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RESEARCH ARTICLE

The Relationship between Characteristics of Cerebospinal Fluid and Tuberculous Meningitis detected using Real Time PCR

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ABSTRAK
Latar belakang: Morbiditas dan mortalitas yang ditimbulkan karena meningitis Tuberkulosa (TB) sangat besar, sementara penegakkan diagnosis masih menjadi kendala karena konsentrasi bakteri di dalam *liquor cerebrospinalis* (LCS) terlalu sedikit. Kenaikan protein, penurunan glukosa, dan pleositosis sel *mononuclear* yang dominan sering digunakan untuk mendiagnosis infeksi TB, namun hasilnya masih inkonsisten.
Tujuan: untuk menganalisis hubungan antara beberapa karakteristik LCS dengan meningitis TB.
Metode: Penelitian observasional, menggunakan desain *cross sectional*, dengan sampel sebanyak 27 orang penderita meningitis dan atau meningoencephalitis TB, dengan *Thwaites diagnostic scoring* ≤ 4, diperoleh dari catatan medis. Diagnosis definitif meningitis TB ditegakkan berdasarkan pada pengecatan Ziehl Neelsen dan atau kultur TB, dan atau *polimerase chain reaction* (PCR) dengan target amplifikasi pada IS6110 (*insertion sequence* 6110). Karakteristik LCS meliputi peningkatan protein, penurunan glukosa, pleositosis dengan sel mononuclear predominan, dan abnormalitas LCS diperiksa di laboratorium mikrobiologi molekuler FK UNDIP/RSUP dr Kariadi Semarang. Analisis statistik yang digunakan adalah Rasio Prevalen (RP).
Hasil: Didapatkan sebanyak 12 (44,4%) dari 27 pasien adalah definitif meningitis TB. Analisis statistik menunjukkan bahwa RP pada peningkatan kadar protein LCS adalah 3,143 (CI 95%: 0,502 – 19,692), penurunan kadar glukosa adalah 1,750 (CI 95%: 0,501 – 6,112), pleositosis dengan sel mononuclear predominan adalah 2,5 (CI 95%: 1,238 – 5,048), sedangkan abnormalitas LCS adalah RP 2,2 (CI 95%: 1,087 – 4,454).
Simpulan: hasil penelitian ini menunjukkan bahwa pleositosis dengan sel mononuclear predominan serta abnormalitas LCS mempunyai korelasi positif dengan definitif meningitis TB.

Kata kunci : meningitis TB, karakteristik CSF, pleositosis, *real time* PCR

ABSTRACT
Introduction: *Tuberculosis (TB) carries a high morbidity and mortality, however, its diagnosis can be difficult due to low concentration of bacteria in the cerebrospinal fluid (CSF). Although increased CSF protein, decreased CSF glucose; cerebrospinal fluid (CSF) pleocytosis with mononuclear cells predominance are commonly used to diagnose TB infection, they show inconsistent results.*
Objectives: *To analyze the relationship between several characteristics of the CSF and TB meningitis.*
Methods: *This is an observational research, with a cross sectional design, 27 patients with TB meningitis and or meningoencephalitis with Thwaites diagnostic score ≤ 4 obtained from mecidal records. The definitive diagnosis of TB meningitis was made using the Ziehl Neelsen (ZN) staining and or culture TB, or polymerase chain reaction (PCR) amplification with the target in IS6110, Ziehl-Neelsen staining (ZN) culture method. Characteristics of CSF including increased CSF protein, decreased CSF glucose, cerebrospinal fluid (CSF) pleocytosis with mononuclear cells predominance and the CSF abnormalities were evaluated in the laboratory of molecular microbiology of Medical Faculty, Diponegoro University/dr Kariadi General Hospital, Semarang. The statistical analysis used was prevalent ratio (RP).*
Results: *Over 27 patients, 12 (44.4%) were diagnosed with definite TB meningitis. Statistical analysis showed that prevalence ratio of the increased levels of protein CSF was 3.143 (95% CI: 0.502 to 19.692), decreased glucose levels was 1.750 (95% CI: 0.501 to 6.112), pleocytosis with a predominance of mononuclear cells was 2.5 (CI 95% : 1.238 to 5.048), whereas the CFS abnormality was RP 2.2 (95% CI: 1.087 to 4.454).*
Conclusion: *This study has shown that pleocytosis with mononuclear cells predominance and CFS abnormalities have a positive relationship with definite TB meningitis.*

Keywords: *TB meningitis, CSF characteristics, Pleocytosis, real time PCR*

INTRODUCTION

Tuberculous Meningitis (TBM) is an acute inflammation caused by the bacteria *Mycobacterium tuberculosis* (TB) in the membranes covering the brain and spinal cord known collectively as the meninges (PARK *et al.,* 2000). This infection carries a high

morbidity and mortality (Agrawal and Nadel, 2011; Mandell *et al.,* 2000). *Mycobacterium tuberculosis* (TB) is the most common cause of death compared to any other viruses or bacteria (40-50% vs. 5-10%). To decrease TBM morbidity and mortality requires timely and accurate treatment. Despite the need for accurate and early diagnosis of TB for effective and accurate treatment, the determination of the etiological diagnosis for TBM can not be done rapidly and accurately (Trung *et al.,* 2012).

The definitive diagnosis of TB meningitis can be made based on the identification of acid-resistant bacteria in the CSF with Ziehl Neelsen (ZN) staining or TB culture (Deshpande *et al.,* 2007a), but this can be difficult because of the low bacterial load CSF. Furthermore, the results of material CSF ZN staining with a low bacilliary load often give a false negative result (Caws *et al.,* 2000), whereas the TB culture takes about 5-8 weeks (Noussair *et al.,* 2009). Polymerase Chain Reaction (PCR) is a rapid, specific and sensitive technique for the detection of TB meningitis (Takahashi *et al.,* 2012). However, successful PCR assay of TB meningitis depends on various factors including storage and transport of samples, DNA isolation and PCR, considering that the primer, solution of PCR, and a thermal cycler also play a role. Moreover, due to sspesific nature of cellular wall of mycobacterium, at the stage of the DNA extraction of besides the shock treatment through the heating and cooling to destroy the cell walls of bacteria, provision of lysozyme followed chemical treatment are required (Thakur *et al.,* 2011; Deshpande *et al.,* 2007a). Oligonucleotide IS6110 an insertion sequence from genome of M. tuberculosis, PCR amplification target was found to be highly specific (about 94%) to mycobacteria belonging to the M. tuberculosis (Caws *et al.,* 2000) and highly sensitivity (85-97%) (Deshpande *et al.,* 2007b; Caws *et al.,* 2000; Maurya *et al.* 2012; Narayanan *et al.,* 2001; Chowdhury *et al.* 2012; Lee *et al.,* 2011).

Characteristics CSF in tuberculosis meningitis were detected by PCR are characterized by increased levels of protein, decreased glucose levels, and pleocytosis with mononuclear cells predominance, still give varying results (Youssef *et al.,* 2006). Various studies have shown that in CSF TB meningitis patients found 16% acellular, 20% -25% had pleocytosis with a predominance of neutrophils, 6% with normal levels of protein, and 25% with normal glucose (Bhigjee *et al.,* 2007). This study aimed to analyze the correlation between CSF protein levels, glucose CSF levels, CSF pleocytosis with mononuclear cells predominance as well as CFS abnormalities and TB meningitis.

METHODS

This is a cross sectional study. All patients with meningitis and or TB meningoencepahalitis with Thwaites' score ≤ 4 (Sunbul *et al.,* 2005), who were hospitalized in Dr Kariadi General Hospital, Semarang between March 1, 2013 to November 30, 2013 Indonesia were entrolled. A definitive diagnosis of TBM was made using PCR amplification targeting IS6110, ZN staining and Mycobacterial culture for detection Mycobacterium tuberculosis complex. Ziehl Neelsen staining and or culture of Mycobacterium tuberculosis and or PCR targeting the IS6110 amplification for the detection of Mycobacterium tuberculosis complex were performed in the laboratory of molecular microbiology FK UNDIP/dr. Kariadi Semarang. Oligonucleotide primers IS6110R: 5'-CGTAGGCGTCGGTGACAAA-3' and IS6110F: 5'-CTAACCGGCTGTGGGTA-3' were used. The extraction procedure used a heating temperature of 56ºC, while the amplification of DNA used a Bio-Rad®iQTM5 real-time PCR. The samples were then centrifuged at 6000 rpm for 15 minutes, using a Thermo Scientific 'Sorvall' Biofuge Primo Centrifuge®. The secondary data were taken from the medical records patient medical including demografic (age, gender) and clinical information (characteristics, thoracic X-rays, CT scans of the head).

CSF characteristics evaluated included increased levels of protein, decreased glucose levels, pleocytosis with mononuclear cells predominance, and CSF abnormalities. Increased protein is defined as the levels of protein> 45 mgdL (Pasco, 2012). A decreased CSF glucose (hypoglycorrhachia) is defined as a glucose level <40 mg/dL A decrease in glucose was defined as glucose levels> 50% compared with serum glucose levels (Pasco, 2012). Pleocytosis with a predominance of mononuclear cells defined as levels of CSF leukocyte total of more than 50 leukocytes / mm 3, with a higher ratio of mononuclear cells from cell polimorfonuclear (Pasco, 2012). CSF abnormality is defined as the presence of three parameters including decreased glucose levels, increased levels of protein and pleocytosis with a predominance of mononuclear cells (Pasco, 2012). Analysis of relationship was conducted with the independent variable of the definite TB meningitis, and dependent variables that includes CSF glucose, CSF protein levels, pleocytosis with a predominance of mononuclear cells and CSF abnormalities. The relationship is expressed in the prevalence ratio (PR). The study has been approved by the Health Research Ethics Committee (KEPK) Diponegoro University Faculty of Medicine and a research permit from the director of the of Dr. Kariadi

General Hospital Semarang.

RESULTS

Between March 1, 2013 to 30, 2013 there were 27 patients with 12 samples with definite TB meningitis. The characteristics of the patients and the results of the microbiology laboratory is reported in Table 1 and Table 2 respectively. The relationship between the dependent variables and the independent variables is presented in Table 3.

DISCUSSION

Contrary to previous study (Pasco, 2012), our study suggests that decreased CSF glucose levels in this study was not associated with definite TB meningitis. This contradicting finding could be due to the fact that elevated levels of protein and a decrease in CSF glucose may be caused by infection of tuberculosis, bacterial

Table 1. Characteristics of the sample

Characteristics of Samples	Definite TB Meningitis		Total n = 27	*p*
	Yes	No		
Age	28 (1-59) *	14 (1-65) *		
Gender				
Male	8 (29.6%)	9 (33.3%)	17 (63%)	1.00
Female	4 (14.8%)	6 (22.2%)	10 (37%)	
Pulmonary tuberculosis radiology				
Yes	6 (22.2%)	6 (22.2%)	12 (44.4%)	.603
No	6 (22.2%)	9 (33.3%)	15 (55.6%)	
Overview CT Scan**				
Yes	10 (37%)	4 (14.8%)	14 (51.9%)	0,011
No	2 (7.4%)	11 (40.7%)	13 (48.1%)	

*** The median value (minimum value-maximum value)**

**** One or more feature of basal meningeal enhancemen, hydrocephalus or infarction.**

Table 2. Results of Staining, culture, and PCR

No.	PCR	Ziehl Neelsen Staining	Mycobacterium culture	No.	PCR	Ziehl Neelsen Staining	Mycobacterium culture
1	-	-	-	15	-	-	-
2	-	-	-	16	-	-	-
3	-	-	-	17	-	-	-
4	-	-	(+) (Slow -growing - nonphotochromogen)	18	-	-	-
5	-	-	-	19	-	-	-
6	-	-	-	20	-	-	-
7	(+)	(+)	(+) (Slow -growing - nonphotochromogen)	21	(+)	-	-
8	-	-	(+) (Slow -growing - nonphotochromogen)	22	(+)	-	-
9	(+)	-	-	23	(+)	-	-
10	-	-	-	24	(+)	-	-
11	-	-	(+) (Slow -growing - nonphotochromogen)	25	(+)	-	-
12	-	-	-	26	(+)	-	-
13	-	-	-	27	(+)	-	-
14	-	-	-				

Table 3. The relationship between the various variables

Information		Definite TB		PR	95% CI
		Yes	No		
Increased protein				3,143	(0.502 to 19.692)
	Yes	11 (91.7%)	10 (66.7%)		
	No	1 (8.3%)	5 (33.3%)		
Lowered glucose				1,750	(0.501 to 6.112)
	Yes	10 (83.3%)	10 (66.7%)		
	No	2 (16.7%)	5 (33.3%)		
pleocytosis mononuclear predominance				2.5	(1.238 to 5.048)
	Yes	5 (41.7%)	1 (6.7%)		
	No	7 (58.3%)	14 (93.3%)		
CSF abnormalities				2.2	(1.087 to 4.454)
	Yes	4 (33.3%)	1 (6.7%)		
	No	8 (66.7%)	14 (93.3%)		
Total		100%	100%		

infections (non-TB) and infections by fungi (Venkatesh *et al.,* 2003). Samples with dual infections (TB and other bacteria) were not excluded due to the small sample size (only 27 samples). Beside a small sample size, this present study is also limited by short study period (9 months). Thus, similiar investigation with a longer time to increase the sample size is needed.

Our study has revealed that CSF pleocytosis with mononuclear cells predominance is patognomonis for TB meningitis. This finding is consistent with that of postulated by previous studies studies (Pasco, 2012; Bhigjee *et al.,* 2007; Rock *et al.,* 2008) despite the other studies showing that in accute meningitis still, neutrophils cells (polymorphonuclear cells) will be dominant (Venkatesh *et al.,* 2003). That Cells of CSF are easily degraded has been demonstrated by a study showing that delays in the processing of samples (> 30 minutes) a significant differences in results (Venkatesh *et al.,* 2003). The findings of this study supports other studies (Rock *et al.,* 2008; Pasco, 2012; Bhigjee *et al.,* 2007) . This study is also limited by the use of the secondary data. Further study using the primary data is needed to increase the significance (Pasco, 2012).

Our study suggests that increased CSF protein is not a patognomonic sign of TB meningitis because several other bacterial, fungal and virus infections can also cause elevated protein level. In fact, elevation of protein due to the degradation of cellular proteins due to damage caused by bacterial, tuberculosis, fungal and viral infections was observed by Venkatesh *et al.* This might also be caused by the inclusion of samples with dual infections.

Although the entry of TB bacteria in the CSF obtained has been shown to be caused by bacteremia by focal infection of the lungs (Rock *et al.,* 2008), our study did not find a significant difference between the chest X-ray with a definite and non-definitie TB meningitis (p = 0.603). This finding is consistent with that of previous study (Pasco, 2012). This may be due to the fact the assay can not detect until anatomical abnormalities develop (DPE Kingsley, 2002). TB patients data in children are often without pulmonary radiology test results (Gie, 2003).

Our study revealed abnormalities CT-scan of the head that is typical for TB meningitis that is significant differences in the basal meningeal enhancement and or infarction or hydrocephalus communicans, between the definite and non-definite TB meningitis with p = 0.011. This finding is consistent with that previous study (Pasco, 2012).

Age definitive TB meningitis in thisstudy mostly at adult age with median (minimum-maximum) of approximately 28 years (1-59). Adulthood is a condition in which the immune system is in the peak of. This situation is contrary to the theory that their TB meningitis caused by immunocompromised so that the infection spreads to other organs. This result may be due to many factors affecting the immune system besides age which are not the scope of this study for exampleaza patient's HIV status (WHO, 2012).

The research is in phase extraction using treatments for shock action the success of a DNA detection depends on the quality of the extraction well. Acid-resistant bacteria with a cell wall composed of

hydrophobic components mycolic acids, which is a specific component of the largest (50%) of the bacterial cell wall constituent (Kleinnijenhuis *et al.,* 2011). Mycolic acids form a thick membrane that surrounds bacteria. Complex shape of the cell wall of bacteria caused difficulty for lysed. DNA extracted from the sample CSF TB with a small bacterial load that requires us to do a special treatment to be able to isolate DNA from TB. Sequence of DNA extracted from samples of CSF are: Shock treatment (heating or cooling) to weaken the bacterial cell wall, giving lysozyme to destroy debris protein, Chemical treatment to lyse the bacterial cell wall, perform DNA purification (purification of DNA), as well as doing elution (dissolution). Shock treatments such as cooling at -20°C and the heating of the suspension for 10 minutes in an appropriate buffer to facilitate lysis of the bacterial cell wall. Lysis of the cell wall can also use detergents for example the use of Sodium Dodecyl Sulfate (SDS), Triton X-100 and lysozyme. Chemical lysis can use Proteinase K and Guanidine HCl. Proteinase K is very useful to eliminate DNA-bound proteins (DNA-bound protein) so as to produce good quality DNA (Thakur *et al.,* 2011).

This study using real time PCR. Some studies showed increased sensitivity with the use of Real-time PCR compared to conventional one. In addition, amplification and detection is once running only, the amount of DNA that have been amplified can be seen at any time (real time), no samples are transferred, and no need to prepare the gel so as to minimize the risk of contamination (Thakur *et al.,* 2011). The use of extraction is better (shock treatments) and the use of more sensitive amplification method (real time PCR) gives good results.

A definitive TB meningitis diagnosis can be made using PCR assay despite negative staining and culture result. There were some specimens that were positive by CSF culture but had negative by PCR. The possible explaination is that primer IS 6110 can oly detect Mycobacterium tuberculosis complex (M. tuberculosis, M. bovis, M. africanum, M. canettii, M. microti, M. caprae, M. pinnipedii). The growth of Mycobacterium is slow-growing - nonphotochromogen but not Mycobacterium tuberculosis complex may still be a growth of M. avium complex, M.celatum, M. ulcerans, M. gastri, M. genavense, M. haemophilum, M. malmoense, M. shimoidei, M. simiae, M. xenopi, M. terrae complex, M. heideibergense, M. branderi, M. triplex, M. Conspicuu (Baron *et al.,* 2007). This is the limitation of this study, because the diagnosis of TB culture can only be made by identification through the formation of colonies, the speed of growth, and pigement formed. Further investigation on a replication of thus study using Biochemical culture identification M. tuberculosis (niacin test) and the use the more complex primer is needed because of the increasing incidence of Non tuberculous Mycobacteria with the increase in TB-HIV cases (WHO, 2012).

CONCLUSION

In conclusion, increased CSF protein levels and a decrease in CSF glucose levels are not associated with TB meningitis but mononuclear pleocytosis and abnormalities CSF associated with TB meningitis.

SUGGESTION

Further studies with a larger number of samples using a biochemical test to identify colonies of M. tuberculosis as well as the use of more complex PCR primers for detecting Non tuberculous Mycobacteria is needed

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