Detection of Mycobacteria in Paraffin-Embedded Ziehl–Neelsen-Stained Tissues using Digital Pathology

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Abstract

Background Tuberculosis is the first infection-related cause of death worldwide. Early diagnosis of paucibacillary tuberculosis represents a challenge, even with direct tissue examination. Digital pathology allows the digital analysis of tissues to identify microorganisms. We aim to develop a program to detect and quantify typical and atypical mycobacteria in paraffin-embedded Ziehl–Neelsen-stained tissues.

Methods Program development: The building of the program, named Pat-Scan, included pathology, systems engineering, and scientific applications. The iScan Coreo Au scanner® was used, and 9 variables were adjusted: Temp Directory Path, Output Directory Path, Server Path, Focus Approach, Focus Mode, AOI Detection Approach, Scan at, No. of Z Layers, and Z Delta. Software parameter settings : Brightness, contrast, sharpness, and red/green/blue. Control module scan and analysis : Ten Ziehl–Neelsen-stained samples were fragmented into 2,000 images and analyzed by a multidisciplinary team to validate the reproducibility of the bacilli images in the tissue, as detected by the software. Results Pat-Scan included software and a scanner that were used to detect and quantify bacilli in paraffin-embedded Ziehl–Neelsen-stained tissues. HD quality image segmentation was performed, and nine planes of the Z-axis were scanned with a 1-micron distance between planes. Image magnification: 40x–80x. Scan time: 10–12 minutes. All samples containing mycobacteria were successfully analyzed by the scanner, and the bacilli could be detected; these results were validated by expert pathologists by microscopy examination, and the presence of bacilli was confirmed in all cases.

Conclusions Pat-Scan allowed the identification and quantification of mycobacteria in paraffin-embedded Ziehl–Neelsen-stained tissues, offering a reproducible diagnostic method that reduces the time for diagnosis and does not affect precision. Further validation is needed for application in the clinical setting.

Background

Mycobacteria can be classified into two major subgroups: typical and atypical. Although they are genotypically different, both have the same phenotypical qualities, which allow the use of the Ziehl–Neelsen staining technique for detection (1). The typical subgroup comprises the *Mycobacterium tuberculosis* complex, which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (2). In addition, approximately one hundred species of atypical mycobacteria, such as *M. avium* (MAC), *M. kansasii*, *M. leprae*, *M. abscessus*, *M. chelonae*, and *M. fortuitum*, are known (3).

On average, it takes between 30 minutes and 2 hours for an expert pathologist to examine a plate with the special Ziehl–Neelsen stain at 40x magnification. Culture methods for these microorganisms take two to three weeks in a liquid medium (4) and four to eight weeks in a solid medium (3). This prolonged time is due to the slow growth of at least half of typical and atypical mycobacteria, which can be inhibited by other bacteria that colonize the human body (3). The performance of the aforementioned tests depends on variables such as sample type (solid or liquid), concentration and amount of bacteria in the sample, type of mycobacterium, and the observer's experience (4). The sensitivity and specificity of the detection performance of the microscopy method are 60% and 47%, respectively, whereas those of the detection performance of the culture method are 22% and 80%, respectively (5).

Mycobacteria identification can be conducted via light microscopy, culture, and/or fluorescence techniques. Although there are more sophisticated methods, these are the most widely used. The success of microscopic detection depends on the capability and training of the observer, and a fair amount of time is needed to search for the mycobacteria.

However, delays in the detection of infectious agents have a negative impact on the mortality of immunocompromised patients, who require treatment to be started early. Furthermore, there are only a small number of published studies that target this topic, especially regarding the use of digital pathology technologies for mycobacteria detection.

The future implementation of a diagnostic digital tool that can systematically make an effective and efficient diagnosis of relevant public health conditions, such as mycobacterial infections, will support diagnosis by pathologists, leading to a fast and possibly precise process. For that reason, our study aimed to implement a new diagnostic method that uses digital technology for the detection and quantification of typical and atypical mycobacteria in paraffin-embedded Ziehl–Neelsen-stained tissues.

Methods

A selection of ten random Ziehl–Neelsen-stained samples from patients diagnosed with a mycobacterial infection in our hospital by conventional microscopy confirmed by an expert pathologist was performed. Of those ten samples, five contained atypical mycobacteria (*M. leprae*), and five typical mycobacteria (*M. tuberculosis*) at different levels of positivity. The samples corresponded to skin tissue (punch biopsy or excisional biopsy) and lung tissue respectively (wedge resection or lobectomy). Ten control samples, corresponding to lung and skin biopsies which were negative for mycobacteria confirmed by expert pathologists, were included for comparison.

The implementation of Pat-Scan (software and equipment) required interdisciplinary work, including pathology, systems engineering, and scientific applications. The iScan Coreo Au scanner[®] is a digital pathology scanner that synchronizes with the Virtuoso[®] software, which supports digital image management. This scanner offers telepathology and high-speed scanning (6) and was used to program and standardize nine variables: Temp Directory Path, Output Directory Path, Server Path, Focus Approach, Focus Mode, AOI Detection Approach, Scan at, No. of Z Layers, and Z Delta. For mycobacteria visualization, image parameters were adjusted as follows: Brightness (70%), contrast (100%), sharpness (100%), red hue (100%), green hue (50%), and blue hue (50%). Subsequently, the 20 complete samples (controls and Ziehl–Neelsen-stained) were prepared and scanned.

Samples were considered positive if they contained at least one atypical or typical *Mycobacterium* bacillus in the entire sample, confirmed either by the pathologist or by the Pat-Scan in 200 images for each sample. In total, 2000 images from the positive cases were processed by the pathology team and the scanner. Furthermore, 200 images were registered for each control, for a total of 2000 images, same as for positive samples.

Microscopic assessment consisted of 5 months of sample evaluation by a pathologist, a cell histotechnologist, a microbiologist, and a scientific-application molecular biologist to validate image reproducibility between the typical and atypical mycobacteria samples and controls (4000 images in total).

Results

With the implementation of Pat-Scan, visualization of mycobacteria was achieved, allowing detection and quantification of paraffin-embedded Ziehl–Neelsen-stained tissues. The tissue was segmented with HD image quality and scanned in 9 planes of the Z-axis with a distance of one micron between the planes. Digital image magnifications of 40x and 80x were obtained with Virtuoso software, and each sample scan took between 10 and 12 minutes to be completed. Figures 1 and 2 are divided into two segments: Segment A shows the Ziehl–Neelsen-stained tissue. Segment B shows the image after the algorithm was applied; the Pat-Scan program erased the background color from the Ziehl–Neelsen stain, allowing the observation of red-stained mycobacteria. All samples (10 out of 10) containing mycobacteria were successfully classified as positive and all samples from the control group were detected as negative by the Pat-Scan scanner.

Discussion

This study represents the initial phase of a diagnostic technique study. We aimed to innovate in the digital pathology field with the purpose of implementing a rapid technique for TB diagnosis. The use of Pat-Scan, which included equipment (scanner) and software, was able to correctly identify all positive samples and controls. Furthermore, it satisfactorily allowed optimization of the visualization time of mycobacteria in all of the paraffin-embedded Ziehl–Neelsen-stained tissues included. This is the first study to use a scanner to digitalize images of this specific type of tissue. Currently, tests such as RT-PCR assay have demonstrated a high degree of sensitivity for pulmonary TB and good sensitivity for extra-pulmonary TB, with a sensitivity of 82% and specificity of 99% for typical mycobacteria, and of approximately 70% and 99% for atypical mycobacteria, respectively (7). Despite of the high yield of this test, it is still not available in all medical centers in developing countries and requires transportation of samples to reference hospitals for their assessment, increasing healthcare costs.

The concept of using image analysis to classify and detect microorganisms was introduced in the late 1990s (8). Veropoulos et al. (9, 10) claim to be the first to attempt computer-assisted detection using auramine–rhodamine-stained sputum smears. They used Fourier descriptors, a classic method of shape recognition, in grayscale. Somoskovi et al. (11) built an automatic microscope connected to a computer and compared this method to the traditional method of mycobacteria detection for examining sputum samples. They concluded that mycobacterial detection was similar in terms of sensitivity and specificity and that their method took less study time. A review of the available literature reveals that the first study employing automated digital diagnostic aids using Ziehl–Neelsen stain was published in 2006 (12, 13) in a master's degree thesis of two Cape Town University students in South Africa. The investigators used

sputum as the study sample and an algorithm that enabled the automatic focusing of the microscope according to the observed color matrix.

In 2010, in a first attempt at image digitalization, Paul Tadrous (14) used Ziehl–Neelsen-stained tissues and a high-resolution camera to visualize mycobacteria. The software used modifications of color and contrast. In 2012, the previous method was applied (15), comparing three different software packages with modifications to the shape, perimeter, area, and maximum and minimum distances between the pixels of internal and external edges to classify the images as tuberculosis or non-tuberculosis, with a precision of 88.37%. A very recent study was published in 2017 (16), comparing several microscopes to identify which one delivered the best images. The investigators used a Gaussian model to apply various techniques that filter background interference, with a precision of >90%. Additionally, a study in China used a convolutional neural network model to recognize tuberculosis bacillus; this study used 201 samples for the test set, and the results were confirmed by pathologists, finding a sensitivity of 97.94% and a specificity of 83.65% (17).

The use of technological aids for the optimization of health care is an increasingly common practice. The inclusion of programs that improve diagnostic accuracy in pathology has the potential to achieve earlier diagnoses, which represents a benefit for patients, since a rapid and accurate diagnosis of tuberculosis is key to manage the disease and prevent its transmission. Even if various studies have been published regarding digital pathology, there is still insufficient evidence to implement a change in the routine process for mycobacteria detection. Although our algorithm is still not able to differentiate between typical and atypical mycobacteria, the following step will be validation of the technique with additional improvements in size discernment for this purpose.

Conclusions

This study offers a diagnostic alternative that reduces the time invested in confirmatory diagnosis, with high precision and reproducibility. The Pat-Scan program is attractive because it enables mycobacterial detection in relatively less time (approximately ten minutes) and offers an alternative for paucibacillary samples. These findings need to be complemented with further studies that seek to assess the performance of Pat-Scan as a diagnostic tool in the clinical setting.

List Of Abbreviations

HD	High Definition
M	Mycobacterium
MAC	Mycobacterium avium
ZN	Ziehl–Neelsen

Declarations

Ethics approval and consent to participate

This manuscript was written in compliance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki Declaration. We have approval of the Ethics Committee in Biomedical Research (Comité de Ética en Investigación Biomédica “IRB-EC” in spanish) from Fundación Valle del Lili University Hospital. This is supported in letter No. 040 of 2018. Act No. 05 March 14th of 2018, which is available if needed with the Corresponding Author.

Consent for publication:

It does not apply to this manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. This manuscript has not been published and is not under consideration for publication elsewhere. Additionally, all of the authors have approved the contents of this paper and have agreed to the journal’s submission policies.

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Authors' contributions

All authors have read and approved the manuscript, and significantly contributed to this paper. LFS: Conception and design, acquisition and analysis, interpretation of data, literature review, manuscript writing and correction, final approval of manuscript. JEB: Literature review, manuscript writing and correction, final approval of manuscript. JM: Literature review, manuscript writing and correction, final approval of manuscript. AS: Acquisition and analysis, interpretation of data, final approval of manuscript. GM: Acquisition and analysis, interpretation of data, final approval of manuscript. VZR: Manuscript

writing and correction, final approval of manuscript. LFT: Conception and design, literature review, manuscript writing and correction, final approval of manuscript.

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Figures

Figure 1

Digital analysis of atypical mycobacteria in the skin tissue at 40x.

Figure 2

Digital analysis of typical mycobacteria in lung tissue at 10x.

Figure 3

Ziehl-Nielsen stain. Typical mycobacteria at 60x. Detected in 37 minutes using light microscopy.

Figure 4

H&E. Lung. Granulomatous chronic inflammation with caseous central necrosis (necrotizing granulomas).

Figure 5

H&E. Lung. Multinucleated giant cells surrounded by a ring of lymphocytes.