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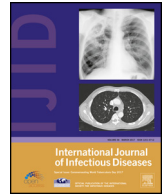
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## Review

## Exploring tuberculosis by molecular tests on DNA isolated from smear microscopy slides



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## SUMMARY

Tuberculosis (TB) is an infectious disease of global public health importance caused by *Mycobacterium tuberculosis* complex. The disease has worsened with the emergence of multidrug-resistant (MDR)-TB strains. The timely diagnosis and treatment of TB remains a key public health priority, and laboratories have a critical role in the rapid and accurate detection of TB and drug resistance. Molecular assays based on nucleic acid amplification techniques have been developed for the rapid, sensitive, and specific diagnosis of TB, with the ability to determine the drug sensitivity status. These molecular techniques are now available or are being implemented in developing countries. However, traditional microscopy and culture methods cannot yet be replaced; the molecular assays can be applied in parallel with these tests for the diagnosis of TB or for drug susceptibility testing. Performing such molecular tests is often restricted by constraints with regard to sputum sample storage and safe transportation from remote health centres to central laboratories. Since smear slides are performed routinely for the diagnosis of TB in most TB diagnostic laboratories, they are readily available and could be the ideal tool to transport sputum for further molecular tests. The aim of this review was to provide a comprehensive survey on the use of smear slides for both TB diagnosis and the molecular test approach. Based on the literature, stained smear microscopy slides can be a safe system for the transportation of sputum specimens from remote health centres to reference TB laboratories for further molecular TB or MDR-TB detection, and could help in the rapid diagnosis and therefore timely management of TB patients.

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## 1. Introduction

Tuberculosis (TB) is a serious public health problem in developing countries and has been worsened by HIV co-infection and the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis*. MDR-TB is caused by strains that are resistant to at least rifampicin (RIF) and isoniazid (INH); XDR-TB is caused by strains that are resistant to RIF and INH, and have also acquired resistance to fluoroquinolones and to one of the second-line injectable drugs: kanamycin, capreomycin, or amikacin.<sup>1</sup>

In most peripheral-level laboratories and health centres in low-income countries, TB treatment is started based on acid-fast

bacillus (AFB) smear microscopy examination, which is the traditional and most commonly used first diagnostic test to screen for pulmonary TB; culture and drug susceptibility testing (DST) cannot be performed routinely as these are mostly done in reference laboratories with modern equipment.<sup>2</sup> While AFB smear microscopy can quickly provide the clinician with information on the patient's TB status, drug resistance is generally suspected only in the case of treatment failure or relapse. There may, therefore, be a risk that resistant *M. tuberculosis* strains will spread among patient contacts, which can be a hurdle to the success of TB control programmes.<sup>3</sup> Thus, the rapid detection of MDR-TB is essential for the early and adequate treatment of these patients.

Novel molecular technologies are now available for the rapid screening of TB drug resistance. The World Health Organization (WHO) has endorsed several molecular tests, such as the GenoType MTBDRplus line probe assay (LPA) and GeneXpert MTB/RIF, to detect MDR-TB.<sup>1</sup> In many countries, especially those with limited

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resources, these molecular tests are only available in reference-level laboratories. In lower-level laboratories, basic diagnostic tests such as smear microscopy examination are offered, and patients or their specimens are referred to reference-level laboratories for further molecular tests.

Due to the presence of genetic markers and the rapid development of genomics, molecular tests are also powerful tools for use in molecular epidemiology studies and for tracing drug-resistant strains. However, these molecular assays require DNA extraction from either *M. tuberculosis* isolates or ideally from sputum samples. Performing such molecular tests is often restricted by constraints of both (potentially infectious) sputum sample storage and safe transportation from remote health centres to central laboratories. Furthermore, the high cost and logistics of providing proper sputum transportation, as well as the return of test results to the peripheral centre and the patient, may also be a challenge. Different transport systems such as filter cards (FTA, Genocard, etc.),<sup>4,5</sup> preservative reagents and ethanol<sup>6</sup> are now available for biological sample storage and transportation at room temperature. However these storage systems are not yet used as routine in low-income countries. However, since smear slides are prepared for microscopy examination in almost all TB diagnostic laboratories, they are readily available and could therefore be the ideal tool to transport sputum for further molecular testing.

This article reviews the literature on the utilization of smear slides obtained for histopathology and/or microscopy diagnosis, for the extraction of mycobacterial DNA and molecular analysis, including molecular diagnostics, drug resistance testing, and genotyping.

## 2. Smear slide systems that have been used for other microorganisms

The first microscope was probably invented around 1300 AD. In the 19<sup>th</sup> century, improvements in microscope technology allowed further exploration of the cellular and microbial worlds. Microscopes then became available to the biologist, and microscope slide mounts rapidly developed into collections, which were kept in the possession of private individuals, universities, hospitals, and other institutions. In addition to slides made by museum staff, private collections were also transferred to museums for safe-keeping, particularly from institutions that no longer had the resources to properly care for such collections.<sup>7,8</sup>

One of the most common ways to observe microorganisms microscopically is to stain the different structures with selective dyes. However, microscopic diagnosis using staining techniques, for instance Gram staining or Giemsa staining to search for bacteria or parasites, respectively, does not usually allow the long-term conservation of samples.<sup>9,10</sup> Moreover, while genomic DNA has been recovered efficiently from untreated glass slides,<sup>11</sup> recycling such glass slides for the DNA extraction after a long-term storage would appear difficult without the usual fixation techniques that may damage the bacterial cells and the DNA.

Pure alcohol (ethanol) is a good fixing agent but it is as toxic as some other fixatives. The combination of alcohol and acid (in particular acetic acid), denatures proteins and DNA. A low pH is detrimental to microbes, due to the denaturation of essential macromolecules such as proteins and the acidification of the cytoplasm, which disrupts enzymatic reactions and membrane potentials. Acids destroy the covalent bonds that hold the base pairs together in a strand of DNA, and alcohol precipitates DNA.<sup>10,12</sup>

The situation is quite different for the AFB due to their resistance to acid and alcohol. AFB staining techniques could benefit from these chemical properties for long-term sample transportation, DNA conservation, and/or archiving of the glass

microscopy slides. Among the microbial specimen conserved on glass slides that have been detected by AFB staining techniques is *Cryptosporidium*: single *Cryptosporidium* oocysts from faecal smears were efficiently detected after having been archived on glass slides for up to 2 years.<sup>13</sup> The high efficiency of DNA isolation from these slides demonstrates the usefulness of archival and type collection slides for molecular biology and molecular taxonomy purposes. DNA from *Cryptosporidium* (oocysts) and *Cyclospora* (oocysts) in faecal smears archived on glass microscope slides stained by conventional AFB method, has been extracted successfully and genotyped.<sup>14</sup> Moreover, this is useful for long-term archiving regarding DNA isolation from museum and type collection slides of microsporidia.<sup>13,15</sup>

With regard to the mycobacteria, the detection of AFB from stained slides by PCR has been reported to have advantages over conventional microscopy and serological methods. In situ PCR combined with histopathological examination was reported to be useful for the confirmation of leprosy in early or doubtful cases in 13/20 patients under 16 years of age in India.<sup>16</sup> Kamal et al. confirmed 72% of leprosy cases using in situ PCR on slit skin smears in paediatric patients with early leprosy compared to only 20% diagnosed by slit smear.<sup>17</sup> Natrajan et al. also reported direct in situ PCR on a 530-bp fragment of DNA encoding a 36-kDa antigen of *Mycobacterium leprae* using skin biopsies of lesions to improve the diagnosis in 70.6% of early cases and 60% of suspected cases among histopathologically unconfirmed adult leprosy cases.<sup>18</sup>

The molecular diagnosis of leprosy using the AFB technique with Ziehl-Neelsen-stained (ZN) microscopic slides requires an optimized method of DNA extraction. In a study comparing four DNA extraction methods (phenol-chloroform, Chelex 100 resin, and the commercial kits Wizard Genomic DNA purification and QIAamp DNA Mini) from ZN slides for nested-PCR, Ruiz-Fuentes et al. obtained good results for leprosy, with a high bacteriological index (BI) with all four methods, although only the Qiagen method gave a positive PCR result with BI-negative slides.<sup>19</sup> Their results demonstrated the feasibility of molecular tests to improve the diagnosis of leprosy with ZN slides. Therefore ZN slides can readily be sent to reference laboratories for later molecular analysis; these slides may be useful not only for diagnosis, but also for the application of other molecular techniques. With regard to the DNA extraction methods, further investigations are needed to assess the very long-term stability of DNA on AFB smear microscopy slides and to optimize the conservation method for biobanking, since microbiology and pathology procedures are variable.<sup>20</sup> Moreover, the environmental conditions of conservation and archiving should be monitored regularly – temperature, light, humidity, desiccation, and freezing can have deleterious effects on many mountants in the long term, such as those in archived museum collections.<sup>8</sup>

## 3. Smear slide systems used for the detection of drug resistance and genotyping of *M. tuberculosis*

### 3.1. DNA extracted from slides for TB diagnosis

Smear microscopy is known to be a rapid method for diagnosing TB; however, its specificity and sensitivity remain low. Moreover, the sensitivity of smear microscopy using AFB staining is limited to 10<sup>4</sup> mycobacteria per millilitre. Combining smear microscopy with the molecular detection of bacilli by PCR is more sensitive and specific compared with the conventional methods.<sup>21</sup>

Extracting DNA from microscopy slides and providing sufficient material for PCR was facilitated by the method developed in 1991 by Walsh et al.; they successfully used Chelex combined with boiling for the extraction of DNA for PCR-based-typing from forensic material.<sup>22</sup> The relative simplicity of the procedures and

the reduction in the number of steps for sample preparation help reduce the chance of introducing DNA contamination.<sup>22</sup>

Vago et al., in 2000, used ZN-negative cytology slides from patients with extrapulmonary TB and HIV and evaluated the usefulness of a nested PCR to detect *M. tuberculosis*.<sup>23</sup> DNA was extracted after removing the coverslip and gently scraping the cytological sample from the slides. They demonstrated that sufficient DNA was obtained from the slides for PCR without any significant loss of integrity and they could detect *M. tuberculosis* on archival cytology slides. Likewise when no fresh specimen was available for the diagnosis of cervical TB lymphadenitis, PCR screening of IS6110 by PCR was achieved using archival Papanicolaou-stained fine-needle aspiration smears with a sensitivity of 50% and specificity of 100%.<sup>24</sup>

In 2004, Tansuphasiri et al. demonstrated a sensitivity of 86.3% and specificity of 100% for IS6110-target amplification by silica-based filter with DNA extracted from ZN sputum smear slides that had been stored for more than 1 year.<sup>25</sup>

Slides prepared from lesions of animals have also proved useful.<sup>26</sup> Reppas et al. extracted DNA from archival and prospective lesion slides and then used a nested PCR targeting the 16S–23S internal transcribed spacer to identify mycobacteria.<sup>26</sup> Likewise, Taqman PCR was used to differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria on archival AFB smear slides.<sup>27</sup> In another study in Brazil, *oxyR* pseudogene amplification followed by allele-specific sequencing provided evidence that stored ZN slides can be used to distinguish *Mycobacterium bovis* from other mycobacteria of the *M. tuberculosis* complex.<sup>28</sup>

In 2013, De Almeida et al. evaluated six different DNA extraction methods for the detection of *M. tuberculosis* by PCR amplification of IS6110, including DNA extraction by Chelex from cultures of *M. tuberculosis* and smear slides.<sup>29</sup> They found that the Chelex + NP-40 method for DNA extraction provided a good quantity of interference-free DNA, mainly in samples with low concentrations of genetic material, which supports its use in the molecular diagnosis of TB. The evaluation of an in-house IS6110-based PCR for TB diagnosis from smear-positive slides showed 52.3% sensitivity, 100% specificity, and positive and negative predictive values of 100% and 89.7%, respectively, proving the possibility of confirming the TB diagnosis.<sup>29</sup>

In Brazil in 2014, Carniel et al. assessed the performance of PCR (IS6110) in DNA extracted from slides prepared for microscopy to diagnose TB at a reference health unit for TB treatment in the Amazonian rainforest area, a difficult to access region with a high TB prevalence.<sup>30</sup> They demonstrated that an in-house PCR using DNA extracted from ZN slides was a feasible alternative method for the detection of *M. tuberculosis*, with a shorter turnaround time for results and an increased case detection rate. They showed that this method could offer a new approach for accurate TB diagnosis, especially in remote settings where culture is not available.

### 3.2. DNA extracted from slides for the detection of drug resistance

To tackle the delay in detecting drug resistance and to strengthen the surveillance of drug resistance, performing PCR-based molecular tests on DNA collected on TB diagnostic slides may allow the rapid detection of resistance and provide a retrospective perspective of the resistance rate. In 2001, Patnaik et al. developed a technique of eluting DNA directly from sputum dried on slides. Using this DNA, they performed PCR for the detection of *M. tuberculosis*, followed by sequencing of the *rpoB* gene to detect RIF resistance. The results were compared to those of culture and AFB smear microscopy.<sup>31</sup> While this study demonstrated the necessity of using a nested PCR for the sequencing of nucleic acids eluted directly from slides, the technique was faster than culture and had a greater sensitivity

than AFB microscopy, and provided information on the genetic drug resistance status.

Mokrousov et al. described a multiplex allele-specific PCR assay (MAS-PCR) that simultaneously detects mutations at several positions of the *emB* gene – a gene that confers the majority of ethambutol resistance in *M. tuberculosis*. They demonstrated the feasibility of this test on DNA extracted from auramine-stained sputum slides.<sup>32</sup> The same method proved useful for the detection of mutations in the *katG* gene, which is responsible for the majority of INH-resistant clinical isolates.<sup>33</sup> In 2003, Mokrousov et al. also described a PCR assay targeting *rpoB* mutations to detect RIF resistance.<sup>34</sup> They evaluated this method using DNA samples extracted from AFB microscopy-positive sputum smears. The lysates were subjected to a Chelex extraction derived method. While these studies were performed only for confirmed smear microscopy-positive cases (smear grade of category '1+' or more), they showed that using DNA extracted from slides generally yielded good amplification to target mutations associated with drug resistance, with sensitivity similar to that for purified DNA from cultured cells.

In the same year, Van Der Zanden et al. extracted DNA from ZN-stained slides to determine RIF resistance by identifying mutations in the *rpoB* gene using reverse line blot hybridization.<sup>35</sup> More interestingly, this approach showed the feasibility of performing a retrospective study using DNA extracted from ZN-stained slides to detect resistance and to genotype strains without the need for viable culture or the need to ship biohazardous materials. The authors also performed *rpoB* sequencing of the DNA extracted from the ZN slides; however, this still required a nested PCR. In 2007, Suresh et al. improved the method by developing a single-tube nested PCR for the rapid detection of *M. tuberculosis* complex and RIF resistance through sequencing using DNA directly from ZN-stained sputum smears.<sup>36</sup> They demonstrated that this method was suitable for the rapid detection of RIF in AFB-positive ZN-stained slides obtained from patients suspected of having MDR-TB. DNA present in ZN-stained smears could be extracted using a simple, rapid, and economic procedure.<sup>36</sup>

With the aim of evaluating the capacity of the WHO-endorsed molecular assays to detect resistant TB using DNA extracted from smear microscopy slides in Madagascar (2011), the present study group investigated the use of DNA extracted from stained sputum smears for the detection of RIF and INH resistance with the commercial GenoType MTBDRplus assay (Hain Lifescience, Germany).<sup>37</sup> This study showed that the performance of the GenoType MTBDRplus assay on DNA extracted from the AFB slides was similar to that found in previous studies with DNA extracted from clinical specimens.

In India in 2013, Bhutia et al. examined the possibility of using DNA extracts from smear-positive slides with the INNO-LiPA Rif.TB assay for the early detection of *M. tuberculosis* and RIF resistance.<sup>38</sup> DNA was extracted from AFB-positive sputum slides using the GenElute bacterial genomic DNA kit and ethanol precipitation. The INNO-LiPA Rif.TB assay proved useful for DNA extracted from smear-positive slides. Smear slide preparation could replace sputum transportation in cetylpyridinium chloride (CPC), reducing the biohazard and controlling the transmission of MDR-TB in the community.

In 2015, the present study group performed a retrospective field study to evaluate the performance of four systems in enabling the transportation and storage of samples for use in the molecular detection of drug resistance using the GenoType MTBDRplus assay.<sup>39</sup> Two hundred *M. tuberculosis* strains spotted on slides were selected and the DNA extracted using the Chelex method. The GenoType MTBDRplus assay showed good sensitivity and specificity for the detection of resistance to RIF and INH in *M. tuberculosis* strains using DNA extracted from the slides. It appears

that the detection of TB and resistance using the GeneXpert MTB/RIF assay on DNA extracted from AFB smear microscopy slides has not yet been performed. However, assuming similar diagnostic value for both GenoType MTBDRplus and GeneXpert MTB/RIF,<sup>40</sup> it would be interesting to investigate their performance on such biological material.

### 3.3. DNA extracted from slides for strain fingerprinting

The feasibility of archiving DNA on slides and the possibility of performing molecular techniques in retrospective studies using smear microscopy slides, allowed the use of the methods for molecular epidemiology of *M. tuberculosis* strains described in the above sections to be used with IS6110 as a genetic marker. In 1998, van der Zanden et al. described the detection and classification of *M. tuberculosis* complex strains from stained microscopy preparations and in sections of paraffin wax-embedded tissues.<sup>41</sup> They showed that the simultaneous detection and strain differentiation of *M. tuberculosis* by spoligotyping was possible in clinical samples prepared using current methods for microscopy and histopathological analysis, without the need for culture. In 2005, Gori et al. evaluated the usefulness of genotyping clinical specimens including sputum, bronchoalveolar lavage fluid, bone marrow aspirate, faeces, cerebrospinal fluid, and urine on AFB-positive slides.<sup>42</sup> The material was scraped from positive slides prepared for ZN staining. They showed that the rapidity of the spoligotyping method used in detection and typing could make it useful in the management of TB in the clinical setting.

In 2007, Suresh et al. demonstrated that the identification of *M. tuberculosis* was possible by *mpt64*-targeted PCR from archival (5–11 years) ZN slides.<sup>36</sup> They were also able to differentiate the strains by spoligotyping.<sup>36</sup> In 2012, Gomgnimbou et al. demonstrated the possibility of performing high-throughput spoligotyping on a Luminex 200 device with AFB-positive slides.<sup>43</sup> Using this method, they found a prevalence of *Mycobacterium africanum* of 20% in Burkina Faso.

Most of the studies performed using DNA extracted from smear microscopy for sequencing required a nested PCR to amplify the targeted template quantities. Performing whole genome sequencing (WGS) on DNA extracted from smear microscopy has not yet been performed, probably due to the fact that these WGS methods require high quality and quantity DNA. However, the development of high-throughput sequencing has paved the way for fast and accurate tools that will allow WGS on DNA extracted from smear microscopy slides.

## 4. Discussion

In most high TB burden countries with limited resources, sputum smear microscopy is used as the first method for TB diagnosis; this technique is simple, fast, and cost-effective. However, its specificity and sensitivity remain low, and the reproducibility of AFB slide observation results depends on human factors (the technician), laboratory expertise, and the sensitivity of the technique. Nevertheless, smear microscopy slides have the advantage that they can be stored and transported at room temperature, thus preventing biosafety issues caused by possible accidents during transportation and the logistical challenges, which is not the case when transporting biohazardous sputum samples that need freezing temperatures.

The improvements made in DNA extraction methods, notably the use of the Chelex method combined with other techniques (which has the advantage of simplicity), along with improvements in the quality of the DNA extracted (that do not affect further molecular tests) has allowed the genetic characteristics of the strains on sputum smear microscopy slides to be studied. Once the

DNA has been isolated, it is stable, allowing other molecular tests to be performed, such as PCR and sequencing. Due to the simplicity of DNA extraction from material scraped off ZN smear microscopy slides, there is also no need for special infrastructure. When applied to TB sputum smear microscope slides, the technique can be used to detect TB, distinguish between *M. tuberculosis* and other mycobacteria, detect drug resistance mutations, or genotype the strains. While the IS6110 sequence has usually been the PCR target of choice for genotyping on ZN slides, other genetic markers such as the DR sequences used for the spoligotyping have also been described. Furthermore, coupling these amplification techniques with sequencing is also feasible, but most often still requires a nested PCR.

Molecular techniques have the advantage of being much faster than culture-based methods and decrease the delay for TB diagnosis. Studies have shown that PCR using DNA extracted from ZN slides is a feasible alternative for the detection of *M. tuberculosis* and decreases the turnaround time for results. The possibility of obtaining DNA from smears used in remote settings could be a good alternative for the more rapid diagnosis of TB and drug resistance. Published studies have demonstrated that DNA recovered from slides can be used to diagnose and genotype TB, and to detect drug resistance. This system could be a good diagnostic alternative for TB diagnosis in remote areas. It could also allow new infection to be distinguished from reactivation in relapse cases when the slide from the first infection has been stored. It could also be used for the surveillance of drug resistance by national TB control programmes in low-income countries, where storage and the transportation of clinical specimens are limited.

The smear microscopy slides made and collected or archived prior to the development of AFB techniques represent a huge library and an extraordinary source of information on the global history and evolution of TB transmission, resistance, and spread. These could be exploited and unveiled using molecular techniques. The majority of published information on DNA from smear microscopy slides is based on retrospective studies. The possibility of performing molecular typing followed by gene sequencing with stored slides will allow retrospective molecular epidemiology analysis. For instance, in the context of the current One Health concept, it could be used to determine the prevalence of *M. bovis* in a population living in high bovine TB incidence setting to evaluate the burden of this zoonosis on public health. More basic research studies on strain genotypes circulating within a country, phylogeny, and phylogeography can now be facilitated and are possible, as the use of archival ZN slides of up to 11 years has been reported.<sup>36</sup>

However, there are several limitations. The sensitivity of molecular tests has been shown to vary from less than 50% to more than 80%, depending on the study and the methods employed. While an increase in the rate of TB detection in patients and improvements in the detection of drug resistance can be achieved by combining molecular techniques on DNA from smear microscopy slides, operational research is still needed to improve and standardize the systems and to evaluate the effectiveness of implementing such systems in the field through the measurement of the incidence and impact in the field. The system in which smear microscopy diagnosis at the peripheral-level laboratory is confirmed by molecular tests at the reference health centre will speed up information exchange without the requirement for any specific upgrade in infrastructure or materials. Also, since we are now in the era of genomics, the feasibility of new sequencing technologies applied to stored slides is a new challenge. With the rapid development of high-throughput sequencing techniques, obtaining whole genome sequences from microscope slides is a question of time and is getting closer day by day. A huge step forward with the development of metagenomics would be the possibility of

studying the microbiome associated and stored with *M. tuberculosis* on those slides.

In conclusion, stained smear microscopy slides can be a safe system for the transportation of sputum specimens from remote health centres to reference TB laboratories and allows further molecular TB or MDR-TB detection. This could help in the rapid diagnosis and therefore timely management of TB patients. The feasibility of molecular typing with slides will also allow large-scale drug resistance surveys and molecular epidemiology studies. However, this system still needs studies on cost-effectiveness to evaluate its feasibility in low- and middle-income countries for national TB programmes. With the rapid development of next-generation sequencing tools and techniques and decrease in costs, this may represent a powerful future molecular storage system tool.

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