International Journal of Mycobacteriology 4 (2015) 1–6

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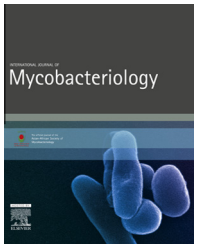

Review

Microscopy as a diagnostic tool in pulmonary tuberculosis

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ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form
17 December 2014
Accepted 20 December 2014
Available online 2 February 2015

Keywords:
Microscopy
Tuberculosis
Ziehl–Neelsen
Mycobacteria
Fluorescence

ABSTRACT

Tuberculosis continues to cast a huge impact on humanity with its high incidence and mortality, especially in developing countries. For tuberculosis case detection, microscopy continues to be indispensible, given its low cost, rapidity, simplicity of procedure and high specificity. Modifications have attempted to improve the sensitivity of microscopy which include: concentration methods such as centrifugation, N-acetyl cysteine–sodium hydroxide, bleach, ammonium sulfate or chitin. Furthermore, classical Ziehl–Neelsen (ZN) staining has been subjected to varying carbol fuchsin concentrations or replaced by Kinyoun staining, fluorescent microscopy or immune-fluorescence. Currently, light emitting diode fluorescence is recognizably the most plausible method as an alternative to ZN staining.

Contents

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Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2014.12.006

Introduction

Tuberculosis (TB) continues to intimidate the human race since time immemorial as a severely debilitating disease. The socio-economic burden of TB has been the subject of much concern, and major efforts are under way to try to achieve its control. In 2012, an estimated 8.6 million cases developed TB and 1.3 million died from the disease [1]. Directly Observed Treatment Strategy (DOTS) was formally introduced in 1997 and involved documentation and surveillance of TB and brought about a degree of control [2]. Here, emphasis is given to TB diagnosis by identification of acid-fast bacilli (AFB) on un-concentrated sputum (direct smears) with Ziehl–Neelsen (ZN) staining [3].

The historical perspective

In the 19th century in Eastern Germany, physician and scientist Robert Koch (1843–1910) established bacterial techniques to diagnose bacterial infections. On the evening of March 24, 1882, Robert Koch presented his landmark lecture with a statement on tuberculosis: "If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plagues, cholera and the like. One in seven of all human beings dies from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more". He demonstrated the presence of the rod-shaped bacterium, Mycobacterium tuberculosis (MTB), by the staining methods invented by him. He used various adaptations of the staining methods of Carl Weigert in smear microscopy. Subsequent to Robert Koch's discovery, several other researchers (Ehrlich, Ziehl, Rindfleisch, and Neelsen), intending to improve on Koch's method, introduced modifications to the reagents and used carbolic acid (phenol) as the mordant. Paul Ehrlich developed the alum hematoxylin stain and demonstrated the tubercle bacillus in 1886. Ehrlich's method was further modified first by German bacteriologist Franz Ziehl (1859–1926) who modified the procedure by using carbolic acid (phenol) as the mordant. Subsequently, pathologist Friedrich Neelsen (1854–1898) kept Ziehl's mordant, but changed the primary stain to the basic fuchsin (first used by Ehrlich in 1882). This method became known as the Ziehl–Neelsen method in the early to mid-1890s and is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria [4]. In this method, heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells. The use of heat in this method has been the reason that this technique is called the "hot staining" method. The Ziehl–Neelsen method has endured as a reliable and effective way to demonstrate the acid-fast bacteria [4]. Simultaneously, in Denmark, Hans Christian Gram developed a method for broadly distinguishing bacteria into two groups on the basis of a particular staining characteristic. However, Mycobacteria are gram positive, but many species stain poorly even after the prolonged heating. In 1915, Kinyoun published a method that has become known as the "cold staining" method because the heating step was removed in favor of using a higher concentration of the carbol-fuchsin primary stain [4].

Utility with the road blocks

Most National TB control programs in developing countries are implementing direct sputum microscopy primarily for tuberculosis case detection [1]. Though culture is more sensitive than microscopy, in developing countries, diagnosis is primarily based on AFB microscopy owing to its simplicity, less cost and rapidity. It is highly specific for MTB, which appear as long, curved and beaded. The Non-Tuberculous Mycobacteria (NTM) may appear as short, straight bacilli with no specific morphology [2].

The MTB forms tight ropes called cords in liquid media which can be identified on AFB smear. Cord formation has been used for presumptive identification of MTB as compared with the MOTT as it is rapid, sensitive and low-cost compared with the conventional identification system [5].

ZN staining has a low sensitivity of 22–43% for a single smear. Maximum sensitivity has been found to be up to 60% under optimal conditions when compared with that of cultures [6,7].

The threshold of detection of AFB in sputum samples under optimal conditions is found to be between 10^4 and 10^5 bacilli per ml. The yield is often decreased further under program conditions due to technical and operational constraints [8]. The sensitivity is even lower in pediatric and human immunodeficiency virus (HIV)/AIDS patients who usually present a pauci-bacillary picture [9,10]. Children under 12 years of age with pulmonary TB rarely produce sputum and are usually unable to expectorate voluntarily. When sputum samples cannot be obtained, gastric aspirate samples are used for detection and isolation of MTB. Even though AFB stain of sputum is positive in up to 75% of adults with pulmonary TB, fewer than 20% of children with TB have a positive AFB smear of sputum or gastric aspirate [10]. A total of 412 adults with culture-proven pulmonary tuberculosis were studied, of whom 185 (44.9%) were HIV sero-positive and had a significantly lower sputum smear positivity than HIV sero-negatives (68% versus 79%, $p < 0.05$) [9].

The collected sample

If pulmonary TB is suspected, specimens originating from the respiratory tract should be collected, i.e., sputum, induced sputum, broncho-alveolar lavage or a lung biopsy. Earlier, for the diagnosis of pulmonary TB, three first-morning spu-

tum specimens (not saliva) obtained after a deep, productive cough on non-consecutive days had been recommended as endorsed by the International Union against Tuberculosis and Lung Disease (IUATLD) and the World Health Organization (WHO) [8].

Several studies have shown, however, that the value of the third sputum is negligible for the diagnosis of TB, as virtually all cases are identified from the first and/or the second specimen.

In the study by Yassin et al. it was reported that 99% of the cases were identified from the first and second specimens [11]. In another study from Turkey, it was found that 97% of AFB are detected in the first sputum sample with only 3% in the second smear and none in the third smear [12]. Reducing the number of specimens would have multiple advantages by reducing the work flow of over-burdened laboratories, reducing cost and inaccessibility to the population.

Apart from the number of samples collected, sputum specimens must be classified in the laboratory with regard to their quality, i.e., bloody, purulent, muco-purulent or salivary.

Improving sensitivity

Sample processing procedures

Besides proper collection of sputum samples from suspected pulmonary TB patients, the preparation of good, uniform, thin smears and staining of smears with high quality staining reagents is imperative in precise reporting on microscopy.

Processing of samples by centrifugation generally leads to higher yield by concentrating the bacilli. In a review on sputum processing methods, 14 studies (culture used as the reference standard) investigated the impact of sputum processing by centrifugation usually on microscopy. In addition, a chemical was used such as either bleach or sodium hydroxide. Sputum processing yielded a mean of 18% (95% confidence interval [CI]: 11–26%) increase in sensitivity as compared with direct smear [13]. In one such study, the sensitivity of AFB smears was increased from 28.6% using the direct method to 71.4% (HS–SH) and 66.7% (NALC–NaOH) using DC methods. Both concentration techniques were highly comparable for AFB smear compared with un-concentrated direct smear [7]. A rare study, however, found sensitivities of direct and NALC–NaOH concentration methods to be similar [14].

Bleach processing has been reported to facilitate identification of bacilli by providing a clearer microscopy field through digestion of mucus and debris and concentrating bacilli through centrifugation or sedimentation. Some studies have found bleach sedimentation to be inexpensive, quick, effective and a simple method to improve the yield of smear microscopy [15]. In one of these studies in a high prevalence of HIV setting, bleach sedimentation microscopy detected significantly more positive cases than did direct smear microscopy, 26.7% versus 21.7%, respectively [15]. Although the bleach method has been used routinely only in three countries, studies suggest the evaluation and introduction of the bleach method in settings where Mycobacterial culture is not performed routinely [16]. In addition, the Stop TB Partnership Retooling Task Force has also identified bleach sedimentation as one of the three promising approaches to improving the sensitivity of sputum smear microscopy, especially in high-burden countries.

The sedimentation studies have investigated the effect of overnight sedimentation or short sedimentation time of 30–45 min with bleach or ammonium sulfate with culture as the reference gold standard [17]. The average increase in sensitivity is 23% (median 28%; range 2–34%) using overnight sedimentation, while in studies with short sedimentation times the average increase was more modest at 9% (median 1%; range 0–36%). Specificity was comparable to direct sputum microscopy [13]. However, the question that remains unanswered is whether the above-mentioned methods will yield similar results if carried out in peripheral laboratories in low-income countries? Concerns evident are: feasibility of centrifugation with irregular power supply, limited resources; additional cost of chemicals, inadequate training and potential biohazards posed.

Chitin has also been tried for better recovery of MTB by mucous digestion and sedimentation. Chitin molecules resemble cellulose closely and both N-acetyl-L-cysteine and chitin have an acetyl-amine group in their structure. Since $NHCO–CH_3$ is responsible for the mucolytic effect of cysteine, chitin might induce the same effect in sputum. In a pioneering study, 16%, 15.1% and 14% of samples were found to be positive for MTB by N-acetyl-L-cysteine concentration, chitin-treatment and NaOCl liquefaction methods, respectively, compared with MTB detected by direct sputum microscopy (9.5%) [18]. Specific details are given in Table 1.

Table 1 – Sensitivity of smear microscopy after various sample processing techniques.

Name of investigator	Method used	Percentage sensitivity	Year
Steingart et al.	Sedimentation using bleach or sodium hydroxide; review	18% (95% confidence interval; CI: 11–26)	2006
Ganoza et al.	Hypertonic saline–sodium hydroxide	71.4% (95% confidence interval; CI: 52.1–90.8)	2008
Ganoza et al.	N-acetyl L-cysteine–sodium hydroxide	66.7% (95% confidence interval; CI: 46.5–86.8)	2008
Cattamanchi et al.	N-acetyl L-cysteine–sodium hydroxide	52% (95% confidence interval; CI: 44–61)	2009
Bonnet et al.	3.5% Domestic bleach	26.7% (95% confidence interval; CI: 23.3–30.2)	2008
Singhal et al.	Phenol ammonium sulfate (PhAS) method	85.5% (95% confidence interval; CI: 83.2–87.6)	2013
Farnia et al.	N-acetyl L-cysteine–sodium hydroxide	16.1% (95% confidence interval; CI: 12.8–19.9)	2002
Farnia et al.	Chitin treatment	15.1% (95% confidence interval; CI: 11.9–18.9)	2002
Farnia et al.	Sodium hypochlorite liquefaction	14.7% (95% confidence interval; CI: 11.5–18.4)	2002

Table 2 – Sensitivity of smear microscopy with different staining reagents.

Name of investigator	Method used	Percentage sensitivity	Year
Deun et al.	1% carbol-fuchsin	83%	2005
Deun et al.	0.3% carbol-fuchsin	78%	2005
Deun et al.	Kinyoun	78%	2005

Reagent concentrations

Different concentrations of carbol-fuchsin have also been tried to increase smear sensitivity. In one such study, ZN staining was done using different concentrations of carbol-fuchsin such as 1%, 0.3% and 0.1% (Table 2). The sensitivity of the test was comparable when 1% and 0.3% basic fuchsin was used; however, sensitivity was reduced significantly using 0.1%. Using this reagent at less concentration can reduce costs which can have large-scale implications. Hence, this concentration has been proposed by WHO and IUATLD, although RNTCP guidelines continue to recommend 1% [19].

Modifications in microscopy

Different microscopic alternatives include fluorescent microscopy, Kinyoun staining and modified carbol-fuchsin staining. As per a systematic review of 45 relevant studies, fluorescent microscopy has been found to be on an average 10% more sensitive than conventional microscopy (95% CI: 5–15%) and almost 98% specific [20]. However, initially fluorescent microscopy was not widely implemented due to high cost, frequent burn-out of expensive mercury vapor lamps, continuous power supply necessity, and need of a dark room [20]. The advent of light emitting diode (LED)-based fluorescent microscopes have overcome these short-comings and are now being increasingly used. In a study from the National Reference Laboratory (NRL), New Delhi, sensitivity and specificity of LED microscopy, mercury vapor fluorescence and light microscopy were 83.1% and 82.4%, 78.5% and 87.5%, 81.6% and 83.5%, respectively. Mean reading time of LED was three times faster than ZN [21]. Presently, Auramine O staining-based LED has replaced conventional ZN microscopy in 200 Designated Microscopy Centers (DMC) of medical colleges operating in collaboration with India's Revised National Tuberculosis Control Programme (RNTCP) in 2012 [22].

Improved MTB detection methods have been attempted in immune-fluorescence format as well. In a study, anti-MTB antibody was used as the primary antibody followed by antibody binding protein (Protein A) labeled with Tris (2,2-bipyridyl) dichlororuthenium (II) hexahydrate (RuBpy)-doped silica nano-particles to generate a fluorescent signal for microscopic examination. The use of the fluorescent nano-particles reveals amplified signal intensity and higher photo-stability than the direct use of conventional fluorescent dye [23]. These techniques are detailed in Table 3.

Smear reading

Manner and quality of smear reading has a major impact on the result of sputum smear microscopy. A prospective observational study over a 6-month period found that the median routine examination time for sputum slide was found to be 2 min 6 s (interquartile range 1:30–2:30). Blinded reexamination of all slides for 10 min significantly increased the number of positive smears from 82 to 116 ($p = 0.0083$), and overall case detection from 28 to 48 patients ($p = 0.011$). Thus, by ensuring that smears are examined for the recommended duration, at least 5 min or 100 fields may be a simple and low-cost way to improve case detection [24].

Quality assurance

The results of sputum AFB microscopy are known to be influenced by various factors, including the proficiency to read smears by microscopist as mentioned above. The need for training laboratory technicians has a major bearing on the quality of sputum AFB microscopy [25]. In an Indian study by the Tuberculosis Research Center (TRC), SNRL, the proficiency of Senior Laboratory Technical Supervisor technicians (STLS) undergoing 15-day training in reading AFB sputum smears was conducted. On day 1, each trainee was given a set of smears for reading which were repeated on day-15 of the training without being told about the identity of the smears. The sensitivity to read sputum AFB smears by fresh STLSs with little or no experience increased from 75% to 94% during the carefully planned training program. The study highlighted the importance of training in improving the microscopy results [26].

The quality of reagents and staining procedures is also a quality determining factor. In another Indian study from TRC Chennai, 73 AFB-negative sputum smears systematically

Table 3 – Sensitivity of smear microscopy with various microscopy variations.

Name of investigator	Method used	Percentage sensitivity	Year
Bhalla et al.	LED microscopy	83.1%	2013
Bhalla et al.	Mercury vapor microscopy	82.4%	2013
Bhalla et al.	Light microscopy	78.5%	2013

selected were rechecked before and after re-staining with the same AFB staining method. AFB not observed in any of the 73 AFB negative slides before re-staining were observed on 30 slides after re-staining. These were diagnosed as environmental Mycobacteria by an experienced microbiologist. The authors concluded that proper storage of slides, preparation of staining reagents with distilled water, washing slides with clean water and using clean immersion oil are essential for preventing contamination [27].

In another study by Rie et al., the effect of a short training course for technicians and the distribution of new microscopes on the quality of smear microscopy in 13 primary health care laboratories from Congo were performed based on the international EQA guidelines. The EQA guidelines were suggested to be useful for implementation in resource-poor settings [28].

Microscopy in National Tuberculosis program

IUATLD had organized a workshop for experts in sputum smear microscopy in August 2005, in which it was suggested that the ZN staining guidelines need to incorporate a wider margin of error for widespread application under field conditions; the utmost importance must be given to quality assurance with a commitment from the National TB programs and other health authorities; countries need to invest in the purchase of high quality microscopes, laboratory supplies and allocate sufficient resources for rechecking and supervision; personnel without specific laboratory schooling can be trained to respond to the immediate need for TB microscopy services to avoid a human resource crisis [29].

As per RNTCP guidelines in India, microscopy forms the mainstay tool in the management of TB. Diligent recording of the laboratory registers is a central concept wherein a laboratory serial number is assigned to each patient, rather than to the sputum specimen. Entire details of the patient are entered, including treatment history and follow-up at regular intervals in order to assess the treatment outcome and accordingly modify regimens. Earlier, for quality assurance of microscopy, all positives and 10% of negative slides among the total slides examined in a calendar month in a Designated Microscopy Center (DMC) were checked in an unblinded fashion by the STLS during onsite supervisory visits, which was substantially labor intensive. In 2002, the lot quality assurance sampling (LQAS) method was suggested in order to recheck the minimum number of slides examined in DMCs for assessing performance, which was found to be useful for monitoring the performance of AFB microscopy centers by reducing rechecking of the minimum number of slides and yet giving a good assessment of quality in microscopy centers [30].

Conclusions

Sputum smear microscopy for the diagnosis of pulmonary TB has stood the test of time. In resource-limited countries, microscopy will remain the primary means of microbiological diagnosis of TB for the foreseeable future. Presently, the LED-based fluorescent microscopy technique has been established as the best alternative to ZN-staining. The modifications in sample collection, sample processing methodology and staining techniques can further expand the base of this age-old diagnostic modality.

Conflict of interest

None delared.

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