Tuberculosis control programmes all over the world are currently using DOTS strategy. Our Revised National Tuberculosis Control Programme (RNTCP) is based on classifying the patients according to the treatment they have taken earlier and detection of acid-fast bacilli (AFB) by direct microscopy\(^1\). Nearly 125 yr ago, Ziehl and Neelsen separately contributed to the evolution of the staining method which is now popularly known as Ziehl-Neelsen (ZN) staining\(^2\). Its colder version, Kinyoun’s staining, is 87 yr old\(^3,4\). Due to its clinical importance this simple method continues to be the centre of debate and also several improvements including the protocols based on fluorochrome staining\(^3-7\) have been introduced. For reviewing these developments, several issues are important:

(i) **Proper sampling**: The demonstration of AFB by Ziehl-Neelsen/fluorochrome staining depends upon the presence of sufficient number of bacilli in the specimen. Usually, the specimen is sputum, only in a section of the cases, it could be an aspirate/biopsy from the involved side. It is very important that a proper and adequate amount of specimen is collected and transported. For this purpose, several guidelines about the number of specimens/type of specimen and method(s) of collecting the same are available\(^3,4\).

(ii) **Transport of the specimens**: In many situations, the same specimen is used for both smear microscopy as well as culture of mycobacteria. As the specimen may not be transported or processed on the same day, it is common practice to use preservatives like CPC (cetyl pyridinium chloride). It has been observed that quaternary ammonium compounds may affect the acid-fast character of AFB, thereby decreasing the detection rates\(^8\). Exposure to unbuffered formalin also affects acid fastness\(^9\), but it may not matter in case of detection of AFB directly from clinical specimens when this fixative is not used. As such, the duration and method of preservation should be given adequate attention for achieving optimal results.

(iii) **Detection of acid-fast bacilli when culture is not required**: In the programme, and where the facilities do not exist, culture for mycobacteria is not done. In such situations, compounds like sodium hypochlorite/bleaching powder are recommended to kill the mycobacteria so that the specimen could be safely handled by the technicians/health care workers. It has been reported that exposure of specimen to sodium hypochlorite for 15 min followed by centrifugation\(^10\) or passing through polycarbonate filter\(^11\) yield good results. There are reports that treatment with bleaching powder may adversely effect the acid fast character.\(^12\) It would thus be important to be aware of limitations of this approach. The study by Selvakumar et al\(^13\) in this issue utilized carbol-fuchsin to fix and stain the bacilli in the deposit from sputum so that it becomes non hazardous. This appears to be a good approach theoretically and does not appear to be compromising on the sensitivity either. However, specificity may be affected if other organisms present in the specimen take up the stain.
(iv) Detection of acid-fast bacilli in smears when culture for mycobacteria is required: To achieve this simultaneously the sputum needs to be processed for homogenization, decontamination and concentration. Decontamination is not required if the specimen is from sterile sites such as CSF, blood, biopsy or aspirate. Usually, sodium hydroxide based Petroff’s method or its modifications (N-acetyl-L-cysteine- NaOH, NaOH + benzalkonium; NaOH +oxalic acid, etc) are used to process the specimens\textsuperscript{3,4,14,15}. Use of N-acetyl-L-cysteine (NALC) improves the release of bacilli by better mucolysis\textsuperscript{14}. Appropriate concentration and duration of exposure to sodium hydroxide remains critical for isolation which, however, may not matter much for detection of AFB. Another approach of CB-18 concentration has been observed to improve the detection of acid-fast bacilli\textsuperscript{16}. Recently, a new universal solution (USP) has been published which appears to be good when investigations like smear microscopy, culture and also gene amplification assay(s) are contemplated simultaneously\textsuperscript{17}.

(v) Staining method(s): Ziehl-Neelson’s staining is traditionally based on heating the carbol-fuchsin on the smears followed by decolourization with acid or acid-alcohol, and finally counterstaining with methylene blue or malachite green. If the technician is well trained and does not overheat, this technique has been reported to give satisfactory results. The heating method can be substituted by cold Kinyoun’s method where higher concentration of phenol and basic fuchsin are used\textsuperscript{14}. There have been reports that this cold Kinyoun’s method may be less sensitive as compared to ZN hot staining method\textsuperscript{18,19}. It will not be fair to pass judgments and individual laboratories should themselves standardize and compare these methods in their own set-ups. Even the quality of stain itself may lead to variations. Besides these acid fast staining methods, flurochrome staining using auramine/ rhodamine stains has also become a popular approach wherever fluorescent microscopes are available\textsuperscript{4}. Not only the fluorescent microscopy has been described to be more sensitive than ZN staining\textsuperscript{20,21}, but it is also more convenient and less strenuous to the technicians. The same smear can be stained by both ZN as well as fluorescent staining\textsuperscript{4}. Flurochrome stained smear may be overstained with ZN, after examination without removing the auramine. However, smears are no longer suitable for fluorescent staining if these have already been stained with ZN earlier.

(vi) Sensitivity of smear microscopy: It is well known that smear microscopy is not a very sensitive tool to detect mycobacteria as 5- 10,000 bacilli per/ml are required to get reproducible results\textsuperscript{5}. Below that level, it is a matter of chance of finding a organism in the specimen. It is for this reason examination of at least three specimens is recommended to detect AFB.

(vii) Specificity: It is well known that AFB could be a nocardia or any species of mycobacteria that could be a pathogen, opportunistic pathogen or a saprophyte. Up to May 2006, there were 119 known species and 11 sub species of mycobacteria\textsuperscript{22} and only 30 of them are obligate or opportunistic pathogens. Of all mycobacteria, Mycobacterium tuberculosis is the commonest mycobacterium causing disease in India. However, in a section of the cases, it could be a colonizing mycobacteria. To confirm aetiological relationship with a non tuberculous mycobacteria as a causative organism of mycobacterial disease, a very cautious approach is necessary. It is only the index of suspicion and repeating the testing of the specimens which can lead to a reasonable sure conclusion.

Conclusion/ future perspective

Smear microscopy has evolved significantly during the last century. Today depending upon financial situation one may use simpler versions or even automated stainers. Despite major technological advances this still continues to be an important tool to diagnose and monitor the
treatment in tuberculosis. We need to gain more experience on whether the molecular methods could be used/complementarily for diagnosing paucibacillary diseases.

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