Research Article

Comparative evaluation between KOH and PAS stain of fungal examination result on lung Tuberculosis patients' sputum with positive Ziehl-Neelsen stain

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ABSTRACT

Mycosis is often misdiagnosed as Tuberculosis (TB) with negative sputum smear results or Tuberculosis recurrent. In the last two-decade, mycosis increases dramatically as HIV and immunocompromised incidence rates increased. Even though, the prevalence data of mycosis in TB patient is less researched, the identification using KOH often give a false negative result. To compare the result of fungal identification using KOH and PAS staining. This study is observational analytical using a cross-sectional design with 29 TB samples that have been diagnosed with Ziehl-Neelsen (ZN) stain positive in RSUD Dr. Soetomo. The sputum of every sample was colored with KOH and PAS and observed by the researcher and microbiology analyst. The results of this study were 22 sputa identified with Candida sp positive and 12 sputa with non-Candida positive using KOH staining. Meanwhile, using PAS staining, there were 25 sputa identified as Candida sp positive and 22 sputa as non-Candidapositive. The congruency test between KOH and PAS has a fair result for both identifying Candida sp (κ = 0.298; significance = 0.069) and non-Candida (κ = 0.240; significance = 0.095). The identification of candida and non-candida in TB patients using KOH and PAS has a fair congruency result. The PAS staining has better identification results both in identifying candida and non-candida rather than KOH staining.

INTRODUCTION

Pathogenic fungal is a microorganisms that can cause disease in humans because fungi grow cosmopolitically with human life both on land and in water (Kawilarang, 2018). Fungal infections are called mycoses. Mycosis in the respiratory system can occur in the upper respiratory tract and lower respiratory tract. The upper airway mycosis is most often found as fungal sinusitis, while the lower airway mycosis is often misdiagnosed as negative-smear pulmonary tuberculosis (TB) or recurrent pulmonary TB. This is due to the lack of pathognomonic clinical symptoms and radiological characteristics that are typical for these two diseases and inadequate examination facilities in mycology laboratories (Mathavi et al., 2014).

In the last two decades, lung mycosis even increased dramatically with the increasing number of patients with immunosuppressed states (José & Brown, 2012). Several kinds of lung pathogenic fungi such as *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii*, *Paracoccidioides brasillensis*, *Blastomyces dermatitidis* and *Cryptococcus neoformans* while the fungal that often cause opportunistic infections are *Candidiasis sp*. and *Aspergillosis sp* (Brooks et al., 2013; Sukamto, 1994; Latgé & Chamilos, 2019).

Laboratories examinations that are routinely carried out to diagnose mycosis are KOH and Parker ink. KOH and Parker ink do not produce good color contrast, so it requires an experienced examiner for interpreting results well. PAS staining is one kind of fungal stain that is not common to be done (Hauber & Zabel, 2009). PAS staining will give magenta-purple color to glucose, which is commonly found in starch, cellulose, mucin, chitin, reticular, fibrin, collagen, fungi, and parasites (Bancroft & Stevens, 1990). This is why PAS staining can clarify observers in identifying fungi's morphology. Based on that problem, this study aimed to determine the prevalence of pulmonary fungal infection in pulmonary TB patients as well as know the comparison between the result of KOH and PAS staining in fungal examinations.
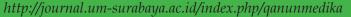
METHODS

This study was observational and analytical with a cross-sectional design study. This study has been ethically approved by *Komite Etik Penelitian Kesehatan RSUD Dr. Soetomo Surabaya* under the ethical clearance certificate number of 1273/KEPK/VII/2019. The sample of this study was 29 patients who were diagnosed with positive-ZN TB during May – July 2019 that met the inclusion and exclusion criteria. The inclusion criteria of this study were: (1) patients with minimally 2 ml extra sputum in the sputum pot collected in Microbiology Laboratorium RSUD Dr. Soetomo either for diagnosing or following up TB's treatment; (2) patients who were diagnosed with positive-ZN TB. Meanwhile, the exclusion criteria of this study were: (1) patient with extra sputum less than 2 ml in the sputum pot collected in Microbiology Laboratorium RSUD Dr. Soetomo; (2) patients who collected sputum mixed with saliva; (3) patient refused to be included in this study.

Patients that met the criteria were collected as the study's subjects with consecutive sampling. These samples were created into one KOH specimen and one PAS specimen. The authors, one laboratory analyst, and one Microbiology consultant (as a reviewer) observed the specimens and recorded the fungi genus. The data collected was then displayed in tables and analyzed with SPSS (using Chi-Square) to count the Kappa score of congruencies.

RESULTS

Of 29 samples in this study, 16 of them were male and 13 others were female. The characteristic of the subject was described in Table 1.

Table 2 below shows that PAS staining can help observers to identify the presence of fungi in specimens better than KOH staining. KOH staining can only help the identification of Candida in 22 samples, whereas by PAS staining, Candida was identified in 25 samples. Not much different, the identification of non-Candida fungi with KOH staining can only identify 11 samples, while with PAS staining as many as 22 samples were identified as positive non-Candidamycosis.

Table 1. The characteristic of subjects.

Characteristic	ZN Score		
	1+	2+	3+
Gender			
Male	7 (24.1%)	6 (10.7%)	3 (10.3%)
Female	3 (10.3%)	10 (34.4%)	0 (0%)
Age			
20 – 29 years old	0 (0%)	4 (13.8%)	1 (3.4%)
30 – 39 years old	2 (6.9%)	1 (3.4%)	0 (0%)
40 – 49 years old	1 (3.4%)	1 (3.4%)	0 (0%)
50 – 59 years old	3 (10.3%)	4 (13.8%)	1 (3,4%)
60 – 69 years old	3 (10.3%)	3 (10.3%)	1 (3.4%)
70 – 79 years old	1 (3.4%)	3 (10.3%)	0 (0%)

Table 2. The congruency between KOH and PAS staining of fungal examination result

ZN Score	Type of Fungi	KOH		PAS	
		1st observer	2nd observer	1st observer	2nd observer
1+	Candida	8 (27.6%)	8 (27.6%)	8 (27.6%)	8 (27.6%)
	non-Candida	4 (13.8%)	4 (13.8%)	8 (27.6%)	8 (27.6%)
2+	Candida	12 (41.4%)	12 (41.4%)	15 (51.7%)	15 (51.7%)
	non-Candida	8 (27.6%)	8 (27.6%)	13 (44.8%)	13 (44.8%)
3+	Candida	2 (6.9%)	2 (6.9%)	3 (10.3%)	3 (10.3%)
	non-Candida	1 (3.4%)	1 (3.4%)	2 (6.9%)	2 (6.9%)
Total	Candida	22 (75.86%)	22 (75.86%)	25 (86.21%)	25 (86.21%)
	non-Candida	12 (37.93%)	11 (37.93%)	22 (75.86%)	22 (75.86%)

Table 3. The congruency analysis of KOH and PAS for Candida identification

			PAS		Total
			Candida positive	Candida negative	
KOH	Candida positive		21	1	22
	Candida negative		5	2	7
		Total	26	3	29

Table 4. The congruency analysis of KOH and PAS for non-Candida identification

			PAS		Total
			Candida positive	Candida negative	
KOH	Candida positive		11	1	12
	Candida negative		11	6	17
		Total	22	7	29

Based on the congruency analysis, it is obtained that the Kappa coefficient value of Candida identification using KOH and PAS is 0.298 with a significance of 0.069. This value means that KOH and PAS staining has fair congruency to identify Candida.

On the other hand, it is obtained that the Kappa coefficient value of non-Candida identification using KOH and PAS staining is 0.0240 with a significance of 0.095. This value means that the KOH and PAS staining is also having fair congruency to identify non-Candida fungi.

DISCUSSION

Diagnosing mycosis is not only based on clinical symptoms but also requires laboratory examination. Microbiological laboratory examination is routinely used as it can approach. Fungal diagnosis accurately by direct microscopic examination of the patient's specimen. This examination is also a fast, useful, and effective examination to identify the fungi's genus or even species by observing the hyphae or pseudohyphae (Thompson et al., 2021).

KOH is a routine stain used to do the direct examination of fungi. This solution is a clear solution that can dissolve fat, lyse epithelium, and dissolve proteins other than fungi. The fungi's element will survive the KOH solution because fungi have chitin and glycoprotein in their cell wall. Therefore, the recommended KOH concentration in microbiological staining is 10% - 30% so that the fungi's elements that will be examined do not dissolve and produce false negative examination results (Nugroho, 2013).

In this study, the results of the 20% concentrated KOH examination conducted by authors can provide identification results of 21 samples with Candida positive and 11 samples with non-Candida positive. The results of identification using KOH staining are still lower than the results of identification when using PAS staining, where 25 Candida-positive and 22 non-Candida positive were obtained.

The better results of PAS staining identification are due to several factors. The first factor is that

PAS staining can give glycogen color in fungi so that the structure of the fungi can be seen more clearly. This can't be obtained from KOH where KOH staining doesn't contain dyes that can clarify the structure of fungi. In addition, PAS staining is not as volatile as KOH staining. In the PAS staining process, the specimen is immersed in 90% alcohol before being stained to maintain the cell's integrity. This 90% alcohol solution can keep the specimen for 90 days or 3 months with a fixed or unchanged cell structure. Otherwise, in KOH staining, samples that have been stained for more than 15 minutes may evaporate and affect the results of identification, where the sample is not as clear as the examination carried out before evaporation occurs.

The results of fungi identification using KOH staining that can't identify well are also obtained in previous studies. KOH staining does not produce contrasting colors between the fungi and the background (Noviandini et al., 2017). Previous research on identifying fungi using KOH staining mentioned that KOH staining will show fungi's elements on transparent on a transparent background which causes KOH staining identification and requires experienced observers to be able to identify or interpret the result properly. This condition might be improved by adding Parker ink on KOH staining, even though, it was also mentioned fungi's element may have poor or even no absorption of Parker ink that making it more difficult to detect fungi's structure (Tambosis & Lim, 2012). The lack of quality of KOH staining and Parker ink is the presence of bluish precipitates (bluish debris) around the fungi's element that may confuse the true fungi's element as it should (Lim & Lim, 2008).

From this study, the authors also found that the analysis of congruency between KOH staining and PAS staining is fair enough to identify both Candida and non-Candida fungi, with respectively Kappa value of 0.298 (significance of 0.069) and 0.024 (significance of 0.095). This Kappa value supports this study hypothesis, that there is a match between the result of Candida and non-Candida examination using KOH and PAS staining in the sputum of pulmonary TB patients with positive ZN in RSUD Dr. Soetomo.

The low congruency result between KOH and PAS staining is caused by the difference between Candida and non-Candida findings on both examinations, where PAS staining always show higher findings than KOH staining. This happens because PAS staining can produce pink, magenta, to purple colors on the fungi's tissue surface that contains glycogen and chitin (Kiernan, 1990; Suntoro, 1983).

CONCLUSION

This study showed the identification of Candida and non-Candida fungi using KOH and PAS staining has fair congruency. PAS staining can identify both Candida and non-Candida fungi better than KOH staining. The prevalence of non-Candida fungi in the sputum of pulmonary TB patients with positive ZN stain in RSUD Dr. Soetomo was 22/29 (75.86%) while the prevalence of Candida fungi was 26/29 (89.66%). Based on this study, the PAS staining could be used as an alternative staining in conducting laboratory examinations to establish a more effective and efficient Mycosis diagnosis.

REFERENCES

Bancroft, J. D., & Stevens, A. (1990). *Theory and Practice of Histological Techniques 3rd Edition* (3rd ed.). Churchill Livingstone.

Brooks, G. F., Carroll, K. C., Butel, J. S., & Morse, S. A. (2013). *Jawetz, Melnick, & Adelberg's Medical Microbiology* (26th ed.). McGraw-Hill Medical.

Hauber, H. P., & Zabel, P. (2009). PAS staining of bronchoalveolar lavage cells for differential diagnosis of interstitial lung disease. *Diagnostic Pathology*, *4*, 13. https://doi.org/10.1186/1746-1596-4-13

José, R. J., & Brown, J. S. (2012). Opportunistic and fungal infections of the lung. *Medicine (Abingdon, England : UK Ed.)*, *40*(6), 335. https://doi.org/10.1016/J.MPMED.2012.03.013

Kawilarang, A. P. (2018). *Bahan Kuliah Mikologi* (2nd ed.). Fakultas Kedokteran Universitas Airlangga.

Kiernan, J. A. (1990). *Histological and Histochemical Method; Theory and Practice. 2nd ed* (2nd ed.). Pergamon Press.

Latgé, J.-P., & Chamilos, G. (2019). Aspergillus fumigatus and Aspergillosis in 2019. *Clinical Microbiology Reviews*, *33*(1). https://doi.org/10.1128/CMR.00140-18

Lim, S. L., & Lim, C. S. H. (2008). New contrast stain for the rapid diagnosis of pityriasis versicolor. *Archives of Dermatology*, *144*(8), 1058–1059. https://doi.org/10.1001/ARCHDERM.144.8.1058

Mathavi, S., Shankar, R., Kavitha, A., Sasikala, G., & Priyadharsini, I. (2014). A STUDY ON PREVALENCE OF PULMONARY CANDIDIASIS AMONG TUBERCULOSIS PATIENTS AND USE OF CHROMAGAR IN IDENTIFICATION OF CANDIDA SPECIES. *Journal of Drug Delivery and Therapeutics*, *4*(3), 118–121. https://doi.org/10.22270/JDDT.V4I3.821

Noviandini, A., Suyoso, S., & Astari, L. (2017). *Pemeriksaan Pewarnaan Kalium Hidroksida (KOH) 20% + Tinta Parker TM Blue-Black, Chicago Sky Blue (CSB), dan Kultur Jamur pada Dermatomikosis Superfisialis (Parker ink-KOH stain, Chicago Sky Blue (CSB) stain, and Fungi Culture, for The Diagnosis of Superficial Dermatomycoses)*.

Nugroho, S. A. (2013). *Pemeriksaan penunjang diagnosis mikosis superfisialis. In: Bramono K, Suyoso S, Indriatmi W, Ramali L, Widaty S, Ervianti E. Dermatomikosis superfisialis. Edisi ke-2. Jakarta: Badan Penerbit FKUI; 2013. Page: 154-66*.

Sukamto. (1994). Pemeriksaan Jamur Bilasan Bronkus pada Penderita Bekas TB Paru. *Repository USU*.

Suntoro, S. H. (1983). *Metode Pewarnaan Histologi dan Histokimia*. Bhratara Karya Aksara.

Tambosis, E., & Lim, C. (2012). A comparison of the contrast stains, Chicago blue, chlorazole black, and Parker ink, for the rapid diagnosis of skin and nail infections. *International Journal of Dermatology*, *51*(8), 935–938. https://doi.org/10.1111/J.1365-4632.2011.05222.X

Thompson, G. R., Le, T., Chindamporn, A., Kauffman, C. A., Alastruey-Izquierdo, A., Ampel, N. M., Andes, D. R., Armstrong-James, D., Ayanlowo, O., Baddley, J. W., Barker, B. M., Lopes Bezerra, L., Buitrago, M. J., Chamani-Tabriz, L., Chan, J. F. W., Chayakulkeeree, M., Cornely, O. A., Cunwei, C., Gangneux, J.-P., … Pasqualotto, A. C. (2021). Global guideline for the diagnosis and management of the endemic mycoses: an initiative of the European Confederation of Medical Mycology in cooperation with the International Society for Human and Animal Mycology. *The Lancet. Infectious Diseases*, *21*(12), e364–e374. https://doi.org/10.1016/S1473-3099(21)00191-2