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ORIGINAL ARTICLE

A Modified Ink Staining Procedure for Cryptococcus Detection of the Cerebrospinal Fluid by Microscope

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SUMMARY

Background: **The pathogen detection rate of traditional ink staining procedure (TISP) is low.**
Methods: **A modified ink staining procedure (MISP) was created to accumulate pathogen by using cell slide centrifuge. Items of RBC, WBC, and cryptococcus were observed.**
Results: **There were 79.98 ± 54.94 RBC and 126.98 ± 36.39 WBC in one MISP microscopic field whereas there were only 3.35 ± 2.41 RBC and 6.15 ± 1.85 WBC in one TISP microscopic field in the same sample (*200). There was statistical difference between those two methods (p = 0.000). There were 40.78 ± 13.23 mL cryptococcus in CSF processed by MISP whereas there were only 2.10 ± 1.10 mL cryptococcus in the same CSF processed by TISP. There was statistical difference between those two methods.**
Conclusions: **The modified ink staining procedure contributes to the separation of cells and pathogens (such as cryptococcus).**
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KEY WORDS

meningoencephalitis, cryptococcus, cell slide centrifuge, cerebrospinal fluid

LIST OF ABBREVIATIONS

CSF - cerebrospinal fluid
TISP - traditional ink staining procedure
MISP - modified ink staining procedure
CrAg - cryptococcal antigen

INTRODUCTION

Cryptococcus is a cause of fatal meningoencephalitis that kills 250,000 people worldwide each year [1]. The finding of pathogens in the brain parenchyma or cerebrospinal fluid is the gold standard of meningoencephalitis diagnosis. However, biopsy from brain tissue cannot become the major laboratory examination method because of its injury potential which cannot be accepted by both the patient and family. Therefore, the microbi-

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ological test of CSF is the most reliable method as a substitute for biopsy to diagnose meningoencephalitis. CSF direct microscopic examination such as traditional ink staining should centrifuge at high speed first with common centrifuge because of its small volume and low concentration of pathogen, cell, protein, lipid, and non-protein nitrogen so as to increase the biological factor concentration in the sample. According to the research of Schwarz [2], the traditional CSF detection method and its improvement would cause large amounts of cell loss. Even the two-step centrifuge collection method invented by Schwarz himself also led to a 10% minimum cell loss although it was applied widely in clinic [2,3]. The pathogenic microorganisms and protein with less relative molecular mass would lose more in the process of centrifuging thus resulting in the low detection positive rate in the CSF pathogen detection. Furthermore, the CO_2 in the untreated CSF sample would release into the atmosphere easily, lack of buffer and long-time delay would all result in a PH change. The PH of CSF would rise from 7.32 - 7.36 to 7.80 or more in a few seconds, thus the cell and pathogenic microorganisms could survive an extremely short time. To increase the pathogen or encephalitis marker positive rate detection in the small volume CSF sample, the constituent loss must be controlled in the sample processing so as to maintain the character of pathogen, cell, protein, and other substances [4,5]. Therefore, we should decrease the breakage and loss in the process of centrifuging as far as possible. In this study, we used cell slide centrifuge primarily to concentrate CSF, then detected the cells under microscope. It was an efficient way to improve the positive ratio for detection of pathogenic microorganism (such as cryptococcus).

MATERIALS AND METHODS

Criterion of admission and exclusion

The diagnosis for cryptococcal meningoencephalitis was done according to the criteria proposed by Gang Zhao and Steiner [6]. Exclusion criteria include encephalitis caused by sexually transmitted disease, tumor, acute disseminated encephalomyelitis (ADEM), multiple sclerosis (MS), and other central neural system diseases.

Sample source

The encephalitis group includes 40 patients hospitalized in the Clinic Neuroscience Center, The Seventh Affiliated Hospital, Sun Yat-Sen University and Guangdong Province Hospital of TDM from May 2016 to March 2021, 25 males and 15 females, aged from 31 to 88 years, with an average of 56 years. The control group included 40 hospitalized fracture patients from the department of orthopedics without system disease, 27 males and 13 females, aged from 20 to 40 years, with an average of 29 years. Exclusion criteria included symptoms of fever, headache, insomnia, mental disorder in the last 15 days, and an obvious abnormal head imageological examination.

Methods

Major reagent and instrument

Wright-Giemsa and ink dye were purchased from BASO corporation in Taiwan. Cryptococcal Antigen (CrAg) lateral flow assay reagent kits were provided by IMMY Inc, USA.

Sample preparation and examination

Two milliliter samples of CSF was collected through spinal tap and examined according to asceptic principles strictly in biosafety cabinets. 1) TISP [4,5-7]: 1 mL CSF was centrifuged at 2,000 g for 10 minutes, then the supernatant was discarded and a smear was made of the sediment, followed by the addition of the appropriate amount of ink. 2) MISP: 500 μL CSF was added into the precipitation pump, using cell slide centrifuge and centrifuged at 400 g for 3 - 8 minutes until most of the liquid was separated, then the precipitation pump was disassembled and the filter paper removed; a marker pen was used to draw a circle on the back of the slide surrounding the sediment. 3) 1 μL ink was added to the sediment and mixed to observe within the circle under the microscope. All samples were tested by IMMY CrAg lateral assay simultaneously.

Observed items

RBC, WBC, Cryptococcus, CrAg.

Statistical analysis

All data were analyzed by SPSS 19.0 software with Fisher's exact probability test and paired *t*-test.

RESULTS

RBC, WBC of CSF

There were 79.98 ± 54.94 RBC and 126.98 ± 36.39 WBC in one MISP microscopic field whereas there were only 3.35 ± 2.41 RBC and 6.15 ± 1.85 WBC in one TISP microscopic field in the same sample (*200, Figure 1). There was statistical difference between those two methods ($p = 0.000$, Table 1).

Cryptococcus of CSF

No cryptococcus was found in the control group processed by TISP and MISP. No cryptococcus was found in 8 encephalitis group specimens processed by TISP. Cryptococcus was found in another 32 encephalitis specimens processed by TISP, and cryptococcus was found in all encephalitis specimens processed by MISP. There were 40.78 ± 13.23 mL cryptococcus in CSF processed by MISP whereas there were only 2.10 ± 1.10 mL cryptococcus in the same CSF processed by TISP. There was statistical difference between those two methods ($p = 0.001$, Table 1, Figure 2).

Table 1. Comparison of CSF indicators treated by TISP and MISP *.

Group	n	RBC (/HPF*200)			WBC (/HPF*200)			Cryptococcus (mL)		
		TISP	MISP	p-value	TSHP	ISHP	p-value	TSHP	ISHP	p-value
Control	40	1.68 ± 1.21	37.05 ± 23.80	0.000	0.18 ± 0.50	3.48 ± 7.20	0.004	0	0	/
Case	40	3.35 ± 2.41	79.98 ± 54.94	0.000	6.15 ± 1.85	126.98 ± 36.39	0.000	2.1 ± 1.10	40.78 ± 13.23	0.000
p-value		/	/		0.000	0.000		0.001	0.000	

*** Fisher's exact probability test and paired *t*-test.**

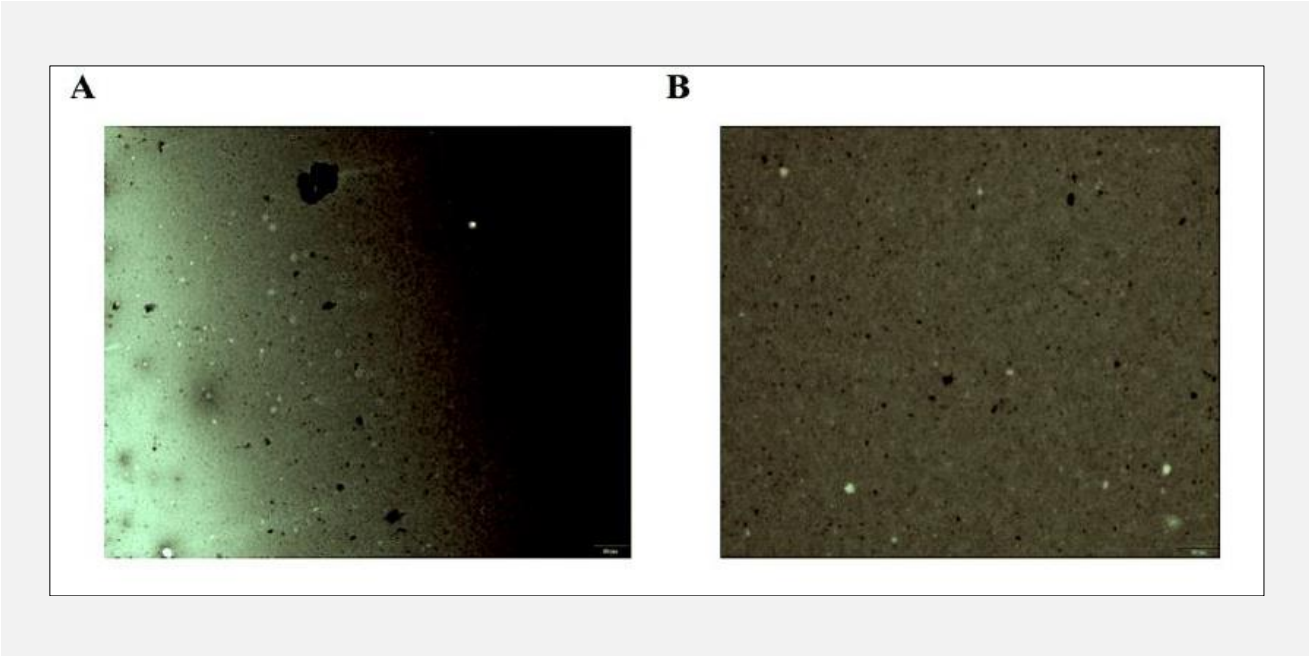

Figure 1. CSF RBC and WBC test.

(A) There were 46 RBC and 2 WBC in one MISP microscopic field, (B) there were only 2 RBC in one TISP microscopic in the same sample field * 200.

CrAg of CSF

CrAg tests were positive in 40 encephalitis specimens, and the control group specimens were all negative.

DISCUSSION

Cryptococcosis is caused by both species of the Cryptococcus species complex (Cryptococcus neoformans and Cryptococcus gattii). Individuals with impaired cell-mediated immunity are at greatest risk of infection. Cryptococcus is one of the most common opportunistic infections in AIDS patients.

Cryptococcal meningoencephalitis laboratory diagnosis is often based on the observation of the characteristic mucopolysaccharide capsule by means of the India ink preparation method [8,9] and the presence of Cryptococcus is confirmed by culture or CrAg, detection of CrAg in serum and CSF has been extensively utilized with very high sensitivity and specificity.

The method of observation for finding the characteristic mucopolysaccharide capsule in CSF is traditional ink staining. CSF is centrifuged at high speed first with a common centrifuge because of its small volume and low concentration of pathogen so as to increase the biological factor concentration in the sample.

The aim of using a cell slide centrifuge before microscopic examination is to concentrate the cell and pathogen in CSF. After this handling, all of the 80 specimens (including the control group) could see more cryptococcus, RBC, and WBC.

Compared with the TISP, the MISP not only concentrat-

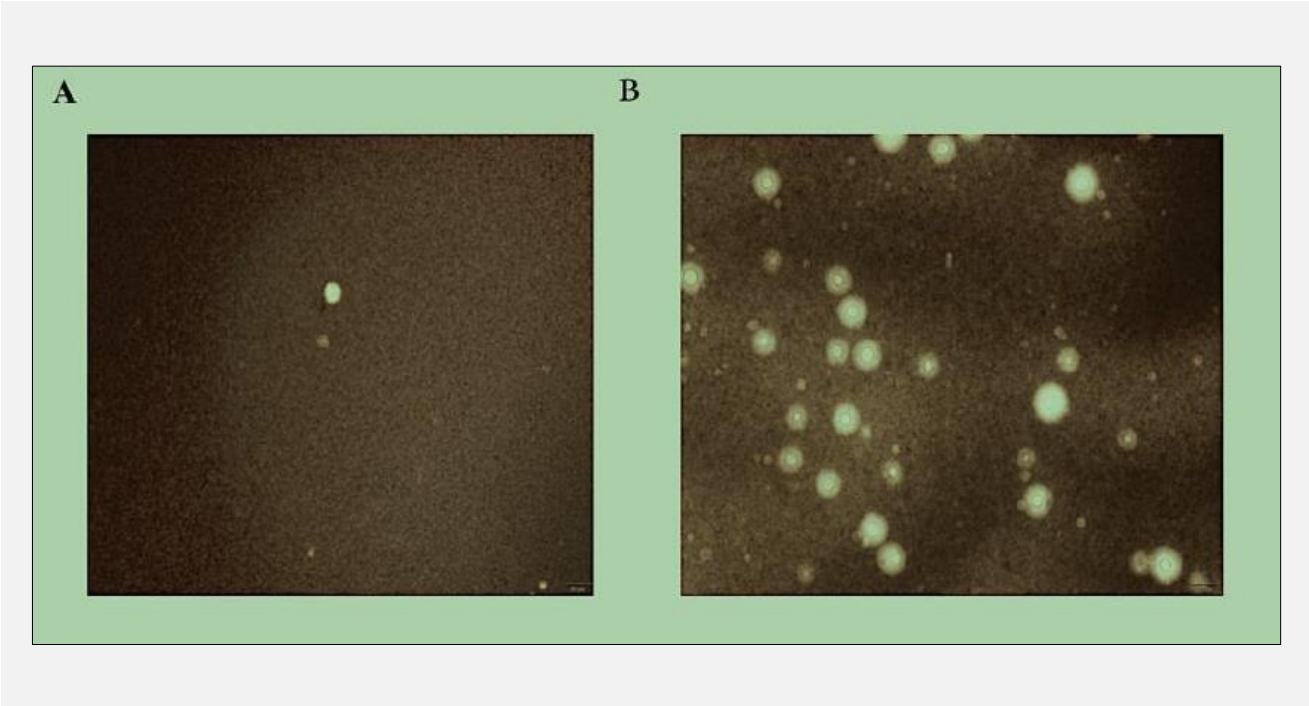

Figure 2. (A) Only 1 cryptococcus was found in one microscopic field processed by TISP * 200, (B) 23 cryptococcus were found in one microscopic field of the same specimen processed by MISP * 200.

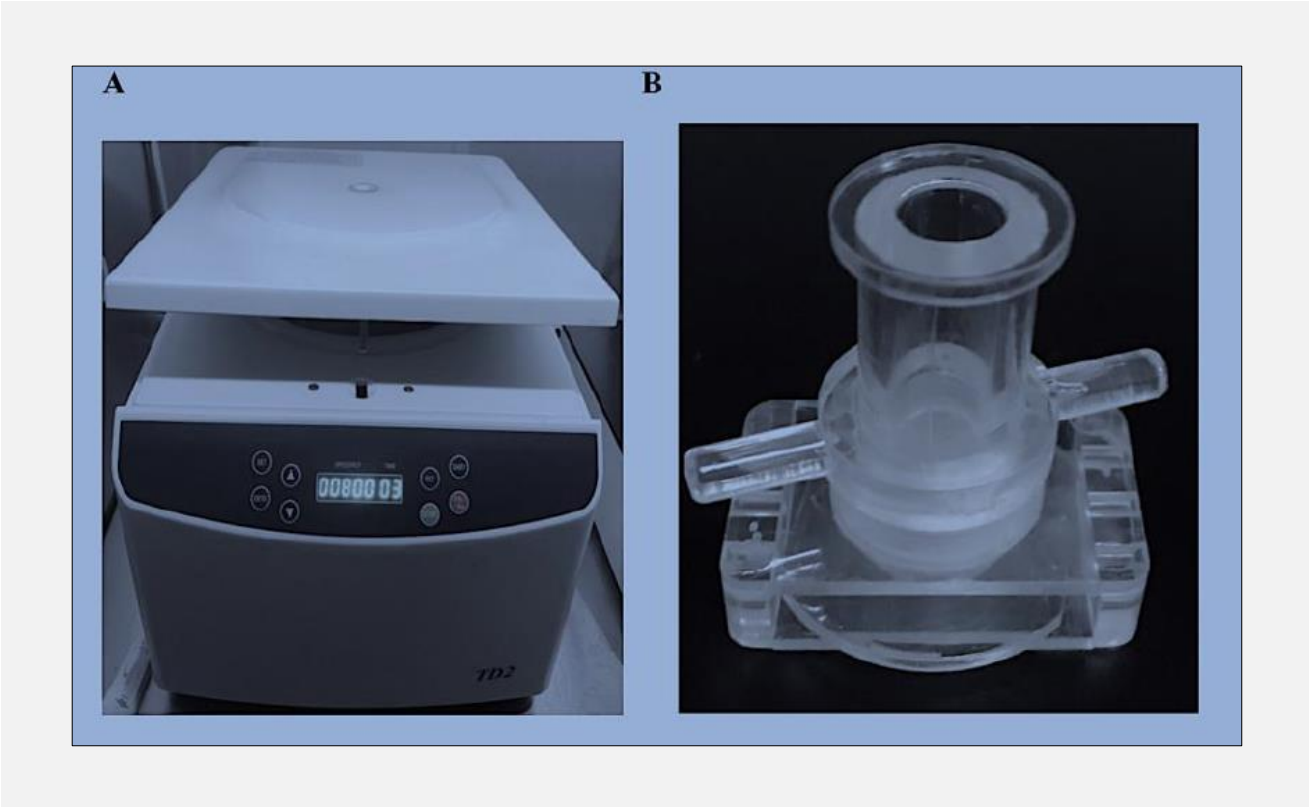

Figure 3. (A) cell slide centrifuge, (B) precipitation pump.

ed but also reduced the breakage of cells and pathomicroorganisms during the centrifuging process. According to Kleine [3], cells are lost during centrifuging and washing. This was the cause of higher test positive rate in the cryptococcal meningoencephalitis CSF.
To summarize, the MISP process concentrated the cells and pathogens, with lower centrifuge speed, reducing cell breakage, maintaining cells, pathogens and form, so that it could increase the positive test rate of pathogens (such as cryptococcus organism) and cells. The small quantity of total sample might have some influence on the result because it was hard to collect CSF, so it is necessary to increase the number of clinic patients for further study.

CONCLUSION

The MISP which used cell slide centrifuge contributes to the separation of cells and pathogens (such as cryptococcus).

Declaration of Interest:
The authors declare no conflict of interest.

References:

1. Williamson PR, Jarvis JN, Panackal AA, et al. Cryptococcal meningitis: epidemiology, immunology, diagnosis and therapy. Nat Rev Neurol 2017 Jan;13(1):13-24 (PMID: 27886201).

2. Schwarz G. Cytomorphology and Cell Yield in a New Cytocentrifugal Technique Allowing the Collection of the Cell-free Supernatant. Lab Med 1991;15(2):45-50. https://doi.org/10.1515/labm.1991.15.2.45

3. Kleine TO, Hackler R, Meyer-Rienecker H. Classical and modern methods of cerebrospinal fluid analysis. Report on the First All-German Symposium of the Society for Laboratory Medicine (FRG) and the Study Group for CSF Analysis and Clinical Neurochemistry of the Society for Psychiatry and Neurology (formerly GDR) in Marburg a. d. Lahn, October 5 - 6,1990. Eur J Clin Chem Clin Biochem 1991;29:705-14 (PMID: 1764547).

4. Kluge H, Wieczorek V, Linke E, Zimmermann K, Witte OW. Atlas of CSF Cytology 2007; New York, Thieme, 7 - 8.

5. Wildemann B, Reiber H, Oschmann P. Laboratory Diagnosis in Neurology, Stuttgart Thieme 2010; 127-8.

6. Steiner I, Budka H, Chaudhuri A, et al. Viral encephalitis: a review of diagnostic methods and guidelines for management. Eur J Neurol 2005;12(5):331-43 (PMID: 15804262).

7. Teunissen CE, Petzold A, Bennett JL, et al. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology 2009;73(22):1914-22 (PMID: 19949037).

8. Land GA. (ed.). 1992. Mycology, p. 6.0.1 - 6.12.4. In HD Isenberg (ed.), Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.

9. Warren NG, Shadomy HJ. 1991.Yeasts of medical importance, p.617 - 629. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.