A Comparison of Sputum Examination for Acid Fast Bacilli by Modified Cold Stain and Ziehl-Neelsen Stain for Screening of Pulmonary Tuberculosis

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Abstract : **A comparative study of the conventional Ziehl-Neelsen (ZN) stain and a new modified cold (MC) stain was carried out to evaluate the efficiency of this staining method in sputum examination for acid fast bacilli (AFB). The MC technique was simplified by avoiding the need for heat and combining the stage of counterstaining to overcome the problems of aerosolized phenol and the more laborious heating method. Of the 392 sputum samples examined, 84 were positive and 297 were negative on both staining techniques, with an agreement of 97.2%. In comparison with culture as the gold standard for the diagnosis of tuberculosis, the ZN stain exhibited a sensitivity, specificity, positive and negative predictive values and efficiency of 68.9, 97.4, 92.1, 87.8 and 88.8%, respectively. The same values for the MC stain were 70.6, 97.8, 93.3, 88.4 and 89.5%, respectively with no statistically significant differences ($P > 0.05$) between the 2 methods. The MC stain was also as reliable as the ZN stain in retaining the color of the stained slide after prolonged storage; an agreement with the first reading was 90% after 4 weeks storage and 80% after 16 weeks storage. The staining reagents had a long shelf life; with agreement between both staining methods of 100% at every time of re-stocking aliquots. To apply this new MC stain for future use at the peripheral level of the health care system, we made a survey using questionnaires sent to 200 hospitals. Most of the respondants accepted that the MC stain was easier to perform, more comfortable and much less expensive than the ZN stain. Altogether, these factors make the MC stain suitable for its use as a practical and rapid sputum staining test for screening of patients with pulmonary tuberculosis and for assessment of their treatment.**

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เรื่องย่อ : **เปรียบเทียบการตรวจเสมหะด้วยการย้อมสีทนกรดวิธีเย็นดัดแปลงกับวิธีซีลเนลเซนเพื่อการตรวจคัดกรองผู้ป่วยวัณโรคปอด**

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การศึกษาเปรียบเทียบประสิทธิภาพของเทคนิคการตรวจเสมหะโดยการย้อมสีทนกรดด้วยวิธีมาตรฐานคือวิธีซีล-เนลเซน กับการย้อมสีวิธีเย็นที่ได้ดัดแปลงขึ้นใหม่ เพื่อใช้ในการคัดกรองผู้ป่วยวัณโรคปอดเบื้องต้น การย้อมสีทนกรดวิธีเย็นนี้ได้ดัดแปลงเทคนิคให้ง่าย ไม่ต้องลนไฟช่วยในการติดสี และลดขั้นตอน โดยการรวมขั้นตอนการล้างสีและการย้อมทับเป็นขั้นตอนเดียวกัน ซึ่งง่ายและสะดวกกว่า และยังลดปัญหาความยุ่งยากรวมทั้งปัญหาไอระเหยของฟีนอลที่เกิดจากการลนไฟโดยวิธีของซีล-เนลเซนที่ใช้กันอยู่ในขณะนี้ได้ ในการตรวจเสมหะผู้ป่วยใหม่ จำนวน 392 ตัวอย่าง พบว่าทั้งวิธี ซีล-เนลเซน และวิธีเย็นดัดแปลงให้ผลบวกกับเสมหะ 84 ตัวอย่าง และผลลบ 297 ตัวอย่าง โดยที่ผลของทั้ง 2 วิธี มีความสอดคล้องกันถึงร้อยละ 97.2 และเมื่ออาศัยผลการเพาะเชื้อเป็นเกณฑ์มาตรฐานในการวินิจฉัยวัณโรคปอดในผู้ป่วยใหม่ พบว่า วิธี ซีล-เนลเซน ให้ค่าความไว ความจำเพาะ ค่าคาดหวังของผลบวก ค่าคาดหวังของผลลบ และประสิทธิภาพ คิดเป็นร้อยละ 68.9, 97.4, 92.1, 87.8 และ 88.8 ตามลำดับ ในขณะที่วิธีเย็นดัดแปลงให้ค่าดังกล่าว คิดเป็นร้อยละ 70.6, 97.8, 93.3, 88.4 และ 89.5 ตามลำดับ ซึ่งพบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) สำหรับความคงทนของสีย้อมบนสไลด์เมื่อย้อมโดยวิธีเย็นดัดแปลง รวมทั้งสารละลายของสีต่าง ๆ ที่ใช้ในการย้อมเสมหะ สามารถเก็บไว้ได้นานเหมือนกับของวิธีซีล-เนลเซน ผลการสำรวจการยอมรับของผู้ปฏิบัติจากแบบสอบถามจำนวน 200 ตัวอย่าง พบว่าส่วนใหญ่ยอมรับว่าวิธีเย็นดัดแปลงเป็นวิธีการย้อมที่ง่าย สะดวก และประหยัดกว่าวิธีซีล-เนลเซน ดังนั้นวิธีเย็นดัดแปลงที่ได้พัฒนาปรับปรุงขึ้นนี้จึงนับว่าเป็นวิธีการย้อมเสมหะเพื่อหาเชื้อทนกรดที่รวดเร็วและน่าจะเหมาะสมในทางปฏิบัติโดยเฉพาะสำหรับห้องปฏิบัติการระดับภูมิภาค เพื่อใช้ในการคัดกรองผู้ป่วยวัณโรคปอดเบื้องต้น รวมทั้งใช้ในการประเมินผลการรักษาในผู้ป่วยวัณโรคปอดได้

INTRODUCTION

The emergence of epidemic multiple-drug-resistant strains of *Mycobacterium tuberculosis* particularly in HIV-positive individuals in conjunction with the upward trend of reported cases of tuberculosis worldwide[1,2] including Thailand[3] represents a major public health problem. Thus, we urgently need improvements in the implementation of existing strategies for tuberculosis control, with particular emphasis on early diagnosis and delivery of effective treatments. At present, the diagnosis of tuberculosis is most commonly made by using microscopy and culture. In Thailand, tuberculosis laboratory services are organized down to the district hospital level providing smear microscopy at all levels, but culture

examination and sensitivity tests are only available at the central level, some TB centers, and provincial hospitals.

For those reasons, microscopic examination for acid fast bacilli (AFB) has been the mainstay for the immediate clinical diagnosis of pulmonary tuberculosis while the results of sputum cultures are pending. Due to its simplicity and low cost, sputum examination by direct microscopy is still the method that is widely used throughout the country for the primary diagnosis of pulmonary tuberculosis, case finding, and for assessment of treatment. The usual staining technique has been the Ziehl-Neelsen (ZN) method which is the most common laboratory technique for staining AFB and is accepted as a conventional method. However, this method requires controlled heating for its success and there are certain disadvantages, for example, multiple stages of staining, it is cumbersome and produces some discomfort when the phenol is aerosolized.

In our preliminary study, we described an improved acid-fast staining technique namely "modified cold (MC) stain" in sputum examination for the primary diagnosis of tuberculosis.[4] This staining procedure uses the same staining solution as the conventional ZN, without increasing the concentration of basic fuchsin-phenol staining solution. The complexity of the staining stage is reduced by not heating and combining the stage of counterstaining thus making it easier, faster and safer as it does not require aerosolized phenol. In comparison with culture as the "gold standard" for primary tuberculosis diagnosis, the results demonstrated that the method was efficient because of high sensitivity and specificity.

In this present study, we further evaluated the efficiency of this MC stain in comparison with the ZN stain for sputum examination of AFB, and studied the stability of staining reagents and the stability of stained slides for reexamination after storage at different times. In addition, the acceptability of this MC stain technique to be used routinely in the future by laboratory workers at the peripheral level of the health care system was also surveyed by using a questionnaire.

MATERIALS AND METHODS

This study is a screening test using specimens obtained from newly diagnosed suspected tuberculosis patients at Bangkok Central Chest Clinic. These patients were over 15 years old with chest symptoms that were suggestive of suspected tuberculosis. The sample size used for this study was 392 cases. Before the study was performed, the researcher was trained and required further standardization in the reading of unknown slides (blind) until he/she achieved the same high level of ability as an experienced microscopist.

Specimen collection

Either spot or collection sputum was taken before any medication was given. The quantity obtained was at least 3 ml. All specimens were divided into 2 categories by randomized allocation as follows : Category I, if the labelled specimens were odd numbers, they were stained by ZN before MC stain; and Category II, if the labelled specimens were even numbers, they were stained by MC before ZN stain.

Smear preparation

Two slides were prepared by smear directly from the purulent or mucopurulent portion of the sputum. Sputum was spread evenly over an area 1 by 2 cm. After the smear was air dried, it was fixed by flaming or placing on the hot plate for a few minutes. Then the fixed smear was stained by the ZN and MC stain.

Ziehl-Neelsen (ZN) stain

The ZN procedure followed the method described elsewhere.[5,6] Firstly, it was stained with carbol fuchsin by flooding the fixed smear with a solution prepared by dissolving 0.3 gm of basic fuchsin in 10 ml of ethanol and then diluting it to 100 ml with aqueous 5% phenol. The smear was gently heated until it steamed with a flame from a Bunsen burner for 5 min. It was then rinsed with water, and was decolorized with 3% acid-alcohol and allowed to stand for 2 min. It was rinsed with water, and counterstained with 0.1% methylene blue for 10 sec.

Then the slide was rinsed with water and air dried before examination. Using a microscope the stained smear was scanned with the X100 oil immersion objective lens for the presence of red thin rods or coccobacilli.

Modified cold (MC) stain[4]

The smear was first stained with carbol fuchsin by flooding the fixed smear with a solution prepared by dissolving 0.3 gm of basic fuchsin in 10 ml of ethanol and then diluted it to 100 ml with aqueous 5% phenol. It was allowed to stand for 10 min, and was then rinsed with water. Following this, the slide was counterstained with modified methylene blue for 2 min (dissolving 1 gm of methylene blue in 20 ml of sulfuric acid, 30 ml absolute alcohol and 50 ml distilled water). Then the slide was again rinsed with water and dried before examination. Using a microscope the stained smear was scanned with the X100 oil immersion objective lens for the presence of red thin rods or coccobacilli.

Report of microscopic findings

Reports of the results of the smear examination included a measure of quantitation, such as the actual number of bacilli seen per field or a 1+ to 3+ rating according to the convention of the American Thoracic Society.[7]

Culture and identification

Sputum samples were decontaminated by swab sputum culture of Nassue[8] and inoculated onto 2 slants of Lowenstein-Jensen (L-J) medium. Slants were incubated at 37°C for 8 weeks and examined weekly for growth. *M. tuberculosis* were differentiated from the other species by their rates of growth, colony pigmentation and morphology, and some biochemical tests.[5]

Stability of the stained slides

To study the reliability of each staining method for retaining color after storage, five smear preparations were made directly from 20 of the sputum specimens (a total of 100 smears). All stained slides in this study were classified by the results on ZN and MC stain. The 20 stained slides for each staining method were divided by reading the results according to the National Tuberculosis Associated of United States of America (NTA) scale, using the scores : N (-), 1+, 2+ and 3+. Stained slides were labeled by a blind method (ZN1 to ZN20 and MC1 to MC20). These slides were examined and the results read again after storing for 4, 8 and 16 weeks, respectively. The results reported for each staining method after storage at each time duration were compared with those results of the first reading.

Stabiltiy of the staining reagent aliquots

In order to assess the reliability of each staining method for stability of the staining reagents after storage at different times, i.e., 0, 2, 4, 8, and 16 weeks, a number of fixed smear slides were made at the same time as the blind unknown sputum samples. For each staining method, five smear preparations were made directly from 20 of the sputum specimens. After fixing the smear as described earlier, they were divided into 5 sets, and each set was subjected to staining with the staining reagents; for example, the first set of fixed smear slides was stained at the first day, the second set was stained after 2 weeks, the third set after 4 weeks, the fourth set after 8 weeks and the fifth set after 16 weeks, respectively. The staining results obtained at different times were compared with those results of the first time.

Acceptance of the staining methods

In order to assess the feasibility for acceptance of the new MC staining method to be used in mycobacteriology laboratories, a total of 200 questionnaires were sent to general hospitals and community hospitals following a simple random technique. The questionnaires consisted of : (i) general data of the respondants, (ii) the thoughts of the respondants with regard to staining sputum for the diagnosis of tuberculosis, and (iii) the acceptance of the staining method by the respondants.

RESULTS

Comparison between the researcher and the experienced microscopist in slide reading of AFB-smear positive and negative is shown in Table 1. Of the 100 unknown sample slides, 53 were classified as positive and 45 as negative by the two readers with an agreement of 98%.

Table 1. Comparison between the researcher and the experienced microscopist in slide reading of AFB smear positive and negative.

Results of the experienced microscopist	Results of the researcher +	Results of the researcher -	Total
+	53	2	55
-	0	45	45
Total	53	47	100

Mc Nemar's $X^2 = 2.00$ df. = 1, P = 0.157
K = 0.9559, Z = 9.537, P < 0.01

Correlation of ZN and MC stains. Table 2 displays the percentages of sputum staining results by the ZN and the MC stains. Of the 392 smear samples examined, 89 (22.7%) were reported positive by the ZN while 90 (23.0%) were reported positive by the MC stain. If classified by grading smear results, the ZN reported 1+, 2+, 3+ and negative were 14.0, 4.3, 4.3 and 77.3%, respectively; while the MC stain were 11.5, 6.1, 5.4 and 77.0%, respectively. The comparative summary of the results is shown in Table 3. Of these tests, 84 were positive and 297 were negative by both staining techniques, with an agreement of 97.2%.

Table 2. The percentages of sputum staining results by the Ziehl-Neelsen (ZN) and the modified cold (MC) stains.

Result by grading	Sputum staining method: ZN stain	Sputum staining method: MC stain
Positive		
1+	55 (14.0%)	45 (11.5%)
2+	17 (4.3%)	24 (6.1%)
3+	17 (4.3%)	21 (5.4%)
Negative	303 (77.3%)	302 (77.0%)
Total	392 (100.0%)	392 (100.0%)

Table 3. Comparative summary of the staining results obtained from 392 smears stained by the Ziehl-Neelsen (ZN) and the modified cold (MC) stains in slide reading of AFB-smear positive and negative.

MC stain	ZN stain		Total
	Positive	Negative	
Positive	84	6	90
Negative	5	297	302
Total	89	303	392

Mc Nemar's $X^2 = 0.09$, d.f. = 1, P = 0.763
K = 0.920, Z = 13.441, P < 0.01

Validities of ZN and MC stains. Table 4 shows the validities of the ZN and the MC stains in comparison with culture for the diagnosis of pulmonary tuberculosis. The ZN stain exhibited the sensitivity, specificity, positive and negative predictive values and efficiency of 68.9, 97.4, 92.1, 87.8 and 88.8%, respectively. The same values for the MC stain were 70.6, 97.8, 93.3, 88.4 and 89.5%, respectively. There were no statistically significant differences in the sensitivities, specificities and efficiencies between the ZN and MC stain (P > 0.05).

Table 4. Validities of the Ziehl-Neelsen (ZN) and the modified cold (MC) stains for primary diagnosis of pulmonary tuberculosis using the culture result as the gold standard.

Staining methods & results	Culture result			Sensitivity	Specificity	Predictive value (%)		Efficiency
	Positive	Negative	Total	(%)	(%)	Positive	Negative	(%)
ZN stain				68.9	97.4	92.1	87.8	88.8
Positive	82	7	89					
Negative	37	266	303					
Total	119	273	392					
MC stain				70.6	97.8	93.3	88.4	89.5
Positive	84	6	90					
Negative	35	267	302					
Total	119	273	392					

Stability of the stained slides. Comparative stability of slides stained by the ZN and the MC and storage at different times is shown in Table 5. The 20 stained slides for each staining method were divided by grading of the AFB smear into negative, 1+, 2+ and 3+; and each grading group consisted of 5 slides. After storage for 4 weeks, both staining methods still gave identical results with an agreement as the first reading of 90% (18 of 20) and under-reading of 10% (2 of 20). After 8 weeks, agreement of the results in the MC group was 95% (19 of 20) which was definitely higher and over-reading was 5% (1 of 20); while agreement of the results in the ZN group was 90% (18 of 20), under-reading was 5% (1 of 20) and missing was 5%. After 16 weeks, the agreement results in the ZN group was 85% (17 of 20), under-reading was 10% (2 of 20) and missing was 5%, while the MC group was 80% (16 of 20), under-reading was 10% (2 of 20) and missing was 10%.

Table 5. Comparison of the results of slides stained by the Ziehl-Neelsen (ZN) and the modified cold (MC) stains after storage at different times.

Result at timing of storage	Result of storage at 0 week									
	ZN stain					MC stain				
	Neg.	1+	2+	3+	Total	Neg.	1+	2+	3+	Total
At 4 weeks										
Negative	5				5	5				5
1+		5			5		5			5
2+		2	3		5		2	3		5
3+				5	5				5	5
Total	5	7	3	5	20	5	7	3	5	20
	K=0.866, Z=6.618, P<0.01					K=0.866, Z=6.618, P<0.01				
At 8 weeks										
Negative	5				5	5				5
1+	1	4			5		5			5
2+		1	4		5			4	1	5
3+				5	5				5	5
Total	6	5	4	5	20	5	5	4	6	20
	K=0.866, Z=6.689, P<0.01					K=0.933, Z=7.205, P<0.01				
At 16 weeks										
Negative	5				5	5				5
1+	1	4			5	2	3			5
2+		2	3		5		2	3		5
3+				5	5				5	5
Total	6	6	3	5	20	7	5	3	5	20
	K=0.799, Z=6.128, P<0.01					K=0.732, Z=5.592, P<0.01				

Stability of the staining reagents. Comparative stability of both staining reagent sets after storage at different times is shown in Table 6. The results obtained at the begining showed that both staining reagent sets had 100% agreement (20 of 20). After storage for 2, 4, 8 and 16 weeks, respectively the agreement results were 90, 85, 85 and 85%, respectively. If obtained by pooling the data under negative and those under 1+, 2+ and 3+ on the other side. Agreement between both staining methods was 100% at every time of re-stocking of aliquots.

Table 6. The frequency of agreement between the Ziehl-Neelsen (ZN) and the modified cold (MC) stains after storing aliquots of staining reagent sets at 0, 2, 4, 8 and 16 weeks.

	ZN stain				
MC stain	**Negative**	**1+**	**2+**	**3+**	**Total**
Week 0					
Negative	5				5
1+		5			5
2+			5		5
3+				5	5
Total	5	5	5	5	20
			K = 1.000, Z = 7.746, P< 0.01		
Week 2					
Negative	5				5
1+		5	1		6
2+		1	1		2
3+				7	7
Total	5	6	2	7	20
			K = 0.860, Z = 6.093, P< 0.01		
Week 4					
Negative	5				5
1+		6			6
2+			2	1	3
3+			2	4	6
Total	5	6	4	5	20
			K = 0.798, Z = 6.038, P< 0.01		
Week 8					
Negative	5				5
1+		5			5
2+		1	3	1	5
3+			1	4	5
Total	5	6	4	5	20
			K = 0.800, Z = 6.174, P< 0.01		
Week 16					
Negative	5				5
1+		5	1		6
2+		1	3		4
3+			1	4	5
Total	5	6	5	4	20
			K = 0.800, Z = 6.128, P< 0.01		

Acceptance of the staining methods by the laboratory workers. For the acceptance of this MC stain technique for routine use in the future by laboratory workers at the peripheral level of the health care system, we surveyed by using questionnaires, and the results obtained were as follows :

(i) General characteristics of the respondants. Completed questionnaires were received from 162 out of 200 hospital laboratories (81%) surveyed. Most of the respondants were females (52.5%), the average age was 32 years old (S.D. ± 5.57). Regarding educational level, a certificate of science was found in the majority (65.4%) and a bachelor degree in 31.5%. As to their work positions, 77.2% were medical laboratorians, 19.8% were laboratory technicians and others (3%) were nurses and employees.

(ii) Training, supervision, work load, and laboratory equipments. 51.2% of laboratory workers had been trained or knew about laboratory method for TB detection and 51.2% had been supervised. The average workload for direct smear detection of TB in sputum was 48 times/month. The most popular method for the staining of sputum for TB detection was ZN stain (89.5%) and Kinyoun stain (10.5%). Many materials and staining reagent aliquots were supplied by the TB Division and Regional TB Centers. Most of the supplied equipments were slides, sputum cups and sets of AFB aliquots and 71.0% of the respondants had no problem in getting supplies or support.

(iii) Thought about staining of sputum for TB diagnosis. Nearly 99% of the laboratory workers thought that TB was a serious infection and 88.3% thought that case finding by direct sputum smear examination was simple and economical. 87.0% of them thought that the most important factor in TB diagnostic test was high sensitivity and high specificity, and 92.6% of them thought that the diagnostic test should be comfortable, simple and economical. 48.8% of laboratory workers thought that the conventional ZN stain had multiple, cumbersome stages and 8.0% thought that the laboratory workers for sputum staining diagnosis had more experience, and more advanced technical skill than they did and 10.5% believed that *M. tuberculosis* could be spread by aerosolized phenol. About 86.4% thought that if they had a new sputum staining method that was efficient and not cumbersome, they would use it instead of the ZN method. After they read and studied the sheet guideline of the ZN and the MC staining methods, 97.5% of laboratory workers were able to understand, 95.1% could use and 63.0% believed it would be unnecessary to have supervision.

(iv) Acceptance for sputum staining methods. 60.5% of the laboratory workers accepted that the MC stain was easier to perform than the ZN stain, and 71.0% of them accepted that the MC stain was more comfortable and much less expensive than the ZN stain. If they must purchase it themselves, 58.6% of them would choose the MC stain. About 53.7% and 69.1% of them accepted both staining methods were more sensitive and more specific, respectively; and 45.7% of them mentioned using the MC stain while 46.9% of them mentioned using both staining methods.

DISCUSSION

One of the most important elements in the control of tuberculosis is the early diagnosis and treatment of patients with pulmonary tuberculosis as they are largely responsible for transmission of the disease. Although presumptive diagnosis of pulmonary tuberculosis can be made on the basis of patient histories and clinical and radiological findings, the definitive diagnosis of tuberculosis continues to depend on the microscopic examination of AFB smears for initial screening and then cultural confirmation. However, culturing requires a prolonged time due to the slow growth of *M. tuberculosis*. It takes about 3 to 6 weeks before a positive culture for this agent can be identified, and it is more expensive, requiring at least a moderately well-equipped laboratory which in Thailand they are available only at the central level, some TB centers, and provincial hospitals; while direct microscopy is the most rapid and cost-effective detection method that can be performed at all peripheral health centers.

Although the sensitivity of direct microscopy is relatively low, requiring about $5x10^3$ bacilli per ml of specimen for detection,[6] the number of tubercle bacilli in pulmonary secretions is directly related to the risk of transmission.[9] The additional advantage of the AFB sputum smear is its close correlation with infectiousness;[10] for examples, smear positive patients are 4-20 times more infectious than smear negative patients; and, if untreated, they may infect 10-15 persons per year. Furthermore, smear-positive patients are much more likely to die if untreated. Thus, microscopy is an important tool for screening patients who may require isolation on admission to a hospital. In many developing countries, direct microscopy of sputum smears continues to be the basic technique for primary diagnosis of pulmonary tuberculosis, case finding, and for assessment of treatment due to its simplicity, rapidity and low cost.

In order to overcome the problems associated with the conventional ZN staining technique which requires controlled heating for its success, which makes the method more cumbersome and uncomfortable because it is necessary to aerosolize phenol, various cold stain techniques have been tried with varying success, eg. the Kinyoun method requires a high concentration of basic fuchsin and phenol or the addition of a detergent; thus avoiding the need for heat, but its disadvantages are that it is uneconomical, time consuming and lost effectiveness due to the instability of the stain and this restricts its use to major centers. Fluorochrome staining is much more sensitive than ZN and is appropriate for use in large laboratories where the workload is excessive. However, the disadvantages of the fluorescence technique are low specificity, high cost of a complete fluorescence microscope unit and handling and maintenance of the optical equipment requires advanced technical skill, thus restricting its use to a small number of laboratories.[10]

In our preliminary study, we described a new modified cold (MC) staining method to be used in sputum examination for primary diagnosis of tuberculosis.[4] The efficiency of this MC stain was further evaluated in comparison to the conventional ZN stain for sputum examination of AFB, and for stability of the staining reagents and stability of the stained slides for reexamination after storage at different times. However, the high quality of reading by the researcher compared to that of the experienced microscopist was achieved before the study by blind reading of unknown slides, and the results in this study showed high agreement between both readers (98%). Nevertheless reading error occured in 2%, where the researcher reported negative but the experienced microscopist reported positive. Reading error by the reader is due mainly to visual or psychological reasons, and occurs in practically all diagnostic clinical and laboratory works. Moreover, under certain conditions the degree of error by over reading as well as under reading varies from one person to another and also within the same individual at different times.[10]

In comparison of sputum examination for AFB as a rapid screening of tuberculosis using culture and biochemical identification of *M. tuberculosis* as the gold standard, the results showed that the ZN and MC stains were able to detect tuberculosis by staining of sputum at a sensitivity as high as 68.9% and 70.6%, respectively. When the results of ZN method were compared with the MC method, there was no statistically significant difference ($P > 0.05$). Although the sensitivity of AFB microscopy was relatively low, its specificity for either the ZN or MC methods was quite high (97.4% and 97.8%, respectively). Since false-positive results were comparatively rare which is similar to previous studies reported by others,[11,12] a positive smear could therefore be relied upon as a good diagnostic indicator.

Besides the problems of favorable sensitivity and specificity, diagnostic laboratories also need acceptable predictive values of the test results. The predictive value for detection is greatly dependent on the incidence of *M.tuberculosis* (MTB)-positive samples. The overall positive predictive values (PPVs) were very good for microscopic examination. A high PPV of AFB smear for *M. tuberculosis* in this study (92.1% by ZN stain and 93.3% by MC stain) was close to the 92% reported by Yajko et al.,[14] despite the high prevalence of *M. avium* complex in respiratory specimens. These data suggested they

could be valid enough in predicting the presence of disease in general population.

The efficiency of the ZN (88.8%) and MC (89.5%) stains also reached the expected satisfactory level because the sensitivity and specificity were high. These data are in close parallel with our previous study of the AFB smear[13] using both fluorochrome and ZN for detection of *M. tuberculosis* with the sensitivity, specificity, PPV, negative predictive value (NPV) and efficiency were 66.4, 96.9, 93.7, 80.7, and 84.5%, respectively.

The MC was also as reliable as the ZN technique in retaining color after storing the stained slide for a long time. In this study, the slides stained by both techniques were stored at room temperature for 4, 8 and 16 weeks, respectively. When comparing the results with the first time (0 week), the data showed agreement on ZN reading of 90, 90 and 85%, respectively while the agreement on MC was 90, 95 and 80%, respectively. This suggests that the appropriate timing for storing stained slides for reexamination should be no longer than 16 weeks; because if storage time is longer the quality and efficiency of stained slides may decrease.

The staining reagents also had a long shelf life, they were stable for at least a month. After the reagent aliquots were stored for 0, 2, 4, 8 and 16 weeks, respectively, the results of both stains obtained by pooling the data for both negative and positive results showed an agreement of 100% for every time it was restocked. This suggests that we are able to stock the aliquots of staining reagents for more than 16 weeks without showing loss of efficiency. The staining reagents remain clear, no precipitation occurs.

In order to apply this new MC staining method for use in the future at the peripheral level of the health care system, we made a survey by using questionnaires sent to laboratory personnels in 200 general and community hospitals for the acceptance of the staining methods. Most of them agreed that the staining of sputum for tuberculosis diagnosis should be more sensitive and specific, comfortable, simple, safe and economical and that it should be unnecessary to have more experience and advanced technical skill. However they agreed that ZN technique had multiple stages and was cumbersome, and accepted that the MC technique was easier to perform (60%), comfortable and economical (71%) and 26-43% believed that the MC stain was more sensitive and specific than the ZN stain, but 53-70% were unsure. However most of them accepted the MC stain because it was easier to perform, and not cumbersome for training.

In conclusion, we have demonstrated that the new modified cold staining method showed high agreement (97.2%) with the results of the conventional ZN staining with no statistically significant differences ($P > 0.05$). In addition, the MC stain was also as reliable as the ZN stain in retaining color and might be used as an alternative to the ZN for sputum staining. Moreover, the MC technique was easier and safer to perform and less expensive than the ZN in terms of laboratory and material and would be useful in a large scale case finding programme. Altogether, these factors make the MC stain suitable for use at the peripheral level of the health care system as a practical and rapid sputum staining for the early diagnosis of tuberculosis and for assessment of the treatment.

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