SOMATO PUBLICATIONS

Annals of Infectious Diseases and Therapy

Case Report

Mycobacterium avium Infection Detected as a Negative Image in Giemsa-stained Sputum Cytology Preparations

Tomohiro Watanabe[1], Hideaki Tanaka[2], Megumi Inaba[3] and Yutaka Tsutsumi[4*]

[1]Cytology Division, Chuken Co. Kumamoto, Koshi, Kumamoto, Japan

[2]Tanaka Clinic, Kashima, Kumamoto, Japan

[3]Department of Respiratory Medicine, Kumamoto Chuo Hospital, Mimami-ku, Kumamoto, Japan

[4]Diagnostic Pathology Clinic, Pathos Tsutsumi, Inazawa, Aichi, Japan

*Address for Correspondence: Yutaka Tsutsumi, Diagnostic Pathology Clinic, Pathos Tsutsumi, 1551-1 Sankichi-Ato, Yawase-cho, Inazawa, Aichi 492-8342, Japan, Tel: +81-587-96-7088; E-mail: pathos223@kind.ocn.ne.jp

Received: 17 March 2021; Accepted: 26 March 2021; Published: 27 March 2021

Citation of this article: Watanabe, T., Tanaka, H., Inaba, M., Tsutsumi, Y. (2021) *Mycobacterium avium* Infection Detected as a Negative Image in Giemsa-stained Sputum Cytology Preparations. Ann Infect Dis Ther, 2(1): 01-05.

Orcid Ids: Watanabe, T: https://orcid.org/0000-0003-3449-1330; Tanaka, H: http://orcid.org/0000-0002-0365-0717; Inaba M: http://orcid.org/0000-0003-1976-6295; Tsutsumi, Y: https://orcid.org/0000-0002-4136-9678

Abstract

A borderline-diabetic 63-year-old housewife, a human T-cell leukemia virus-1 carrier, complained of fever and coughing. Computed tomography scan identified a 50 mm-sized, cavitated mass in the lower lobe of the right lung. To evaluate the nature of the lesion, cytological screening was performed. Giemsa-stained sputum cytology was quite effective for identifying mycobacteria as negative (unstained) bacillary images in the cytoplasm of many neutrophils and some macrophages. A re-staining technique clearly demonstrated acid-fastness of the ghosted rods. Microbial culture and real-time polymerase chain reaction analysis confirmed infection of *Mycobacterium avium*. It is necessary for us to employ Giemsa stain in sputum cytology, particularly when lung infection is clinically suspected.

Keywords: Giemsa stain, *Mycobacterium avium*, Negative staining, Nontuberculous mycobacterium, Sputum cytology

Highlights

- Giemsa-stained sputum cytopreparations demonstrated mycobacteria as a negative image.
- When lung infection is suspected, Giemsa stain should be performed in sputum cytology.
- Giemsa stain can significantly contribute to quick diagnosis of mycobacterial infection.

Introduction

German doctors significantly contributed to the development of acid-fast stain for the diagnosis of tuberculosis [1-3]. In 1882, Robert Koch discovered *Mycobacterium tuberculosis*. Paul Ehrlich invented a special stain for *M. tuberculosis*, the alum hematoxylin stain. In 1882, Franz Ziehl, a bacteriologist, modified Ehrlich's method by employing carbolic acid (phenol) as a mordant. Friedrich Neelsen, a pathologist, further changed the primary reagent to carbol fuchsin in 1884. Ziehl and Neelsen's modifications were thus established as a classical acid-fast stain. Joseph Kinyoun, an American surgeon, modified the Ziehl-Neelsen stain, in 1915, by removing the heating step from the procedure, named as Kinyoun stain. José Faraco in Brazil (1938) and George Fite in USA (1947) developed a meticulous modification, called Fite-Faraco stain, with increased mycobacterial detectability in paraffin sections by using a mixture of two parts xylene/one part vegetable oil for deparaffinization.

Cytological diagnosis of mycobacterial infection has conventionally been made by recognizing epithelioid granulomas with caseous necrosis and by proving acid-fast bacilli with Ziehl-Neelsen's stain. In 1989, Maygarden, et al. [4] first described "negative images" of mycobacteria in modified Wright (Diff-Quik)-stained cytology smears sampled from patients with acquired immunodeficiency syndrome. In 1990, Fisher, et al. [5] documented the same phenomenon in Gram and Giemsa-stained cytological preparations. The usefulness of non-acid-fast stain such as Giemsa stain for detecting mycobacteria in cytological preparations has been reported repeatedly [6-11].

We report herein a female case of opportunistic *Mycobacterium avium* infection of the lung. The use of Giemsa stain for sputum cytology was quite effective for localizing mycobacteria in the cytoplasm of neutrophils and macrophages. A re-staining procedure confirmed acid-fastness of the "ghost" images of long rods.

Case Presentation

A 63-year-old housewife nonsmoker visited Tanaka Clinic, Kashima, Kumamoto, with complaints of coughing and fever lasting for one month. The leukocyte count in the peripheral blood was normal: 6,100/μL (reference value: 3,000–7,800/μL), without association of abnormal cells. Serum C-reactive protein level was elevated to 2.96 mg/dL (reference value: <0.3 mg/dL). She had past history of hypertension, brain infarction and borderline diabetes mellitus. Neither immunosuppressive nor autoimmune disorders were recorded. Serum antibody against human immunodeficiency virus was negative, but the antibody against human T-cell leukemia virus-1 (HTLV-1) was positive (≥1:256). Computed tomography (CT) scan identified a single, 50 mm-sized cavitated subpleural mass in the S6 segment of the lower lobe of the right lung (Figure 1). In addition to microbial culture and the real-time polymerase chain reaction (RT-PCR) test specific for *Mycobacterium tuberculosis*, sputum cytology specimens were evaluated for differential diagnosis, including lung cancer, lung abscess, mycotic infection, tuberculosis, and non-tuberculous mycobacterial infection.

Routine bacterial culture failed to demonstrate causative pathogens, and the RT-PCR test targeted to *M. tuberculosis* was negative. Acid-fast bacilli were not identified in the sputum smear examination. The sputum sample was sent to a cytology laboratory, Chuken Co. Kumamoto, Koshi, Kumamoto, Japan. In the cytology division of Chuken Co. Kumamoto, both wet ethanol-fixed Papa-

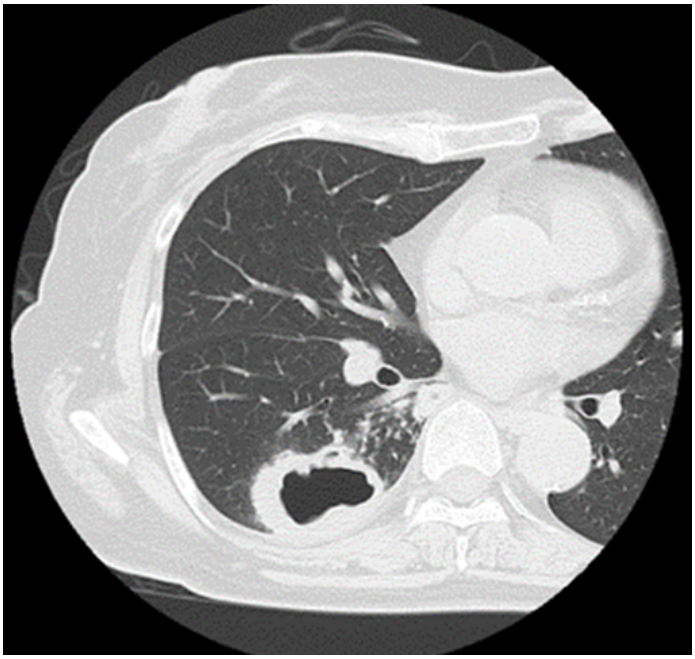

Figure 1: Chest CT scan: A thick walled cavitated lesion measuring 50 mm is seen subpleurally in the S6 segment of the right lung.

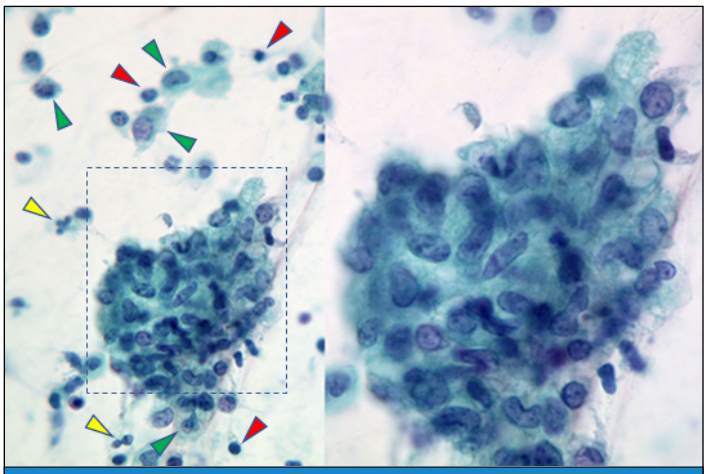

Figure 2: Papanicolaou-stained sputum cytology (right: a close-up view of the dotted square in the left panel): Clusters of epithelioid cells are identified in the inflammatory background with neutrophils (yellow arrowheads), lymphocytes (red arrowheads) and macrophages (green arrowheads).

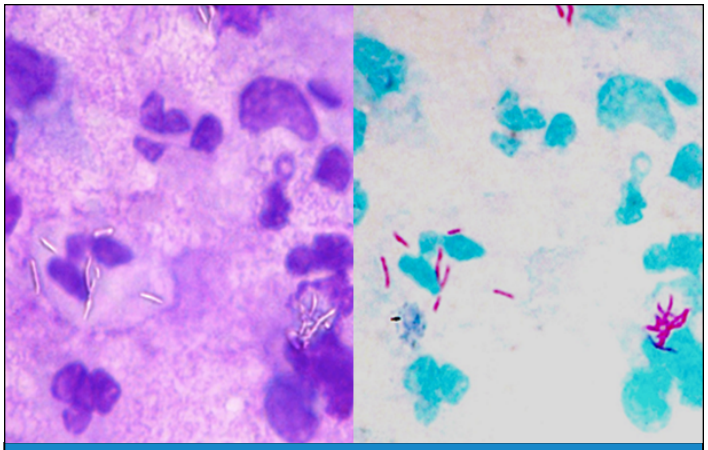

Figure 3: Giemsa-stained sputum cytology (left) and the re-stained picture for Ziehl-Neelsen's acid-fast reactivity (right). Negatively stained bacterial ghosts are seen in the cytoplasm of neutrophils, and the same rods reveal distinct acid-fastness.

nicolaou stain and air-dried Giemsa stain are routinely performed for respiratory cytology practice, particularly when infectious etiology is clinically suspected. In the Papanicolaou-stained sputum preparation, clusters of epithelioid cells with Langhans-type multinucleated giant cells were scattered (Figure 2) in the background of neutrophils, lymphocytes, and macrophages. In Giemsa-stained preparation, long bacilli were seen in the cytoplasm of neutrophils and macrophages as negatively stained ghost images (Figure 3, left). The ghosts were seen predominantly in the neutrophils. The negatively stained bacilli were refractile, when the aperture of the microscopic lens was stopped down. At this point, mycobacterial infection, including tuberculosis, was cytologically indicated, and immediately reported. After sloughing off the cover glass, the Giemsa's dyes were bleached in acid alcohol, and Ziehl-Neelsen's sequence was re-stained on the same glass slide. The refractile bacilli clearly showed acid-fastness (Figure 3, right), definitely confirming the diagnosis of mycobacterial infection.

The patient was then transferred to Kumamoto Chuo Hospital, Kumamoto. Transbronchial lung biopsy indicated nonspecific chronic active inflammation rich in neutrophils. From the biopsied tissue, *M. avium* was identified by mycobacterial culture and the following analysis with Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) by using MALDI Byotyper (Bruker Japan Daltonics division, Yokohama). The RT-PCR test employing specific primer pairs COBAS TaqMan MAI targeted at 16S ribosomal RNA of *M. avium* and *M. intracellulare* (equipmemt: COBAS TaqMan 48, Roche Diagnostics, Tokyo) further detected *M. avium*-specific DNA sequence. Chemotherapy with peroral daily administration of clarithromycin (800 mg/day), rifampicin (450 mg/day) and ethambutol (750 mg/day) was effective. Three months later, CT scan confirmed considerable involution of the cavitated lung lesion to 19 mm in size. The chemotherapy continued for a total of six months, and at present time, no abnormal shadow is observable on the chest X-ray film.

Discussion

Cavitated lung infection of *M. avium* occurred in a Japanese woman, a healthy carrier of HTLV-1 living in Kumamoto, Kyushu island, an endemic area of HTLV-1-related disorders. The mycobacterial infection was quickly diagnosed by Giemsa-stained sputum cytology, whereas acid-fast bacilli were not found in the sputum smear preparations. Papanicolaou-stained preparations demonstrated epithelioid granulomas. The mycobacteria predominantly phagocytized by neutrophils were characteristically Giemsa-unstained: We believe the areas rich in neutrophils should be evaluated for the negative images under the microscope. The re-staining trial beautifully showed acid-fastness of the long rods.

Acid-fast microorganisms (mycobacteria) contain large amounts

of lipid called mycolic acids in the cell wall [12]. This unique cell wall structure of mycobacteria renders them impermeable for dyes (azur-eosin-methylene blue) used in Giemsa stain, which is categorized in so-called Romanowsky stains [4,6,9]. The rods thus appear as negatively stained and refractile ghosts [4-11]. The negatively stained images of mycobacteria should be a very helpful and valuable finding of notice for prompt cytological diagnosis of mycobacterial infection. Awareness of this unique feature prompts us cytologists and cytopathologists to detect mycobacteria in cytology specimens. It is known that the same ghost phenomenon is also encountered in Gram-stained smears and hematoxylin and eosin stain [4,5,10,13,14].

In the present case, the ghost figures in the Giemsa-stained preparation were prominently observed in the cytoplasm of neutrophils. It is known that the granulomas seen in nontuberculous mycobacterial infection may be rich in neutrophilic reaction to form "suppurative granulomas" (featured by neutrophil-rich abscess surrounded by epithelioid cells) [15,16]. The predominance of the negatively stained rods in the cytoplasm of neutrophils may indicate nontuberculous mycobacterial infection rather than tuberculosis, as was so in the current case.

We should emphasize the importance of the use of Giemsa stain, a rapid and cost-effective method, for sputum cytology evaluations particularly in cases clinically suspected of lung infection. Of note is that mycobacteria can be visualized with non-acid-fast routine stain. In the cytology practice, Giemsa stain is far superior to Papanicolaou stain for identifying a variety of microbes, such as fungi, *Nocardia* and bacteria causing lung infections [17]. In the Giemsa-stained preparation, the types and amounts of inflammatory cells are recognizable, and the monotonous growth pattern of pathogens can be confirmed. We strongly recommend that such a search is routinely introduced in the sputum cytology practice, particularly in cases lung infections are clinically suspected.

Conflict of Interest Statement

The authors do not have any conflicts of interest to declare in relation to the present report. There were no sources of funding for reporting of the present case.

Statement of Ethics

The patient provided a written informed consent for the publication of this case report. The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Author Contributions

We declare that all the authors made a substantial contribution to the concept of the case report or interpretation of data and approved the version to be submitted. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content.

References

1. Bishop, PJ., Neumann, G. (1970) The history of the Ziehl-Neelsen stain. Tubercle, 51(2): 196–206.
2. Singhal, R., Myneedu, VP. (2015) Microscopy as a diagnostic tool in pulmonary tuberculosis. Int J Mycobacteriol, 4(1): 1–6.
3. DiNardo, AR., Lange, C., Mandalakas AM. (2016) Editorial commentary: 1, 2, 3 (years) … and you're out: the end of a 123-year historic era. Clin Infect Dis, 62(9): 1089–1091.
4. Maygarden, SJ., Flanders, EL. (1989) Mycobacteria can be seen as "negative images" in cytology smears from patients with acquired immunodeficiency syndrome. Mod Pathol, 2(3): 239–243.
5. Fisher, JF., Ganapathy, M., Edwards, BH., Newman, CL. (1990) Utility of Gram's and Giemsa stains in the diagnosis of pulmonary tuberculosis. Am Rev Resp Dis, 141(2): 511-513.
6. Torlakovic, E., Clayton, F., Ames, ED. (1992) Refractile mycobacteria in Romanowsky-stained bone marrow smears. A comparison of acid-fast-stained tissue sections and Romanowsky-stained smears. Am J Clin Pathol, 97(3): 318–321.
7. Ang, GAT., Janda, WM., Novak, RM., Gerardo, L. (1993) Neg-

ative images of mycobacteria in aspiration biopsy smears from the lymph node of a patient with acquired immunodeficiency syndrome (AIDS): Report of a case and a review of the literature. Diagn Cytopathol, 9(3): 325–328.

8. Iyengar, KR., Basu, D. (2001) Negative images in the fine needle aspiration cytologic diagnosis of mycobacterial infections. Malays J Pathol, 23(2): 89–92.

9. Basu, D., Nilkund, J. (2003) Detecting mycobacteria in Romanowsky-stained cytologic smears. A case report. Acta Cytol, 47(5): 774–776.

10. Prasad, CSBR, Narasimha, A., Harendra Kumar, ML. (2011) Negative staining of mycobacteria. A clue to the diagnosis in cytological aspirates: two case reports. Ann Trop Med Public Health, 4(2): 110–112.

11. Patnayak, R., Chowdhury, I. (2019) Negative images of mycobacteria in cytology. Med J Dr D Y Patil Vidyapeeth, 12(6): 553-554.

12. Marrakchi, H., Lanéelle, MA., Daffé, M. (2014) Mycolic acids: structures, biosynthesis, and beyond. Chem Biol, 21(1): 67–85.

13. Hinson, JM., Bradsher, RW., Bodner, SJ. (1981) Gram-stain neutrality of *Mycobacterium tuberculosis*. Am Rev Respir Dis, 123: 365–366.

14. Trifiro, SAM., Lebel, BF., Rene, P. (1990) Ghost mycobacteria on Gram stain. J Clin Microbiol, 28: 146–147.

15. Bartralot, R., Pujol, RM., García-Patos, V., Sitjas, D., Martín-Casabona, N., Coll, P., et al. (2000) Cutaneous infections due to nontuberculous mycobacteria: histopathological review of 28 cases. Comparative study between lesions observed in immunosuppressed patients and normal hosts. J Cutan Pathol, 27(3): 124–129.

16. Li, JJ., Beresford, R., Fyfe, J., Henderson, C. (2017) Clinical and histopathological features of cutaneous nontuberculous mycobacterial infection: a review of 13 cases. J Cutan Pathol, 44(5): 433–443.

17. Tsutsumi, Y. (2021) Cytological diagnosis of infectious diseases: identification of pathogens and recognition of cellular reactions. IntechOpen: Innate Immunity in Health and Disease, Open access peer-reviewed chapter (60 pages). doi: 10.5772/intechopen.95578.