS: Cross-sectional observational studies with genomic DNA samples from infants with sepsis and non-sepsis which were stored according to the standard storage of genetic materials in the Biomedical Laboratory of Faculty of Medicine Universitas Andalas Padang City, Indonesia. This study is part of a previous study by Rukmono P. Continued with PCR examination, sequencing and bioinformatics analysis.

RESULTS: Only *IL1β* rs1143643 G > A gene polymorphism was associated with the incidence of neonatal sepsis and was statistically significant (p = 0.017). No significant association was found between gene polymorphisms of *BPI* rs4358188 G > T, *CD14* rs2569190 A>G or *MMP16* rs2664349 G > A and neonatal sepsis (p > 0.05).

CONCLUSION: Gene polymorphism of *IL1β* rs1143643 G > A is associated with the incidence of neonatal sepsis.

Introduction

Neonatal sepsis is a health problem because it causes severe morbidity and mortality in neonate intensive care units. The susceptibility of neonates is due to immaturity in the development of an immune response or due to maternal and environmental risk factors that can cause infection [1].

The *World Health Organization* (WHO) estimates 1 million deaths each year from neonatal sepsis and 42% of these deaths occur within the first week of life [2]. The incidence of neonatal sepsis in the United States ranges from 1 to 4 per 1000 live births and from 2.4 to 16 per 1000 live births in Southeast Asia. In Indonesia, the neonatal mortality rate caused by infection and sepsis reached 1 to 10 per 1000 live births and reached 13 to 27 per 1000 live births in babies born under 1500 g [3].

The cause of neonatal sepsis is multifactorial and could have a maternal, neonatal or environmental basis. In recent years, many studies have investigated the association of genetic variation to the incidence of neonatal sepsis. Study into the association of genetic variation to neonatal sepsis is very important because identifying genetic variations in genes that are

involved in bacterial-induced cell responses and those associated with the pathogenesis of sepsis can help clarify the pathophysiology of sepsis in a group of high-risk patients. It is useful for the development of new diagnostic tools and specific management plans for more accurate prediction of patient prognosis [1].

To find treatment solutions for neonatal sepsis incidence, further studies on aspects of the immune system need to be carried out, primarily associated with the inflammatory process. Several genes that are considered to be associated with it and have not been explicitly studied in Indonesia are *Bactericidal Permeability Increasedasing Protein* (BPI), *Cluster of Differentiation 14* (CD14), *Interleukin 1 Beta* (IL1β) and *Matrix Metalloproteinase-16* (MMP16).

This study aims to reveal the association of gene polymorphism of *BPI* rs4358188, *CD14* rs2569190, *IL1β* rs114364003 and *MMP16* rs2664349 with the incidence of neonatal sepsis.

Material and Methods

Study design and research sample

The study is an observational study with a cross-sectional design using genomic DNA extraction samples from sepsis and non-sepsis infants which were stored according to standards for genetic material in the Biomedical Laboratory of FK UNAND. The materials used in the study are part of a previous study by Rukmono P. The Team of Research Ethics Committee approved the study, Faculty of Medicine, Universitas Andalas, Padang No: 532/KEP/FK/2017.

Operational definitions

The variables of this study included several independent variables: Gene Polymorphism of *Bactericidal Permeability Increasing Protein* rs4358188, *Cluster of Differentiation* 14 rs2569190, Interleukin 1β rs1143643 and *Matrix Metalloproteinase*-16 rs2664349; and a dependent variable is neonatal sepsis.

Research procedure

The criteria for neonatal sepsis which is used, is the presence of clinical symptoms of infection confirmed by positive blood culture results indicating bacteremia. The samples are then carried out by PCR examination, electrophoresis, DNA restriction, sequencing, and bioinformatic analysis.

Data analysis

The analysis was performed using the chi-square test to determine the association between gene polymorphism and neonatal sepsis, with a level of $p < 0.05$ considered as statistically significant. Data analysis was carried out by using *STATA 14.2 (Stata Corporation).*

Results

The samples obtained were from 30 neonates with neonatal sepsis and 30 neonates with unproven neonatal sepsis (controls) (Table 1).

Table 1: Demographic characteristics of study subjects

Variable	Neonates		p
	Proven sepsis (n = 30)	Unproven sepsis (n = 30)	
Gender			
Male	15 (50.00%)	23 (76.67%)	0.061
Female	15 (50.00%)	7 (23.33%)	
Age			
< 3 days	26 (86.67%)	29 (96.67%)	0.353
≥ 3 days	4 (13.33%)	1 (3.33%)	
BW			
≤ 2500 g	9 (30.00%)	12 (40.00%)	0.588
> 2500 g	21 (70.00%)	18 (60.00%)	
APGAR score at 1 minute			
≤ 3	1 (3.33%)	0 (0.00%)	1.000
> 3	29 (96.67%)	30 (100%)	
Gestational age			
28–31 weeks	2 (6.67%)	3 (10.00%)	0.483
32–35 weeks	4 (13.33%)	8 (26.67%)	
36–38 weeks	8 (26.67%)	8 (26.67%)	
≥ 38 weeks	16 (53.33%)	11 (36.67%)	
Maternal fever (≥ 38°C)			
Yes	14 (46.67%)	13 (43.33%)	1.000
No	16 (53.33%)	17 (56.67%)	
Thick-smelling amniotic fluid			
Yes	16 (53.33%)	8 (26.67%)	0.065
No	14 (46.67%)	22 (73.33%)	

Table 1 showed the demographic characteristics, consisting of gender, birth weight, APGAR score at first minute, gestational age, and occurrence of maternal fever and presence of thick-smelling amniotic fluid.

Table 2: Clinical symptoms in study subjects

Clinical symptoms in study subjects	Neonates		p
	Proven sepsis (n = 30)	Unproven sepsis (n = 30)	
Crying			
Strong	23 (76.67%)	26 (86.67%)	0.453
Moaning	6 (20.00%)	4 (13.33%)	
Unreacted	1 (3.33%)	0 (0%)	
Suction reflex			
Strong	14 (46.67%)	19 (63.33%)	0.489
Weak	14 (46.67%)	10 (33.33%)	
Vomiting	1 (3.33%)	1 (3.33%)	
Nothing	1 (3.33%)	0 (0%)	
Seizure			
Yes	1 (3.33%)	1 (3.33%)	1.000
No	29 (96.67%)	29 (96.67%)	
Lethargy			
Yes	13 (43.33%)	6 (20.00%)	0.096
No	17 (56.67%)	24 (80.00%)	
Chest retraction			
Yes	8 (26.67%)	4 (13.33%)	0.333
No	22 (73.33%)	26 (86.67%)	

Obtained more neonates aged < 3 days (86.67%) who suffered sepsis compared to neonates aged ≥ 3 days (13.33%) and more neonates born with

thick-smelling amniotic fluid (53.33%) had proven sepsis although not statistically significant (p > 0.05). Clinical symptoms in study subjects (Table 2).

Table 2 showed the clinical symptoms of the study subjects which included crying, suction reflexes, seizures, lethargy, and chest retraction, and there was no statistically significant association between these characteristics (p > 0.05), (Table 3).

Table 3: Gene polymorphism of *BPI, CD14, IL1β*, and *MMP16*

Type of mutation	Allele	f	%
	AA (*homozygous*)	7	11.67
BPI rs4358188 G > A	GA (*heterozygous*)	23	38.33
	GG (*wild type*)	30	50.00
	GG (*homozygous*)	17	28.33
CD14 rs2569190 A > G	AG (*heterozygous*)	34	56.67
	AA (*wild type*)	9	15.00
	AA (*homozygous*)	16	26.67
IL-1β rs1143643 G > A	GA (*heterozygous*)	29	48.33
	GG (*wild type*)	15	25.00
	AA (*homozygous*)	35	58.33
MMP16 rs2664349 G > A	GA (*heterozygous*)	24	40.00
	GG (*wild type*)	1	1.67

Table 3 known, only three SNPs were found in this study population, namely *CD14, IL1β*, and *MMP16* gene polymorphism, and no *BPI* gene polymorphism was found. Association of genetic polymorphism with neonatal sepsis (Table 4).

Table 4: Association of genetic polymorphism with neonatal sepsis

Gene and allele of the polymorphism	Group				p
	No sepsis	%	Sepsis	%	
BPI rs4358188 G > T					
No mutations	15	25.0	15	25.0	1.00
Mutation	15	25.0	15	25.0	
CD14 rs2569190 A > G					
No mutations	3	5.00	6	10.0	0.472
Mutation	27	45.0	24	40.0	
IL1β rs1143643 G > A					
No mutations	12	20.0	3	5.0	0.017
Mutation	18	30.0	27	45.0	
MMP16 rs2664349 G > A					
No mutations	1	1.67	0	0	1.00
Mutation	29	48.33	30	50.0	

Table 4 showed the four SNPs in the study samples that were proven or unproven to have sepsis. Only gene polymorphism of *IL1β* rs1143643 G > A was associated with the incidence of neonatal sepsis and was statistically significant (p < 0.05). However, no significant association was found between gene polymorphism of *BPI* rs4358188 G > T, *CD14* rs2569190 A > G or *MMP16* rs2664349 G > A and neonatal sepsis (p > 0.05).

Discussion

Identification of genetic variation in neonatal sepsis is essential because infants, especially those with very low birth weights (VLBW) or born prematurely, have immature immune systems and innate immunity to bacterial infections is disrupted. This causes infants to be at risk of sepsis or severe sepsis. Therefore, identification of these genetic variations can help to clarify sepsis pathophysiology in a group of high-risk patients.

About demographic characteristics of the study subjects, no significant association was found between these characteristics and neonatal sepsis. There was no significant association between gender and neonatal sepsis (p > 0.05). This result contrasts with the results of Shivaprasad B's study in which male infants had sepsis risk factors that were 3.1-fold higher than female infants. Shane L. Andi also found a higher incidence of sepsis in term male infants than term female infants, although this association has not been found in premature infants [4]. Sepsis is multifactorial, and inflammatory cytokine effects on sepsis development can be modified by age, gender, and several environmental factors.

In the age group of the study subjects, no significant association was found (p > 0.05). Neonatal sepsis is divided into two categories based on time of onset, namely neonatal early-onset sepsis (EOS) if symptoms occur at < 72 hours, and neonatal late-onset sepsis (LOS) if symptoms occur at ≥ 72 hours. The incidence of neonatal early-onset sepsis is reported to be 0.98 infections per 1000 live births [4]. While the estimated number of LOS cases nationally is unknown.

About the birth weight group, there were no significant differences in the sepsis and non-sepsis groups regarding birth weight (p > 0.05). The incidence of sepsis was inversely proportional to birth weight, namely 10.96 per 1000 live births with a birth weight of 401 – 1500 g, 1.38 for 1501 – 2500 g, and 0.57 for > 2500 g [4].

With regard to the APGAR score at 1 minute, one neonate (3.33%) had an APGAR score of ≤ 3 and 29 neonates (96.67%) a score of > 3 of APGAR score in the sepsis sample group, and 0 (0.00%) neonate with ≤ 3 of APGAR score and 30 (100.00%) neonates with > 3 of APGAR score, with p-value of 1,000, showed no significant differences between sepsis and non-sepsis groups based on APGAR score at 1 minute. This is not by the study conducted by Gebremedhin D, where the APGAR score at 5 minutes and shortly after birth showed a significant association with the risk of neonatal sepsis. Neonates with an APGAR score of < 7 at 5 minutes have a higher risk of developing neonatal sepsis than neonates with an APGAR score of ≥ 7 (OR = 68.9; 95% CI 3.63, 1307.90). Similarly, neonates who cry immediately at birth are 99% less likely to experience sepsis than neonates who do not cry immediately at birth (OR = 124.0; 95% CI 6.5, 2379) [5]. This may be due to the nature of crying, which is a physiological event, and changes associated with this event.

About gestational age, no significant differences were found between sepsis and non-sepsis groups. Based on the literature, the overall case fatality rate of neonatal sepsis is 16%, with an incidence that is inversely proportional to gestational

age: 54% at 22–24 weeks of gestational age, 30% at 25 – 28 weeks, 12% at 29 – 33 weeks, and 3% at greater than 37 weeks [4]. While for the risk of LOS incidence based on gestational age is 36.3% in neonates with a gestational age of < 28 weeks at least having one episode of LOS compared to 29.6%, 17.5% and 16.5% in moderate preterm neonates (29–32 weeks of gestational age), late preterm (33–36 weeks of gestational age) and a term neonates, respectively.

There was no significant association between the maternal fever group during labour (≥ 38°C) and the incidence of sepsis ($p > 0.05$). This was not by the results of previous studies, which found that neonates born to mothers who had a fever during labour had a 6-times greater risk of experiencing sepsis than neonates born to mothers who did not experience intrapartum fever [5], [6]. Intrapartum fever is considered to be an indicator of maternal infection which is often transmitted to the baby *in utero* or as it passes through the birth canal and often causes EOS. Maternal fever without accompanying chorioamnionitis signs can also increase the risk of sepsis but is often accompanied by a cause of noninfectious maternal fever such as dehydration or epidural anaesthesia [7]. According to Verma P et al., some maternal factors that predispose to sepsis include vaginal examinations that are too frequent (23.25%), maternal fever (33.33%) and history of foul-smelling amniotic fluid (24.72%) [8].

In the sepsis group, 16 neonates (53.33%) were from mothers with thick-smelling amniotic fluid and 14 neonates (46.67%) from mothers without a history of thick-smelling amniotic fluid. Whereas in the unproven sepsis group, there were eight neonates (26.67%) from mothers with thick-smelling amniotic fluid and 22 (73.33%) from mothers without a history of thick-smelling amniotic fluid ($p > 0.05$). The results of this study are consistent with two previous studies which reported that foul-smelling amniotic fluid, prematurity, low birth weight, residency, parity, and ANC facility services were not statistically associated with the risk of neonatal sepsis. However, regarding absolute numbers of sepsis cases, there was a higher incidence of thick-smelling amniotic fluid and vice versa [5]. Rawat S. also reported that the discovery of foul-smelling amniotic fluid is evidence of anaerobic bacteria and is one of the predisposing factors for sepsis, but there is insufficient evidence confirm this as an independent risk factor for neonatal sepsis [7].

Clinical symptoms were examined in this study, including crying, suction reflexes, seizures, lethargy and chest retraction. After statistical analysis, no significant differences were found in the group that was proven to have sepsis or not proven to have neonatal sepsis against crying ($p = 0.453$), suction reflexes ($p = 0.489$), seizures ($p = 1.000$), lethargy ($p = 0.096$) and chest retraction ($p = 0.333$). This suggests that both groups had almost the same clinical symptoms, so it does not affect the results of the study.

About *BPI* rs4358188 gene polymorphism in the sepsis group (30 subjects), 12 subjects experienced heterozygous polymorphism (GA) of the rs4358188 *BPI* gene, three subjects with homozygous polymorphisms (AA) and 15 wild-types (GG) subjects. Whereas in the no sepsis group (30 subjects), 11 subjects experienced heterozygous polymorphisms (GA) of the rs4358188 *BPI* gene, four subjects with homozygous polymorphisms (AA) and 15 wild-types (GG) subjects.

BPI is a gene that encodes factors that play an essential antibacterial and anti-inflammatory role and are commonly found in neutrophil azurophilic granules, playing an essential role in defence against Gram-negative infections. Esposito et al. reported that the AG genotype of BPI rs4358188 was associated with a reduced risk of sepsis [4]. However, Abu Maziad's study did not show an association between *BPI* polymorphisms and susceptibility to sepsis [9]. Michalek et al. also reported a negative association between *BPI* SNP and sepsis in children aged 0 – 18 years where the GG genotype of *BPI* rs435188 was associated with increased susceptibility to severe sepsis and adverse perinatal outcomes [10].

In the case of polymorphism in the *CD14* rs2569190 gene, 16 subjects experienced heterozygous polymorphism (AG) of the rs2569190 *CD14* gene, eight subjects experienced homozygous polymorphism (GG), and there were six wild-types (AA) subjects in the proven neonatal sepsis group. In the unproven neonatal sepsis group (30 subjects), 18 subjects were found with heterozygous polymorphism (AG) of the rs2569190 *CD14* gene, nine subjects experienced homozygous polymorphism (GG), and the remaining three subjects were wild-type (AA).

CD14 is a component of lipopolysaccharide receptor molecules and an important recognition receptor that plays a key role in the immune response and inflammation. Previous studies have shown that *CD14* gene polymorphisms are involved in several inflammatory diseases, such as ulcerative colitis and Crohn's disease. Previous reports have shown that the biological association of *CD14* rs2569190 gene polymorphism with the survival of patients with A allele can be caused by a strong pro-inflammatory response in patients, along with higher *CD14* expression [11].

Studies have shown that GG genotype of *CD14* rs2569190 gene is associated with an increased risk of severe sepsis and death in sepsis shock patients undergoing major surgery [1], [11]. Patients with GG genotype of rs2569190 had a shorter probability of survival compared to AA/AG genotype of rs2569190 in 60 days (62.3% vs 50%), and 90 days (62.3% vs 52.6%) ($p = 0.046$) compared to AA/AG genotypes. A to G polymorphism at -159 positions in the region of *CD14* (rs2569190) gene promotion can cause different activities in promoter

arrangement. In this context, the G allele of rs2569190 seems to have implications for decreasing *CD14* transcription regulation and also decreasing mCD14 expression and circulating sCD14 levels [11].

The study conducted by Wang reported that there was no evidence that rs2569190 was associated with susceptibility to sepsis [12]. In 2013, a meta-analysis by Zhang et al. concluded that *CD14* rs2569190 polymorphism was not a relevant risk factor for sepsis and mortality. The authors found that patients with AA/AG genotype had a higher risk of death than patients with the GG genotype (Asian population). This finding is likely to be a result of the small number of studies associated with sepsis mortality that were included in the meta-analysis [13].

In the sample group with *IL1β* gene polymorphism, 20 subjects experienced heterozygous polymorphism (GA) of rs1143643 *IL1β* gene, seven subjects experienced homozygous polymorphism (AA), and the remaining three subjects were wild-type (GG). Whereas in the unproven group of neonatal sepsis totalling 30 subjects, nine subjects were found who experienced heterozygous polymorphism (GA) of rs1143643 *IL1β* gene, nine subjects experienced homozygous polymorphism (AA), and the remaining 12 subjects were wild type (GG).

Interleukin 1β is a key pro-inflammatory cytokine that is produced early in response to microbial invasion and plays an important role in the pathogenesis of sepsis and sepsis shock. These molecules stimulate the production of prostaglandins and nitric oxide, the two vasodilatory mediators found in sepsis.

Esposito et al. reported that the CT and TT genotypes of *IL1β* rs1143643 gene were associated with a significant increase in the overall risk of sepsis [4]. The three polymorphisms of *IL1β* rs1143634, rs1143633 and rs1143643 were associated with the risk of allergic asthma ($p = 0.034$, OR = 1.523; $p = 0.024$, OR = 1.471; $p = 0.044$, OR = 1.420) [14]. The study by Abu Maziad et al. did not find an association between *IL1β* gene polymorphism and sepsis. This discrepancy may be due to differences in the definition of sepsis and its severity, and differences in some general characteristics of the subjects, included ethnicity, compared to those used in Esposito's study [9].

Concerning *MMP16* gene polymorphism, 12 subjects experienced heterozygous polymorphism (GA) of the rs2664349 *MMP16* gene, 18 subjects experienced homozygous polymorphism (AA), and there were no subjects with wild type (GG). In the unproven neonatal sepsis group, totalling 30 subjects, 12 subjects experienced heterozygous polymorphism (GA) of the rs2664349 *MMP16* gene, 17 subjects who experienced homozygous polymorphism (AA) and one wild type (GG) subject.

One of the advantages of the PCR sequencing method the detection of other nucleotides besides the nucleotide targets, for example, SNP *MMP16* rs2616505 A > G, rs11785236 A > G, and rs10504853 T > G; these were also found in this study and SNP has not been studied previously in Indonesia.

The results of this study on increasing MMP expression after exposure to LPS indicate that protease can affect the pathogenesis of endotoxemia so that MMP levels are said to be associated with sepsis severity. In this study, MMP may play a role in the incidence of sepsis because almost all samples had mutations, although it was not statistically significant. MMP is regarded as a time bomb because most of the samples had mutations in both sepsis and non-sepsis patients. Therefore, currently, MMP-based therapy (MMP-inhibitor) is being developed in patients with sepsis and sepsis shock, for example, glucocorticoids, retinoids and progesterone which can suppress MMP expression [4].

MMP16 is a zinc-dependent enzyme, and this element is very important for the normal functioning of the innate or adaptive immune systems [15]. The possibility that MMP genetic variation significantly affects susceptibility to infectious disease in humans is still very small [16]. A consistent discovery to many gene expression studies that patients with pediatric septic shock are characterised by extensive repression of genes that directly participate in zinc balance or directly depend on them to perform normal functions [17], [18], [19], [20].

Esposito et al. reported that the GG genotype of rs2664349 was associated with an increased risk of significant sepsis. This is the first report of the potential effects of *MMP16* genetic variation on sepsis and seems to be in agreement with recent evidence that MMP is, not only a matrix degradation enzyme as previously thought, but also has multiple immunomodulation mechanisms. SNP rs2664349 not only affects pulmonary expression, *MMP16* function and risk of bronchopulmonary dysplasia in preterm infants but also affects to MM2 activation, MMP which plays a core role in monocyte chemoattraction and subsequently affects the response to infectious agents [4], [21].

In conclusion, this analysis confirmed gene polymorphism of *IL1β* rs1143643 G > A is associated with the incidence of neonatal sepsis.

Acknowledgements

The author would like to thank the parties from Biomedical Laboratory, Faculty of Medicine, Andalas University and all parties involved in this study.

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