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Contents

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Viral Shedding in University Students Infected by Influenza A(H1N1)pdm09, Nakhon Ratchasima Province, Thailand, June 2011

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Abstract

Oseltamivir is often prescribed to treat influenza patients, yet its effect on viral shedding among Thai young adults infected with influenza A(H1N1)pdm09 virus remained unclear. During May to June 2011, an influenza A(H1N1)pdm09 outbreak was detected in University S, Nakhon Ratchasima Province, Thailand. A prospective observational study was conducted to define duration of viral shedding and immunologic response in infected students undergoing oseltamivir treatment, and identify factors associated with viral shedding. We enrolled all acute respiratory illness (ARI) patients attending the medical center at University S during 3-7 Jun 2011 with laboratory confirmation of influenza A(H1N1)pdm09 infection by real-time reverse transcription polymerase chain reaction (rRT-PCR). Additional throat swabs were collected and tested daily until rRT-PCR results became negative through two consecutive days. Series of serum samples for hemagglutination inhibition (HI) test were also collected from the individuals. Log-rank test was applied in analysis of association between patients' characteristics and duration of viral shedding. Of 29 sick students enrolled, 45% were males. All were prescribed oseltamivir for five days and none of them were hospitalized. Median duration from onset of symptoms to the last day of viral shedding detected was five days (range 3-9 days). Over 80% of the patients had 4-fold rises of HI titer within 2-3 weeks after onset of symptoms. None of the patients' characteristics were significantly associated with duration of viral shedding. However, persons with delayed antiviral treatment tended to have longer duration of viral shedding. Early oseltamivir treatment probably reduced risks of severe influenza in young adult patients. However, guidelines on infection control need to emphasize on strict hygiene and prevention measures in treated patients for nine days in order to minimize the risks of influenza transmission.

Key words: influenza A(H1N1)pdm09, viral shedding, outbreak, university, Thailand

Introduction

Knowledge on viral shedding from respiratory tract is an important factor in order to minimize transmission in community. In young adults infected by influenza A(H1N1)pdm09 virus with mild symptoms, a study in Singapore showed that mean duration of viral shedding was 6±2 days,[1] while studies in China and the United States reported 4-6 days.[2-4] In Thailand, studies on viral shedding of influenza A(H1N1)pdm09 revealed as five days (range 1-12 days) among military conscripts[5], seven days (range 2-14 days) in a school and 7.5 days (range 3-14 days) in a military camp[6].

Duration of viral shedding of influenza A(H1N1)pdm09 virus in children tended to be longer than that of young adults.[7-9] Moreover, various studies agreed that oseltamivir could effectively reduce the duration of viral shedding and prevent severe complications from the virus.[1,2,10] On the other hand, immunocompromised health status seemed to prolong shedding of respiratory viruses for weeks and even months.[11-13]

Since June 2009, numerous outbreaks of influenza A(H1N1)pdm09 in schools and colleges have been reported in Thailand. On 30 May 2011, influenza A(H1N1)pdm09 outbreak was detected among students in University S, Nakhon Ratchasima Province, the northeastern region of Thailand. In this outbreak, 455 out of 10,515 staff and students were affected, with an attack rate of 4.3% since 22 May 2011. Epidemiological control measures including health education, inhibition of mass gathering and case isolation had been in place on 31 May 2011.[14] All students were informed to visit the medical center of University S for influenza screening by rapid test. If one had positive result by the rapid test, that person was immediately provided with full course of oseltamivir. In Thailand, knowledge on viral shedding of young adult patients infected with influenza A(H1N1)pdm09 and received antiviral treatment remained limited.[5,6] This study was, therefore, conducted among university students infected with influenza A(H1N1)pdm09 and undergoing treatment at the medical center in order to describe characteristics of viral shedding and immunologic response, and identify factors associated with duration of viral shedding.

Methods

Study Design

A prospective observational study was conducted from June to September 2011 among random samples of students from University S. Participants were acute respiratory illness (ARI) cases who had attended the medical center at University S during 3-7 Jun 2011 and had laboratory confirmation of influenza A(H1N1)pdm09 infection by real-time reverse transcription polymerase chain reaction (rRT-PCR). ARI cases were defined as the persons who had any two of four symptoms: fever, runny nose, cough or sore throat. Fever was either measured body temperature of 38°C and above, or self-reported fever. We excluded persons with immunocompromised health status, such as HIV infection, or having steroids or anti-neoplastic drugs. On the enrollment day which was defined as day 0, all participants had a throat swab collected and completed a standardized questionnaire that included demographic characteristics, underlying diseases, height, weight, past history of influenza vaccination, signs and symptoms of respiratory illness, and treatment. Subsequently, throat swabs from each participant were collected daily until two consecutive swabs were negative for influenza A(H1N1)pdm09 virus by rRT-PCR or up to 10 days. Duration of viral shedding was defined as the time period between onset of symptoms and the first date of undetectable RNA by rRT-PCR.

Virological Methods

Throat swab specimens were tested by rRT-PCR for influenza A(H1N1)pdm09. Serum samples were tested for antibodies to influenza A(H1N1)pdm09 virus by hemagglutination inhibition (HI) assay using 0.5% turkey erythrocytes according to the standard protocols.[15] All serum samples were tested using the same batch of turkey erythrocytes to allow precise interpretation of the laboratory results. All the laboratory tests were performed at the Department of Microbiology, Faculty of Medicine, Siriraj Hospital.

Analysis and Statistics

We compared duration of virus shedding by selected characteristics, including gender, body mass index (BMI), underlying diseases, reported symptoms, time of receiving oseltamivir, history of having influenza or influenza vaccine since 2009, and immunologic response. All variables were tested by log-rank test. Moreover, geometric mean titers (GMT) with 95% confidence intervals (95% CI) were calculated from participants' HI titers.

Human Subjects Review

This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Number 755/2010). Written informed consent was obtained from every person participated in the study.

Results

Twenty nine participants with laboratory confirmed influenza A(H1N1)pdm09 infection by rRT-PCR were enrolled. Among them, 13 (45%) were males and median age was 20 years (interquartile range 19-21 years). Allergy was the most commonly reported underlying condition and was reported in five (17%) patients. There were five (17%) participants with history of having influenza or receiving influenza vaccination since 2009, indicating that they might have gained immunity in the past. Fever (86%) was the most frequently reported, followed by cough (79%), myalgia (72%) and sore throat (69%). Total 20 cases (69%) received antiviral treatment within two days after onset of symptoms while median time from onset of symptoms to treatment was one day (range 0-6 days) (Table 1).

Viral Shedding

Among 29 participants, mean duration of viral detection from onset of symptoms was six days (SD 2

days) and median was five days, with the range of 3-9 days (Figure 1).

Table 1. Characteristics of participants with acute respiratory illness (ARI) in University S, Nakhon Ratchasima Province, Thailand, 3-7 Jun 2011 (n=29)

Characteristic	Number	Percent
Male	13	45
Body mass index (BMI) ≥ 23kg/m^2	14	48
Underlying allergy	5	17
Previously healthy	24	83
History of having influenza or receiving influenza vaccine since 2009	5	17
Self-reported symptoms		
Fever	25	86
Cough	23	79
Myalgia	21	72
Sore throat	20	69
Runny nose	20	69
Headache	20	69
Dyspnea	8	27
Diarrhea	1	3
Treatment		
Received antiviral within 48 hours after onset of symptoms	20	69
Hospitalization or death	0	0

Overall, 83% of participants had undetectable influenza A(H1N1)pdm09 virus after day seven. Comparison on duration of shedding by selected patients' characteristics showed that no characteristics were significantly associated with the duration of viral shedding (Table 2).

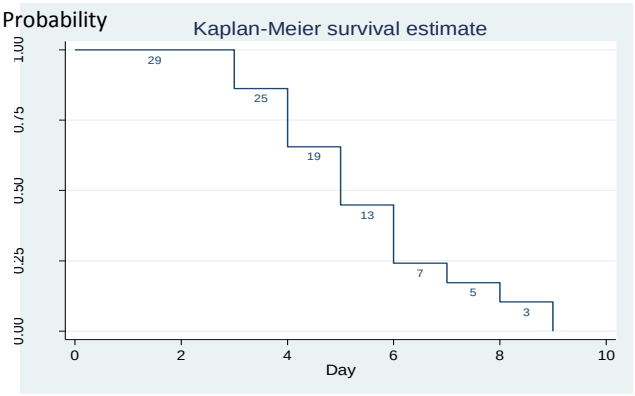

Figure 1. Kaplan-Meier plot showing probability of rRT-PCR positive influenza A(H1N1)pdm09 by days after onset of symptoms in University S, Nakhon Ratchasima Province, Thailand, 3-7 Jun 2011 (n=29)

Geometric Mean of HI Titer (GMT)

All the participants were not enrolled on the same day. Dates of enrollment were 0-6 days after their onset dates. On day 0 or enrollment day, GMT of all participants was 1:6 (95% CI = 1:5-1:7). On day 14 of enrollment, GMT rapidly increased to 1:63 (95% CI = 1:38-1:103), and 4-fold rise was reported on day 14 among 83% of the participants. GMT was 1:69 (95% CI = 1:44-1:106) on day 28 and 1:127 (95% CI = 1:86-1:162) on day 90, which tended to reach a steady stage (Figure 2).

Table 2. Comparison on median duration of viral shedding by selected patients' characteristics in University S, Nakhon Ratchasima Province, Thailand, 3-7 Jun 2011

Variable		Median duration of viral shedding (day)	P-value (Log-rank test)
Gender	Male	5	0.24
	Female	6	
BMI ≥ 23 kg/m^2	Yes	6	0.28
	No	4	
Underlying allergy	Yes	7	0.12
	No	5	
Self-reported fever	Yes	5	0.19
	No	4	
Received antiviral drug within 48 hours	Yes	5	0.09
	No	6	
History of influenza infection or influenza vaccination since 2009	Yes	7	0.44
	No	5	
4-fold rise by HI test within 14 days	Yes	6	0.54
	No	4	

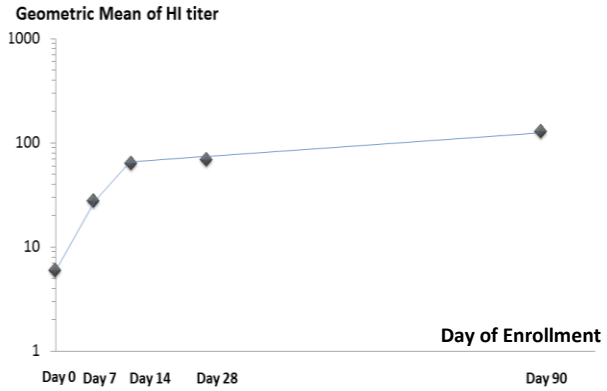

Figure 2. Geometric means of HI titer (GMT) at day 0 (enrollment day), day 7, day 14, day 28 and day 90 (n=29)

Discussions

Median duration of viral shedding of influenza A(H1N1)pdm09 in this university was compatible with the findings from other studies that reported 4-6 days in young adults treated with oseltamivir.[1-4] However, the median duration was shorter than that of the other study conducted in Nakhon Ratchasima Province which revealed as seven days[6]. In comparison of different settings, patients with mild symptoms usually had shorter duration of viral shedding than hospitalized patients.[16,17]

For immunologic response, HI titer rose rapidly 2-3 weeks after the patients were infected by influenza A(H1N1)pdm09. Then, the titer remained constant. By day 90, HI titer increased only slightly from the constant level because titer of some patients (14%) still had not reached 4-fold rise on day 28. In addition, we observed that the patients who had 4-fold rise in shorter time tended to have longer duration of viral shedding probably due to higher viral load. Nonetheless, the test showed no statistical significance and viral load were not measured in this study.

Regarding factors associated with long duration of viral shedding, we observed that duration among those who received antiviral treatment within 48 hours after onset of symptoms was not significantly shorter than those with late treatment (P-value = 0.09). Furthermore, the longest duration of four and six days after their onset dates was observed in two out of three patients receiving late antiviral treatment. On the other hand, viral shedding was extended among those with BMI of 23kg/m^2 and more, underlying allergy and reported fever though the differences were not statistically significant. The statistically insignificant association with patients' characteristics might be due to small sample size.

Even though the actual isolation for seven days could result in effective control of influenza transmission in this outbreak,[14] this study underlined the need to reconsider two additional days to cover 17% of the patients with longer viral shedding.

Limitations

Though we did not obtain the actual first day of viral shedding, this study reported viral shedding as the duration between onset of symptoms and the first date of undetectable RNA virus. Sample size was relatively small since it was not designed to analyze factors associated with long viral shedding. Meta-analysis might be necessary to demonstrate variables associated with viral shedding. Viral load was not measured due to financial limitation. Generalization of the study was limited only to healthy young adult patients receiving oseltamivir. Finally, we were able to follow up all enrolled patients for 28 days. Nonetheless, eight of 29 patients (27%) were lost to follow up for serum collection on day 90.

Conclusions

Duration of viral shedding of influenza A(H1N1)pdm09 among patients treated with oseltamivir in this university was 3-9 days (median five days). Immunologic response was noticed about 2-3 weeks after getting infection and then the HI titer tended to be constant. Early antiviral treatment within 48 hours was identified as a factor that probably reduced the duration of viral shedding, yet not showing any statistical significance. In addition, factors those probably lead to long viral shedding included high BMI, underlying allergy and reported fever. Guidelines on infection control need to emphasize on practice of strict hygiene and prevention measures, such as isolation of treated patients for nine days to minimize risks of influenza transmission, particularly those with obesity or underlying allergy. The study demonstrated the benefit of early antiviral treatment in preventing severe outcome of influenza A(H1N1)pdm09 infection and shortening the viral shedding time.

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Suggested Citation

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Multidrug-resistant Tuberculosis Patients in Bhutan, August 2011 to July 2012

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Abstract

This study aimed to describe the factors associated with multidrug-resistant tuberculosis (MDR-TB) in Bhutan. The study covered all MDR-TB patients admitted to Gidhakom Hospital, Thimphu from August 2011 to July 2012. Data were collected from MDR-TB registers, laboratory registers and inpatient records as well as from interview with patients about demographic characteristics, history of previous anti-TB treatment, compliance and contact history. There were total 19 MDR-TB patients. Majority of the patients were males (63%). Median age was 25 years for males and 30 years for females. Of the 19 cases, 47% and 37% had contacted with TB case and MDR-TB case in the same house respectively. About 74% reported previous history of anti-TB treatment. Among those with previous treatment, 79% did not comply with the directly observed treatment short-course (DOTS). It was found that 20% of new cases and 50% of previously treated persons were resistant to four first-line drugs. In conclusion, previous anti-TB treatment, non-compliance to DOTS and contact with MDR-TB were observed in majority of the cases. We highlighted the importance of early detection of MDR-TB, providing health education on prevention of disease transmission and strengthening DOTS policy. Investigation on household contacts should be given priority to identify and control the spread of TB.

Key words: tuberculosis, multidrug-resistant, directly observed treatment short-course, Bhutan

Introduction

The Bhutan Ministry of Health has made several advances in management of tuberculosis (TB) since 1986.[1] In order to address the TB problem in the country, the National Tuberculosis Control Program (NTCP) was established in 1976. One major strategy was the introduction of directly observed treatment short-course (DOTS) in 1994 and it was implemented nationwide in 2004.[1] In Bhutan, a person diagnosed with TB is usually admitted to a hospital for 1-2 weeks and asked to continue DOTS at home for the treatment. A DOTS provider could be a health worker, a family member or a community member who observed a TB patient taking the daily dose of anti-TB drugs. Treatment success rate for smear-positive TB had been maintained above 90% since 2005. However, the current challenge is the emergence of multidrug-resistant tuberculosis (MDR-TB).[2]

MDR-TB is defined as the infection with strains of *Mycobacterium tuberculosis* that are resistant to at least isoniazid and rifampicin, the two most efficacious first-line anti-TB drugs. Extensively drug-resistant tuberculosis (XDR-TB) is defined as MDR-TB plus resistance to fluoroquinolone and at least one second-line injectable agent of amikacin, kanamycin or capreomycin.[3]

There was no representative data on drug resistance TB in Bhutan. In 2010, the World Health Organization (WHO) estimated that MDR-TB prevalence in the country was around 1.7-2.5% among new TB cases and 17-18% among retreatment TB cases.[2] Since facilities that provided MDR-TB services had been limited in the country, all MDR-TB cases were referred to Gidhakom General Hospital which was located in Thimphu and the only center in the country providing the MDR-TB treatment. The public health laboratory attached to Jigme Dorji National Referral Hospital, Thimphu was the only laboratory in the country for TB drug sensitivity test.

In 2011, MDR-TB was isolated in five of 382 (1.3%) new patients with smear-positive TB and 14 out of 70 (20.0%) retreatment cases.[4] Though the number of MDR-TB cases was not high, emergence of such cases threatened the effort and effectiveness of NTCP in the country.[1] Since no study on MDR-TB was conducted in the past, this study was initiated to describe epidemiological characteristics of MDR-TB

patients, treatment compliance and factors associated with MDR-TB patients. The study results would be useful to the NTCP for MDR-TB control in the country.

Methods

In 2011, total 215 health facilities, including 31 hospitals, were serving 695,822 people in Bhutan. All health services in Bhutan are provided free of charge. Most people can normally access to health care services and facilities when they are sick. Gidhakom Hospital with 60 beds (20 beds for MDR-TB patients) was the only hospital equipped to provide health care to MDR-TB cases. All MDR-TB cases were admitted to this hospital for six months and referred back to health facilities in their villages for follow-up.

We conducted a descriptive study among all MDR-TB patients admitted to Gidhakom Hospital from August 2011 to July 2012. MDR-TB was defined as infection with strains of *M. tuberculosis* that were resistant to at least isoniazid and rifampicin. Inclusion criterion for this study was restricted to MDR-TB patients admitted during the same period of the study. Patients who were unwilling to participate in the study were excluded. Ethical approval was obtained from the Research Ethics Board of Health in Bhutan.

Data collection was conducted by reviewing medical records including MDR-TB registers, laboratory registers and inpatient records for information on previous anti-TB treatment, treatment outcomes and laboratory results. In addition, we interviewed patients using a semi-structured questionnaire for demographic characteristics, clinical symptoms, previous anti-TB treatment, history of contact with TB cases and their knowledge on TB. The data were analyzed using Epi Info (version 3.5.3).[5] Fisher's exact test and chi-square test were used. P-value of 0.05 was considered as statistical significance.

Results

There were total 19 MDR-TB patients identified, with 63% were males. The most common symptoms experienced by MDR-TB patients were cough (91%), chest pain (86%), weight loss (71%), breathlessness (65%), fever (62%) and hemoptysis (62%). Median age was 25 years for males (range 14-65 years) and 30 years for females (range 22-41 years). More males (83.3%) were literate compared to females (42.9%), with p-value of 0.19. Median household income of males was higher than that of females (Table 1).

Of the 19 MDR-TB cases, 14 (73.7%) reported previous history of anti-TB treatment (Table 2). Of

Table 1. Demographic characteristics of MDR-TB patients by gender in Bhutan, August 2011 to July 2012

Characteristic	Male (n=12)		Female (n=7)		Total (n=19)	
	Number	Percent	Number	Percent	Number	Percent
Age (year)						
Median (range)	25 (14-65) years		30 (22-41) years		27 (14-65) years	
≤15	1	8.3	0	0	1	5.3
16-25	4	33.3	1	14.3	5	26.3
26-45	6	50.0	5	71.4	11	57.9
>45	1	8.3	1	14.3	2	10.5
Marital status						
Married	8	66.7	6	85.7	14	73.7
Single	4	33.3	1	14.3	5	26.3
Literacy						
Literate	10	83.3	3	42.9	13	68.4
Illiterate	2	16.7	4	57.1	6	31.6
Occupation						
Employee	8	66.7	1	14.3	9	47.4
Business	2	16.7	1	14.3	3	15.8
Student	1	8.3	1	14.3	2	10.5
Others*	1	8.3	4	57.1	5	26.3
Median household income (Ngultrum)**/month	15,000 (5,000-20,000)		10,000 (5,000-20,000)		10,000 (5,000-20,000)	

* *Including farmers, housewives and monks*

** *50 Ngultrum = 1 USD*

Table 2. Previous anti-TB treatment and underlying diseases in MDR-TB patients by gender in Bhutan, August 2011 to July 2012

Previous anti-TB treatment	Male (n=12)		Female (n=7)		Total (n=19)	
	Number	Percent	Number	Percent	Number	Percent
Previously treated case	10	83.3	4	57.1	14	73.7
Did not follow DOTS treatment*	9	90.0	2	50.0	11	78.6
Missed anti-TB drugs ≥7 days*	4	40.0	1	25.0	5	35.7
Median duration on previous anti-TB treatment* (range)	12 (5-18) months		8 (8-12) months		8 (5-18) months	
Treatment outcome before MDR-TB						
Failure*	5	50.0	1	25.0	6	42.9
Relapse*	4	40.0	3	75.0	7	50.0
Default*	1	10.0	0	-	1	7.1
Drinking alcohol during anti-TB treatment*	6	60.0	1	25.0	7	50.0
Contact history						
Contact with TB case in the same house	6	50.0	3	42.9	9	47.4
Contact with MDR-TB case in the same house	5	41.7	2	28.6	7	36.8
Other underlying diseases including bronchial asthma and diabetes	3	25.0	4	57.1	7	36.8

** With previous anti-TB treatment*

those with previous treatment, 78.6% did not comply with DOTS. In addition, 35.7% of them reported of missing prescribed anti-TB drugs for seven or more days while treatment failure and relapse occurred in 42.9% and 50.0% respectively. Half of them (50.0%) also reported drinking alcohol during the past anti-TB treatments, with most of them were males.

Of the 19 cases, 47.4% and 36.8% reported contact with TB case and MDR-TB case in the same house respectively. During MDR-TB treatment, 83% of the male and 71% of the female cases were aware that TB could be transmitted through droplets and face mask should be used to reduce the chance of transmitting TB to other close contacts. Similarly, 75% of the male and 86% of the female patients knew that MDR-TB could be cured by taking prescribed medicines.

A review of TB data from the same period showed that among 70 retreatment TB cases, there were 10 with treatment failure, 55 relapsed and five defaulted cases. Sixty percent (6/10) of those with treatment failure who developed MDR-TB compared to 13% (7/55) of relapsed and 20% (1/5) of defaulted cases (p-value = 0.004). Overall, 20% (14/70) of these retreatment cases developed MDR-TB during the study period.

Drug sensitivity test of the 19 MDR-TB cases (five new and 14 retreatment cases) showed that 20% of the new cases and 50% of the previously treated patients were resistant to four first-line drugs: isoniazid, rifampicin, streptomycin and ethambutol (Table 3). No patients met the case definition of XDR-TB in this study.

Discussion

This was the first study on MDR-TB conducted in Bhutan. We found that majority of MDR-TB cases were males, having history of previous anti-TB treatment and contact with TB or MDR-TB patients. These findings were compatible with the global tuberculosis report on surveillance and response of WHO.[2]

Table 3. Drug resistant patterns of MDR-TB patients by types of patient in Bhutan, August 2011 to July 2012

Drug resistant pattern	New cases (n=5)		Retreatment cases (n=14)		Total (n=19)	
	Number	Percent	Number	Percent	Number	Percent
2 drugs (isoniazid, rifampicin)	1	20.0	5	35.7	6	31.6
3 drugs (isoniazid, rifampicin, streptomycin)	3	60.0	2	14.3	5	26.3
4 drugs (isoniazid, rifampicin, streptomycin, ethambutol)	1	20.0	7	50.0	8	42.1

P-value = 0.19

Similar studies conducted in Nepal[6], India[7] and Bangladesh[8] reported that majority of MDR-TB cases were males as well. In addition, these studies hypothesized that women were more compliant with treatment and therefore, less likely to develop MDR-TB strains compared to men. This might be true in Bhutan as well since our results showed that MDR-TB women had better treatment compliance than MDR-TB men. The finding of young age group among MDR-TB cases might emphasize on importance of this newly emerging problem in the country. However, lack of laboratory facilities for diagnosing MDR-TB in the past could have missed the cases in the previous years.

Three-forth of our study population had history of anti-TB treatment before they developed MDR-TB, with nearly half of those cases had treatment failure. This finding was consistent with the other reports since previous anti-TB treatment had been widely recognized as a predictor of MDR-TB by different studies.[6-8] Reports from the WHO identified that retreatment was strongly associated with MDR-TB.[9]

Though Bhutan had achieved more than 90% of treatment success rate during past seven years[2] and proportion of previously treated cases who developed MDR-TB was in line with the WHO estimation of 20%, it was still a burden for the country. The poor outcomes from previous treatment could have been caused by poor adherence and non-compliance of DOTS, indicating the problems in implementing DOTS and monitoring effectively in the country. Poor drug adherence could have increased the chance of drug resistance. Studies on DOTS versus self-administered therapy (SAT) for TB patients found that higher cure rate and better sputum conversion rate were achieved among patients on DOTS compared with those on SAT. These studies provided evidences that even in the best SAT, results were inferior to those achieved through DOTS.[9-11]

We found higher percentage of the isolates resistant to the four first-line anti-TB drugs compared to the findings of other studies,[12-14] except in Bangladesh where 65% were resistant to all four first-line drugs.[8] Increasing incidence of resistance to isoniazid and rifampicin has become alarming because these are the most potent bactericidal and sterilizing drugs in the TB control program, and are used in fixed-dose combination of short-course therapy for TB. Therefore, resistance to these drugs could lead to failure of the TB program.

Another finding in this study was the high number of contact with MDR-TB cases before they developed MDR-TB. The studies conducted in other countries found that the household contacts constituted a high risk group for TB.[15-16] Higher rates of MDR-TB among contacts indicated that drug resistant strains were circulating and being transmitted from person to person. Therefore, active case finding among household contacts of MDR-TB patients is important to identify the secondary spread.

Limitations

As the number of cases was small, it might limit the interpretation of detailed analyses. When the participants were asked to recall their behavior, it could subject to recall bias due to difficulty in remembering the past events and their treatment adherence from several years ago, particularly for chronic cases. In addition, drug quality was not explored in this study.

Public Health Actions and Recommendations

Association between MDR-TB and prior exposure to anti-TB treatment necessitates closer monitoring on treatment outcomes of individual patients as well as follow-up for drug resistance. We highlighted the importance of early detection of MDR-TB cases, providing health education on household protection, strengthening DOTS policy and monitoring the management of DOTS at all levels. Investigation on household contacts should be given priority in order to identify and control the spread of TB. Lack of diagnostic facilities for MDR-TB in Bhutan coupled with remoteness and poor transportation system implied that MDR-TB cases could be grossly under-diagnosed. Extension of such facilities to regional hospitals would be helpful for better treatment to reduce the burden of TB and MDR-TB, and also for better estimation of the MDR-TB burden and pattern of anti-TB drug resistance,.

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The Proficiency Level of Microscopists Detecting *Mycobacterium tuberculosis* at Government Health Clinics in Three Selected States of Malaysia, 2009-2010

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Abstract

Sputum smear microscopy is the cornerstone of diagnosing infectious tuberculosis. The presence of microscopic errors may misclassify or misdiagnose cases as non-cases, or vice versa. Substandard performance will compromise the efforts to detect tuberculosis and complicate measures to control. This study aimed to determine the proficiency of microscopists at three selected government health clinics in Malaysia. A cross-sectional study was conducted in 2009-2010. Three states were selected based on their high sputum positivity rate. All microscopists were enrolled and instructed to stain and grade a set of seven predetermined densities of mycobacilli slides. Two independent raters assessed their readings. A total of 100 microscopists and 700 slides were tested. 88.2% of slides were in agreement, with sensitivity of 83.8%, specificity of 94.3%, positive predictive value of 95.2% and false negativity rate of 18.7%. From the low positive slides, 27.5% were graded as negative. Two-third of microscopists achieved the accepted grading proficiency and 37% were scored as good staining. There was a need to intensify training on smear microscopy if the gateway for diagnosing TB in Malaysia was smear microscopy.

Key words: proficiency testing, *Mycobacterium tuberculosis*, Kappa agreement, microscopist

Introduction

In Malaysia, when a patient presents at a health clinic with chronic cough, fever and loss of appetite, the medical doctor will rely on the patient's clinical symptoms, results of sputum smear and chest X-ray findings to make a diagnosis of tuberculosis (TB). Care of TB patients starts with a quality assured diagnosis by identifying *Mycobacterium tuberculosis* in clinical specimens and microscopy of sputum smears.[1]

The cornerstone of the diagnosis of TB is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Grading of the positive smears gives a broad indication on severity of disease and response to therapy.[2] Positivity of these smears is highly dependent on quality of staining, microscope and number of microscopic fields examined. The technique is simple and inexpensive, and detects people with infectious TB which are responsible for most TB epidemics.[3]

In 2010, the World Health Organization (WHO) reported 8.8 millions of TB incident cases (range 8.5-9.2). While the incidence rate has been falling since 2002, the WHO emphasized that laboratory strengthening was needed to be accelerated.[4] As the incidence of TB declined, fewer cases were detected correspondingly and fewer specimens were tested. Thus, maintaining the proficiency in sputum microscopy would become more difficult.

The WHO recommended the countries to implement an External Quality Assessment (EQA) in their TB laboratories to improve their efficiency and reliability of the smear microscopy. There are three components in EQA which included on-site evaluation, blinded rechecking and panel testing.[5]

Since inception of the National Tuberculosis Control Program (NTCP) in 1961, TB ranked as second amongst the reported infectious diseases in Malaysia. The notification rate in 2009 was 64.0 per 100,000 populations and the rate has been plateaued for the past 15 years. As a routine practice of microscopists in TB laboratories, they submitted 100% of their positives slides and 10% sampling of the negatives slides to be read by their supervisors. In 2010, the NTCP embarked on the EQA program using a blinded

rechecking system to replace the routine practice of microscopists in TB laboratories (External quality assessment for National Tuberculosis Program, Ministry of Health, Malaysia. 2011, unpublished report).

The main objective of the study was to determine the proficiency level for detecting *Mycobacterium tuberculosis* from sputum smears by the microscopists at peripheral laboratories in government health clinics.

Methods

A cross-sectional study was conducted in 2009-2010. Three out of 14 states in Malaysia with the highest TB burden and sputum positivity rate were selected. All the microscopists reading TB smears in the health clinics were enrolled. The study protocol was approved by the Medical Research Ethics Committee. A self-administered questionnaire was given to the microscopists to determine the level of working experience, training received, workload, quality of microscope and staining used. Medical officers in the health clinics were examined for red-green color vision defect and vision acuity. A set of seven unstained slides with predetermined densities of *Mycobacterium tuberculosis* was given to each microscopist to stain and grade the slides.

The slides were prepared using the Smithwick and Stratigos technique.[6] The positive slides were prepared from fresh sputum specimens which were not more than two days old with bacillary load of +2 or more acid fast bacilli (AFB), and were divided into three main groups: high positive slides (3+ mycobacillary load), moderate positive slides (2+ mycobacillary load) and low positive slides (1+ and scanty mycobacillary load). The negative slides were prepared from fresh sputum specimens with no bacillary load and were used as diluents for the positive slides. The set of seven unstained slides comprised of three negative slides and four positive slides, which included two low positive slides, one moderate positive slide and one high positive slide. To minimize the bias, the slides were blinded and randomly placed in slide boxes before transporting to the participating microscopists. The microscopists were required to stain using their own Ziehl-Neelsen (ZN) staining materials, record the grading and return the stained slides within two weeks (excluding delivery time) to the reference TB laboratory. The slides were then blinded and scored by two independent reference microscopists (raters). An inter-rater Kappa agreement with 95% confidence interval was conducted to determine the strength of agreement between the raters.

The raters used a point system to assess staining quality of each slide independently. The slides were categorized into acceptable and not acceptable quality. Points were given according to staining characteristics of the slides: two points for magenta red AFB, two points for blue background, one point for pink AFB, one point for very light blue background, one point for presence of scum and one point for very dark background. Slides with score point of three and four were categorized as acceptable, and slides with score point of two and below as not acceptable (Figures 1-5). If there was any discrepancy in staining assessment between two raters, the slides were re-assessed by a consultant reference microbiologist and the result was taken as final.

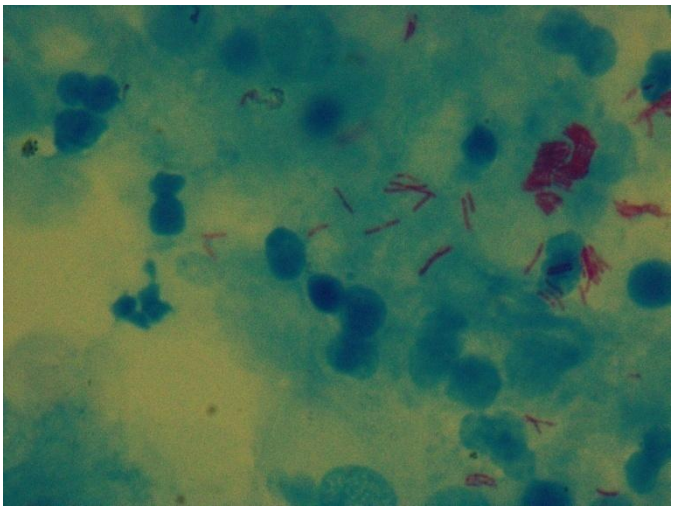

Figure 1. Slide with acceptable staining from sputum smear for *Mycobacterium tuberculosis*

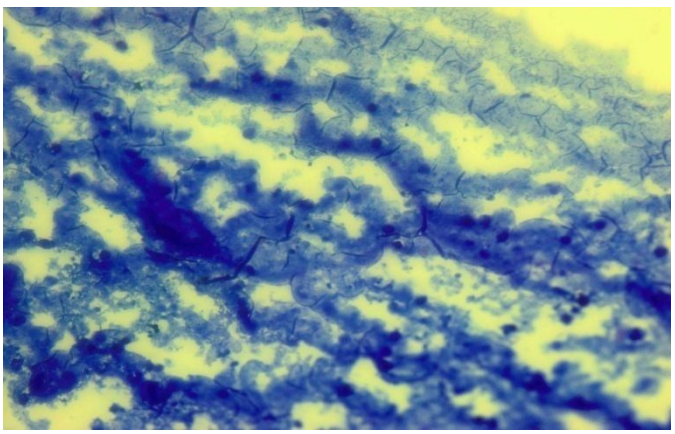

Figure 2. Slide with over-staining of methylene blue (poor staining) from sputum smear for *Mycobacterium tuberculosis*

Similarly to staining, the consultant reference microbiologist also addressed the discrepancies encountered for grading. Each slide was scored 10 marks for ability to differentiate positive and negative slides correctly, and five marks for any quantification error (QE). QE is defined as the

difference of at least two grades when reading the positive slides. A positive slide read as negative was scored as zero mark and vice versa. The criteria table from WHO/IUATLD was used for correctness in proficiency grading.[5] The total score of 80% and above was taken as the acceptable proficiency level.

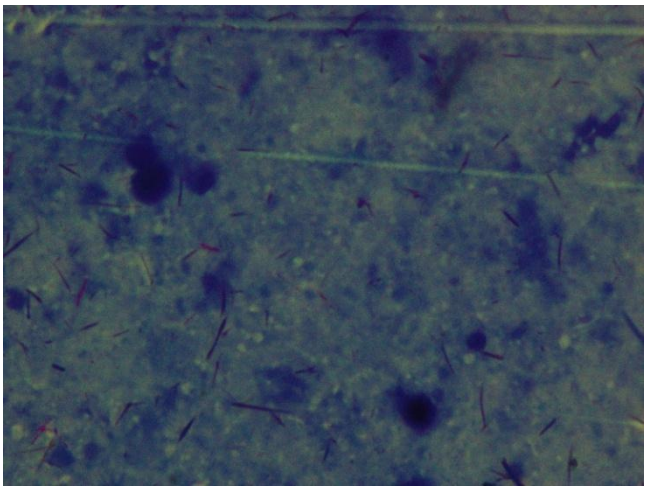

Figure 3. Slide with crystals (underestimation of AFB) from sputum smear for *Mycobacterium tuberculosis*

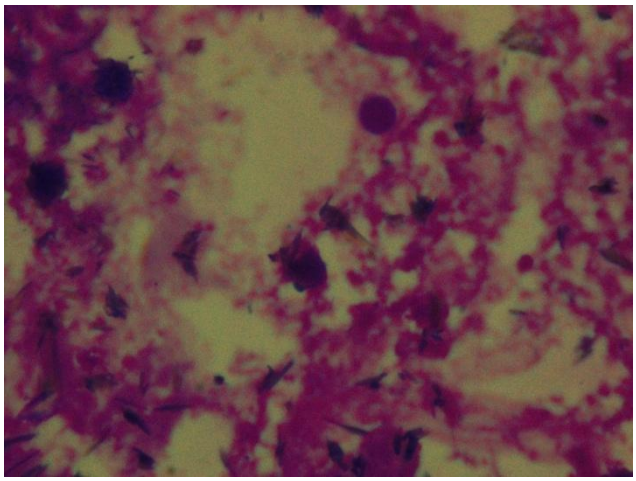

Figure 4. Slide due to over-staining from sputum smear for *Mycobacterium tuberculosis*

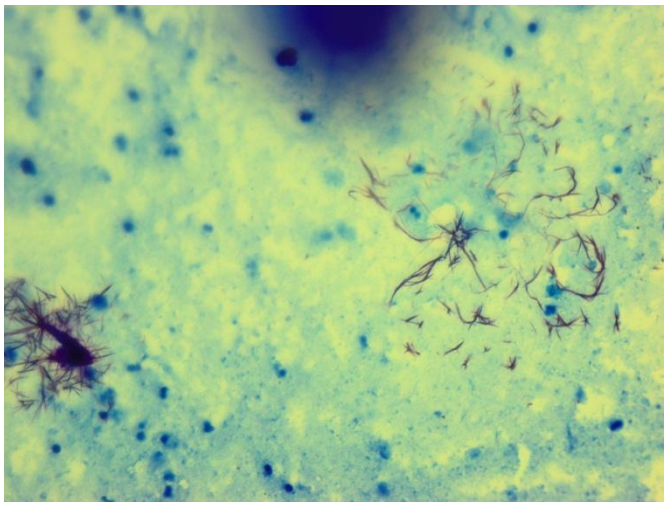

Figure 5. Slide with carbol fuchsin crystals looking like AFB from sputum smear for *Mycobacterium tuberculosis*

The Kappa analysis and data analysis were conducted using SPSS version 15.0 (SPSS Inc., Chicago III).

Results

All 61 health clinics in the three selected states were involved in this study and some clinics have more than one microscopist. All the 122 microscopists registered at the 61 health clinics consented for the study, but only 100 (82.0%) were eligible for the proficiency testing. Two microscopists with red-green color visual defect and 20 microscopists without a complete set of seven slides were excluded. Age range of the microscopists participated in the study was 23-58 years old (Table 1). After excluding the sets with missing and broken slides, total 100 panel slides were tested. The raters scored a total of 400 positive and 300 negative slides independently. The Kappa agreement for grading and staining among the raters was 0.86 (95% CI = 0.8-0.9) and 0.66 (95% CI = 0.6-0.7) respectively.

Table 1. General characteristics of the microscopists reading sputum smears for *Mycobacterium tuberculosis* at government health clinics in 3 selected states, Malaysia, 2009-2010 (n=100)

Variable	Percent
Age range	23-58 years
Experience of 5 years or more in TB work (majority)	48
Maximum smear of 5 or less proceed per day (majority)	48
Good condition of microscope	97
Yearly servicing of microscope	74
Source of staining	
- Self-prepared	6
- Commercially prepared	37
- Centrally prepared	57
Attended in-service training on TB	80

A total of 618 (88.3%) slides were in agreement with the final raters' readings (Table 2). False positivity rate was 4.8% and false negativity rate was 18.7%. Sensitivity of grading from the microscopists compared to that of the raters was 83.8%, with specificity 94.3% and positive predictive value 95.2%. Of 300 predetermined negative slides, 5.6% were graded as positive by the microscopists. Among 400 predetermined positive slides, 16.3% were graded as negative and 12.5% were found to have QE (Table 3). The low positive slides had the highest incorrect reading (27.5%) whilst the moderate positive slides had the highest QE (29.0%) (Table 4). Sixty seven percent of the microscopists were scored as 80% or above proficiency level for grading and only 37% had achieved good proficiency level for staining (Table 5).

Table 2. Performance of the microscopists (n=100) and the raters reading sputum smears for *Mycobacterium tuberculosis* at government health clinics in 3 selected states, Malaysia, 2009-2010

Item				Performance
Performance between raters				
Kappa agreement for grading				0.86 (95% CI = 0.8-0.9)
Kappa agreement for staining				0.66 (95% CI = 0.6-0.7)
Performance between raters and microscopists				
Microscopists' reading		Raters' grading		
		Positive	*Negative*	*Total slides*
	Positive	335	17	352
	Negative	65	283	348
	Total slides	400	300	700
Overall agreement for grading*				88.3%
False positive**				4.8%
False negative***				18.7%
Sensitivity				83.8%
Specificity				94.3%
Positive Predictive Value				95.2%

* Number of negative and positive slides with consistent reading between raters and microscopists

** Grade as negative by rater, but grade as positive by microscopist

*** Grade as positive by rater, but grade as negative by microscopist

Table 3. Performance of the microscopists as graded by the raters reading sputum smears for *Mycobacterium tuberculosis* at government health clinics in 3 selected states, Malaysia, 2009-2010

Predetermined slide	Correct (10 marks)			Incorrect (0 mark)			Quantification error (5 marks)			Total
	Number	Percent	95% CI	Number	Percent	95% CI	Number	Percent	95% CI	
Negative	283	94.3	91.1-96.7	17	5.6	3.3-8.9	Not related	Not related	Not related	300
Positive	285	71.3	66.5-75.6	65	16.3	12.8-20.3	50	12.5	9.5-16.2	400
Total	568	81.1	78.0-83.9	82	11.7	9.5-14.4	50	7.1	5.4-9.4	700

Table 4. Performance of the microscopists graded by the raters using the predetermined positive slides from sputum smears for *Mycobacterium tuberculosis* at government health clinics in 3 selected states, Malaysia, 2009-2010

Positive slide	Correct		Incorrect		Quantification error		Total Number
	Percent	95% CI	Percent	95% CI	Percent	95% CI	
Low positive	69.5	62.6-75.8	27.5	21.4-34.2	3.0	1.1-6.4	200
Moderate positive	64.0	53.8-73.4	7.0	2.9-3.9	29.0	20.4-38.9	100
High positive	82.0	73.1-89.0	3.0	0.6-5.0	15.0	8.6-23.5	100

Table 5. Overall proficiency level of the microscopists for grading and staining quality from sputum smears for *Mycobacterium tuberculosis* at government health clinics in 3 selected states, Malaysia, 2009-2010

Proficiency level	Percent	95% CI
Proficiency level for Mycobacterium grading		
Scored ≥ 80%	67	56.9-76.1
Scored ≤ 79%	33	23.9-43.1
Proficiency level for Ziehl-Neelsen staining		
Scored ≥ 80% (good)	37	27.6-47.2
Scored 79-50% (fair)	40	30.3-50.3
Scored 49-0% (poor)	23	15.2-32.5

Discussion

In diagnosing the infectious pulmonary TB, sputum smear examination is the most important test to diagnose a person with persistent cough.[7] A number of newer TB diagnostic tools were becoming available, but the screening method in Malaysia was still microscopic examination of sputum smear. Consequently, the microscopists' knowledge on AFB morphology, their microscopic skills and staining technique greatly affect the patient care. Thus, reaching and maintaining an acceptable level of proficiency in sputum smear microscopy was imperative for a successful NTCP.

This study showed the false negative rate of 18.7% which, if translated into the working environment, indicated that some patients might not receive appropriate treatment and transmitted TB in general population. In addition, the study revealed that more errors occurred among the low positive slides when the staining was substandard. Poor quality of staining contributes to low detection of tubercle bacilli. This is especially true among the low positive slides. A study in India has shown that occurrence of false negative could be reduced from 58% before training the laboratory technicians on proper staining technique to 22% after training.[8]

Detecting TB at an early stage is of utmost importance and lacking the ability to detect bacilli in smears with low density could result in less effective TB control program. A person with untreated smear positive TB may infect 10-15 people per year, making the identification of these infectious patients as one of the key aspects of TB control.[9]

Sputum AFB microscopy may never reach 100% agreement in reading smears even between the experienced readers.[10] This can be seen from the Kappa agreement between the raters in this study, 0.86 (95% CI = 0.8-0.9) and 0.66 (95% CI = 0.6-0.7) for grading and staining respectively.

QE has no direct impact on treatment and monitoring, but it can indicate the general knowledge of the microscopist on AFB microscopy and skills of using the microscope. Correct quantification can at times be helpful to clinicians for decision making in difficult cases.[5] This study revealed that QE was high (29%) among the predetermined moderate positive slides which might indicate the possibility of microscopists not following the standard procedure for reading the smears. Consistently, under-reading of number of AFB can give useful indication to problem areas in the diagnostic process.

The microscopists were instructed to use their own ZN staining materials available in their laboratories to enable capturing the existing conditions in routine staining practice. In other words, the substandard staining performance might not be due to technique alone, but also might include other limiting factors such as the quality of ZN stains. The maximum number of ZN smears examined per a microscopist on average per day should not exceed 20. If more smears on average are read over a period of time, visual fatigue will lead to deterioration of reading quality. On the other hand, proficiency in reading ZN smears can only be maintained by examining at least 10 to 15 smears on average per week, i.e. a minimum of 2-3 examinations per day.[10,11] This could be one of the possible reasons for the poor performance because the microscopists might have experienced fatigue from other routine works. Inadequate number of smears read per day may also affect the ability to maintain proficiency. Hence, proficiency of microscopists can fluctuate from time to time depending on regular practice as well as other conditions such as fatigue resulting from reading many slides.

Choosing the number of slides for this study was considered as a good representation for the assessment indicator and concurrently not to add unnecessary burden to the existing workload of the technicians in the laboratories. A set of seven slides was taken as appropriate for the raters and other microscopists in various laboratories to process and examine per working day without losing the quality. Literature reviews have shown studies using slides ranging from 6-10 slides per set for panel testing with maintaining the necessary composite of mycobacilli density.[5,9]

To reduce burden for preparation of numerous slides, only one set of unstained slides per microscopist was used in this study as compared to other studies that used both stained and unstained slides. We could only determine two different proficiencies of the microscopists, namely, staining alone and staining with grading together as one entity. Hence, in this study, when a microscopist performed poorly for grading, we could not determine whether he was truly poor at grading which here referred to the cumulative process of staining with grading. In other words, if the same microscopist was given a different slide with good staining quality, his capacity to read the slide might be much better.

Scoring for staining is difficult as it is very subjective. However, we have attempted to identify the weakness in diagnostic process by reading the characteristics and the features presented after staining. Sending

unstained slides for test panels has the advantages of testing several aspects of the staining procedure conducted by the microscopist, including preparation of staining reagents, staining procedures, reading and reporting results.[5]

This panel testing was timely since the NTCP was at the early stages of conducting the EQA rechecking program for all states in Malaysia. The findings of this study could provide a quick assessment among the microscopists from the three states and the template would be useful if similar assessments were to be conducted in other states. In a northern province of South Africa, a proficiency testing was conducted with the aim to solve operational problems and developed an 'intervention plan' for corrective action. Before intervention, the correct result was 85.5% and with the intervention, the result was increased to 97.4%.[11]

Limitations

As with any study, limitations were inevitable and the results from this study might be biased as the microscopists were aware that they were being tested and thus, they would have dedicated more time and effort to get a better performance. Nevertheless, if poor proficiency was found, it implied that the real performance might be even poorer.

Findings and interpretations from this study were only applicable to the microscopists in the three states. These might not be generalized to other states as the resources, workload and manpower were different, and the selection was based on TB prevalence and laboratory burden.

Public Health Actions and Recommendations

We recommended that tests for vision and cataract should be incorporated into the health examination for microscopists aged 40 years and above. This would help to detect any vision impairment among microscopists that may affect their ability to read the ZN stained AFB slides. It would also be a good practice to screen the microscopists serving in TB laboratories for the red-green color vision defect.

We suggested training the microscopists to emphasize the importance of staining and preparation for staining. Regular quality control on staining reagents should be encouraged by using positive and negative control slides before a new batch of reagents is used in the laboratory. As carbol fuchsin and methylene blue may precipitate over time, it is essential that microscopists filter their stains when precipitation occurs as this may affect the quality of their staining.

This study was presented to the policy decision makers, and the eyes tests for vision and cataract among microscopists aged more than 40 years has been incorporated into the health screening for health staff in accordance with the Public Service Circular number 3/2003. As for the existing and new microscopists working in TB laboratories, they have been directed to be screened for red-green color vision defect using the Ishihara Chart which has been available in all health clinics.

Conclusion

This study revealed that only 67% of the microscopists had the acceptable proficiency level for grading and the false negativity was high (18.7%). The low-density predetermined positive slides had the highest percentage (27.5%) of incorrect reading. The aim of staining is to make the bacilli easily visible so that their presence can be detected. Only 37% of the microscopists had achieved good staining level. When there is low staining quality accompanied by low-density of mycobacilli, this further compounds the problem on visibility which results in false negative slides. This is of great concern for intermediate burden countries like Malaysia where most of the suspected TB cases may have low bacillary load at the onset of disease. Due to better health care facilities or better awareness, patients with suspected TB may seek treatment early, but might not be detected by sputum smear microscopy. This may be one of the reasons for late detection of cases and patients being diagnosed as moderate or severe form of pulmonary TB.

The optimum performance in diagnostic process is derived from skillful practice of a series of procedures that begins with sampling and carries through smearing, staining and grading. If there is a weak link in any of the procedures, then it is difficult to attain the desired performance. Identifying the problem area certainly makes the corrective action on easier tasks and ultimately, customizes training according to the needs of the diagnostic system.

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