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

Luz Fernanda Sua
Fundacion Valle del Lili
Jhon Edwar Bolaños
Universidad ICESI Facultad de Ciencias de la Salud
Juliana Maya
Universidad ICESI Facultad de Ciencias de la Salud
Aura Sanchez
Fundacion Valle del Lili
Giovanna Medina
Fundacion Valle del Lili
Valeria Zúñiga-Restrepo
Fundacion Valle del Lili
Liliana Fernández-Trujillo (liliana.fernandez@fvl.org.co)
Fundación Valle del Lili https://orcid.org/0000-0003-0789-9154

Research article

Keywords: mycobacterium, algorithm, data quality, software design

Posted Date: January 22nd, 2020

DOI: https://doi.org/10.21203/rs.2.21514/v1

Version of Record: A version of this preprint was published at Tuberculosis on January 1st, 2021. See the published version at https://doi.org/10.1016/j.tube.2020.102025.

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 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

The implementation of Pat-Scan (software and equipment) requires 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. The brightness, contrast, sharpness, and red/green/blue were adjusted for the images, with a final standardization for adequate mycobacterial visualization. Subsequently, 10 complete samples containing mycobacteria (controls; Ziehl–Neelsen-stained) were prepared and scanned. Furthermore, 200 images were registered for each control, for a total of 2,000 images that underwent assessment over 5 months 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.

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 containing mycobacteria were successfully detected by Pat-Scan and confirmed by microscopic evaluation by expert pathologists.

Discussion

This study represents the initial phase of a diagnostic technique study. The use of Pat-Scan, which included equipment (scanner) and software, allowed the identification and optimization of the visualization of mycobacteria in paraffin-embedded Ziehl–Neelsen-stained tissues, enabling their quantification. This is the first study to use a scanner to digitalize images of this specific type of tissue. The concept of using image analysis to classify and detect microorganisms was introduced in the late 1990s (7). Veropoulos et al. (8,9) 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. (10) 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 (11,12) 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 (13) 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 (14), 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 (15), 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% (16).

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. Although many studies have been published regarding digital pathology, there is still insufficient evidence to implement a change in the routine process for mycobacteria detection.

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.

Funding

Tecnoquímicas S.A Pharmaceutics, through the funding announcement “Convocatoria Tecnoquímicas 2018” which intended to offer funding grants for clinical research in developing countries such as Colombia, provided a £4.699,64 grant for this study. This grant was used for the transportation of the histological blocks from the storage center *Iron Mountain*, located outside the city, to our hospital (Fundación Valle del Lili). Furthermore, it was used to ensure the operation of logistics and medical staff during the study.

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.

Acknowledgement

Not applicable

References

1. Rodriguez G. Mycobacterias [Internet]. 2008. 381-399 p. Available from: http://www.higiene.edu.uy/cefa/2008/micobacterias.pdf
2. Gentry CA. Atypical Mycobacteria. In: Pharmacotherapy Self-Assessment Program (Infectious Diseases, Book 6). PSAP Edito. American College of Clinical Pharmacy; 2005. p. 99–126.
3. Murray P, Rosenthal KS, Pfaller MA. Mycobacterium. In: Microbiología Médica. Madrid: Elsevier; 2014. p. 235–47.
4. Pérez del Molino M, Tuñez Bastida V, García Ramos M, Lado Lado F. Diagnóstico microbiológico de la tuberculosis. Med Integr [Internet]. 2002;39(5):207–15. Available from: http://www.elsevier.es/es-revista-medicina-integral-63-articulo-diagnostico-microbiologico-tuberculosis-13029946
5. Cortés Sierra E. Descripción de técnicas fenotípicas y moleculares para la identificación de. Pontif Univ javeriana. 2009;114.

6. Ventana Medical Systems I. iScan Coreo Au: Whole slide brightfield scanner. 2011.
7. Weeks PJD, Gaston KJ. Image analysis, neural networks, and the taxonomic impediment to biodiversity studies. Biodivers Conserv. 1997;6(2):263–74.
8. Veropoulos K, Learmonth GM, Campbell CE, Knight B, Simpson J. Automated identification of tubercle bacilli in sputum: a preliminary investigation. Anal Quant Cytol Histol. 1999; 21:277–82.
9. Veropoulos K, Campbell C, Learmonth BK, Simpson J. The automated identification of tubercle bacilli using image processing and neural computing techniques. International Conference on Artificial Neural Networks. 1998.
10. Somosközi A, Györi Z, Czoboly N, Magyar P. Application of a computer-directed automated microscope in mycobacteriology. Int J Tuberc Lung Dis. 1999;3(4):354–7.
11. Russell M. Autofocusing and image segmentation in microscopy for automatic detection of tuberculosis in sputum smears. 2006.
12. Mohamed MQ. Implementation of a graphical user interface for tuberculosis detection in sputum smear images. 2006.
13. Tadrous PJ. Computer-assisted screening of Ziehl-Neelsen–stained tissue for mycobacteria: Algorithm design and preliminary studies on 2,000 images. Am J Clin Pathol. 2010;133(6):849–58.
14. Muhammad Khusairi O, Mohd Yusoff M, Jaafar H. Performance comparison of extreme learning machine algorithms for mycobacterium tuberculosis detection in tissue sections. J Med Imaging Heal Informatics. 2012;2(3):307–12.
15. Shah MI, Mishra S, Sarkar M, Rout C. Identification of robust focus measure functions for the automated capturing of focused images from Ziehl–Neelsen stained sputum smear microscopy slide. Citometry Part A J Quant Cell Sci. 2017;91(8):800–9.
16. Xiong Y, Ba X, Hou A, Zhang K, Chen L, Li T. Automatic detection of mycobacterium tuberculosis using artificial intelligence. J Thorac Dis. 2018;10(3):1936–1940. doi:10.21037/jtd.2018.01.91

Figures

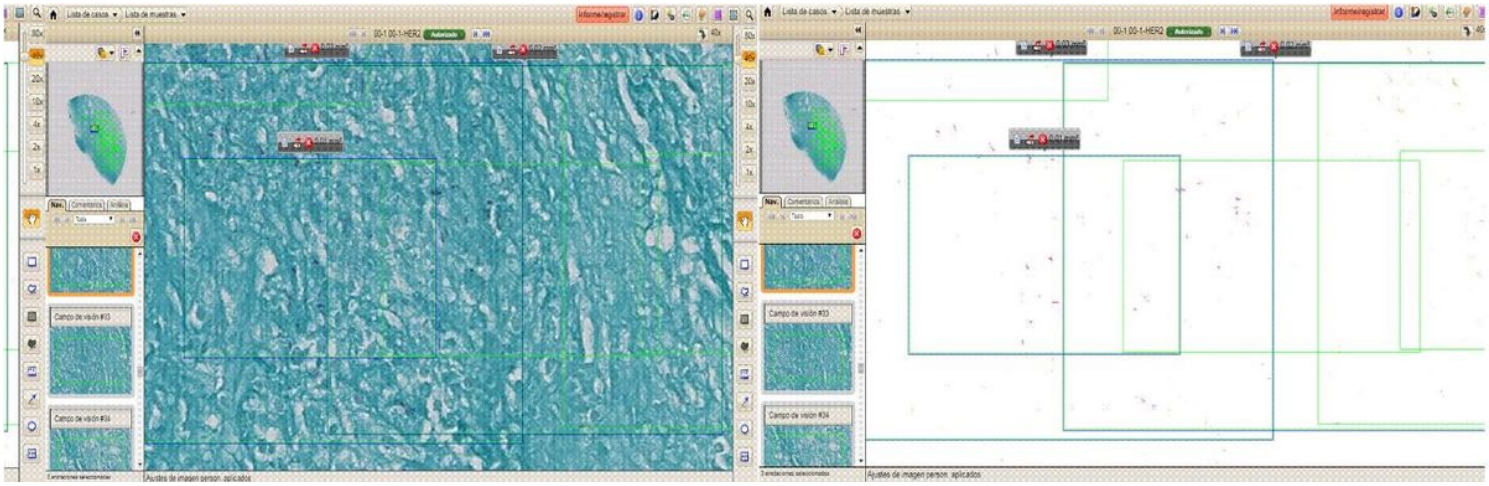

Figure 1

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

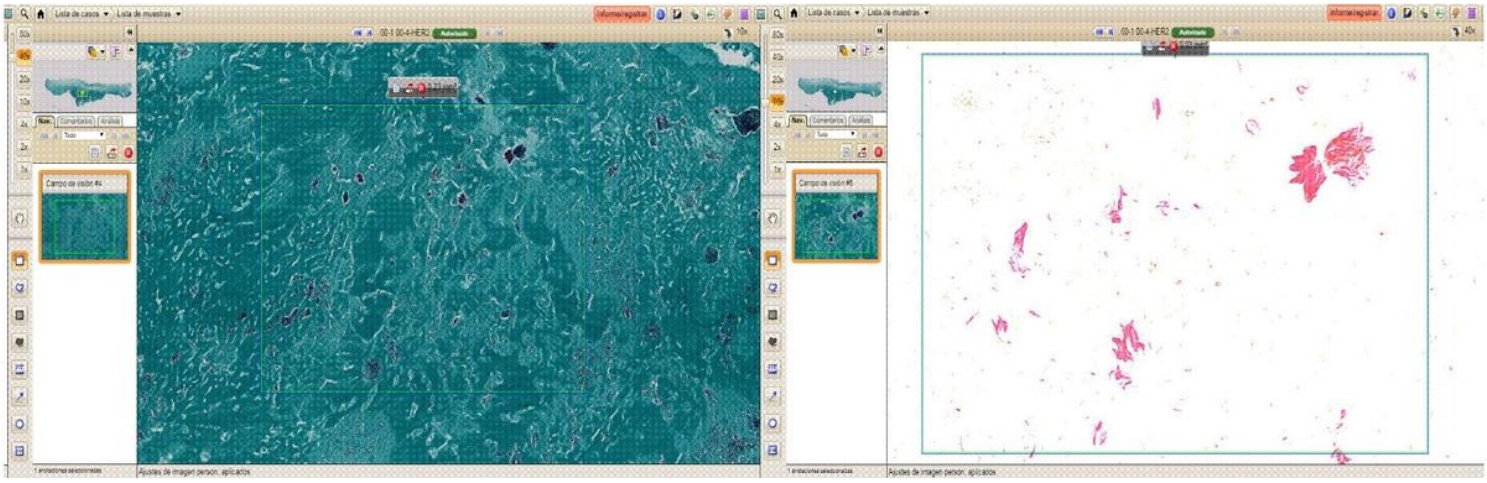

Figure 2

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

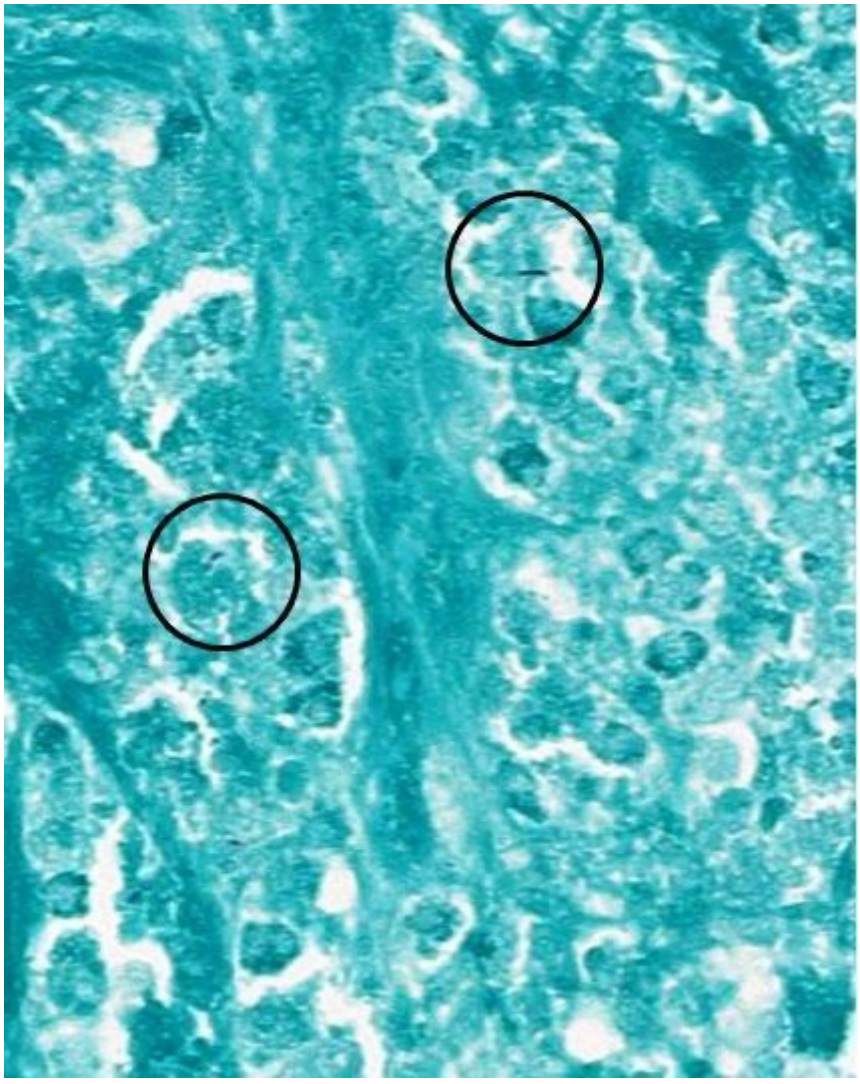

Figure 3

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

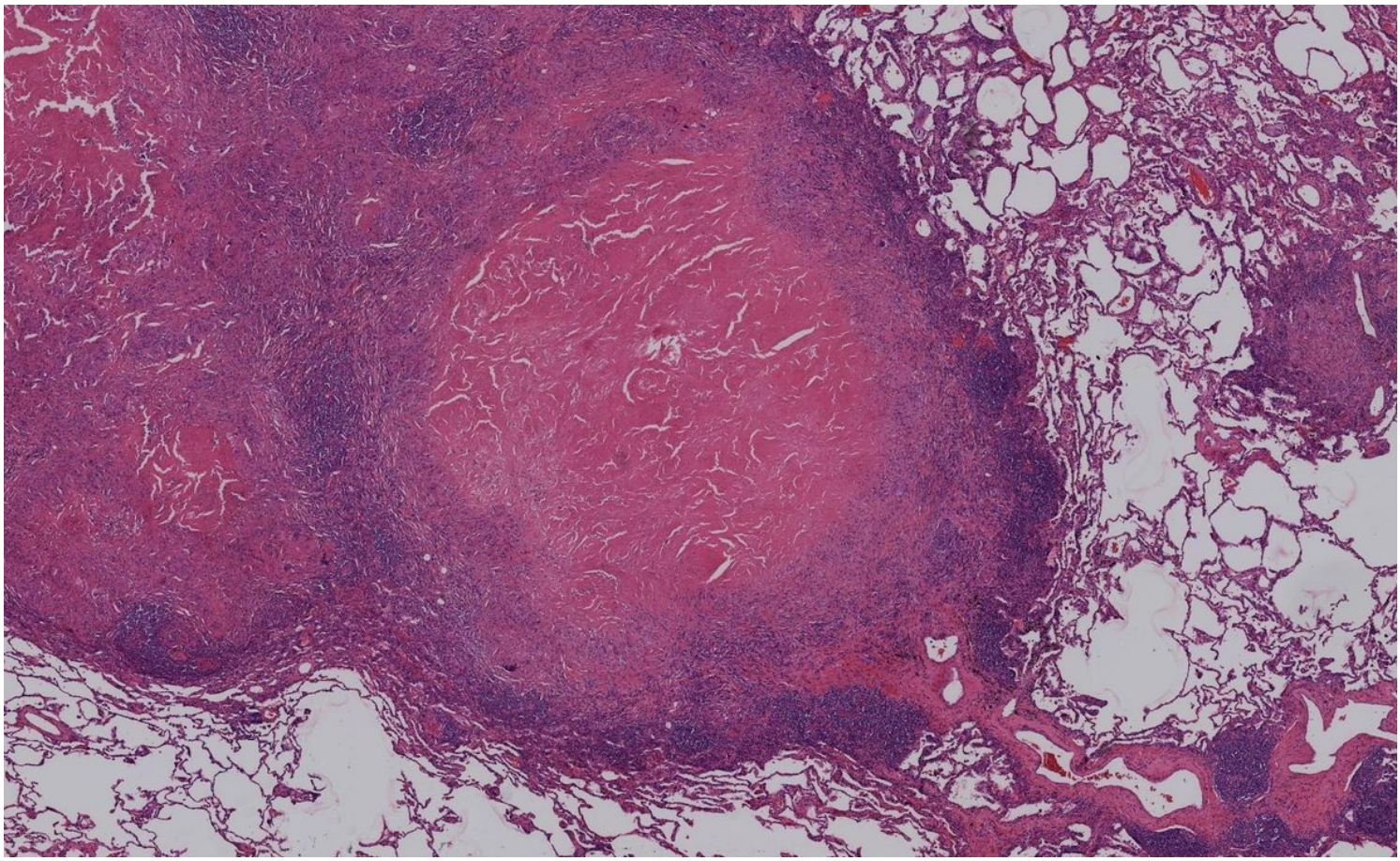

Figure 4

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

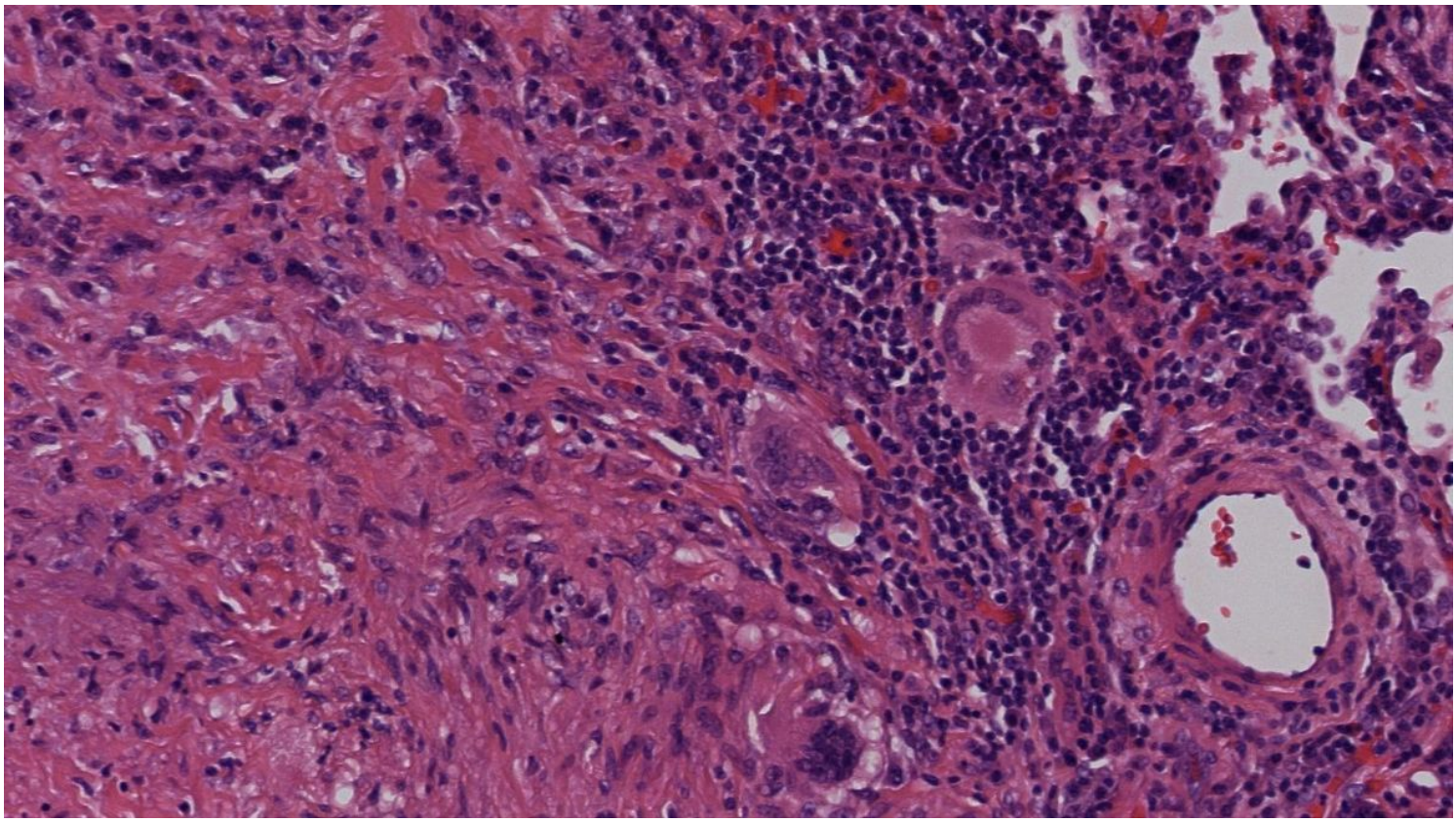

Figure 5

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