14

Three Cases of *Mycobacterium tuberculosis* Infection Initially Recognized by Focus Changing Examination in Gram Staining

Yoshiko Atsukawa[1], Sayoko Kawakami[1], Yasuo Ono[3], Ryuichi Fujisaki[2], Yukihisa Miyazawa[1] and Hajime Nishiya[2]
[1]*Department of Central Clinical Laboratory, Teikyo University Hospital, Tokyo;*
[2]*Department of Internal Medicine, Teikyo University School of Medicine, Tokyo;*
[3]*Department of Microbiology, Teikyo University School of Medicine, Tokyo, Japan*

1. Introduction

Patients with fever and pulmonary symptoms with consolidation shadows on chest X-ray are assumed to have pneumonia, which in most cases is bacterial-induced, and their sputa are first stained with gram stain. In patients with pneumonia, aside from cases with shadows typical of tuberculosis, the first empiric therapy is usually antimicrobial agents.

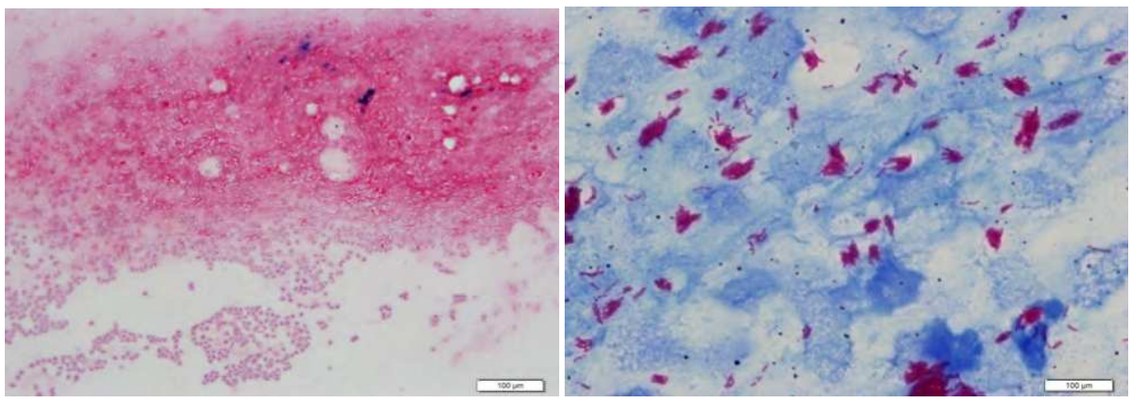
On gram staining of sputum, *Mycobacterium tuberculosis* either is weakly gram-positive or appears as colorless rods or "ghosts" (Hinson et al.,1981;Trifito et al.,1990). However, there is no description of Gram staining as useful staining for *M. tuberculosis* in the textbooks.

If tubercle bacilli could be easily detected in clinical samples with gram staining, it would be possible to detect tuberculosis more promptly and easily, which may contribute to tuberculosis control. In this paper, we present three infective tuberculosis cases in which gram staining easily detected tubercle bacilli before Ziehl-Neelsen (Z-N) staining and diagnosed by polymerase chain reaction (PCR) or culture.

Case 1

A 67-year-old man with hypertension, hyperlipidemia and atrial fibrillation visited our hospital because of lumbago, fever of approximately 38°C and an increased serum CRP level. Five years earlier, he had undergone resection of a prostate tumor. Two years prior to the current admission, thymectomy was performed because of myasthenia gravis and he was administered 20 mg/day prednisolone and 150 mg/day cyclophosphamide. One month earlier, he developed swelling on the back of the right hand. Gram staining of pus from the back of the hand showed many neutrophils with no bacteria when focus was adjusted on the nucleus of neutrophil, but contained gram-positive granular rods with slightly longer focus and brightening colorless rods with slightly shorter focus, suggesting the presence of *M. tuberculosis*.

The bacilli were positive (2+) on Z-N staining(Fig.1,2,3,4). The presence of *M. tuberculosis* was confirmed by PCR.



(a) (b)

Fig. 1. Gram staining and Ziehl-Neelsen staining of the pus in case 1. Gram staining of pus under a hundred-fold focus (a) and Ziehl-Neelsen staining of the pus (b). The sample contained 2+ bacilli on Ziehl-Neelsen staining(X 1000). The presence of *M. tuberculosis* was confirmed by PCR.

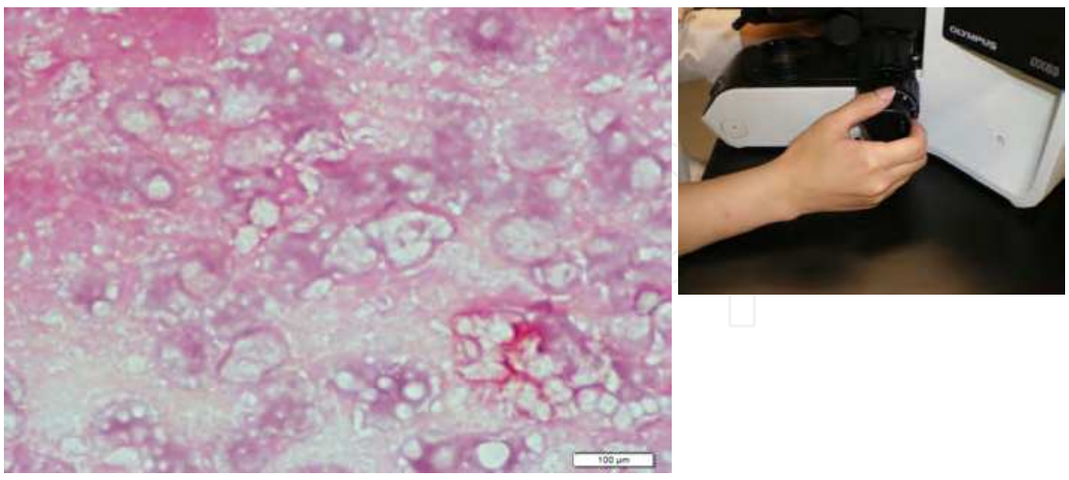


Fig. 2. Gram staining of pus under ordinary focus in case1. Gram staining of pus (X 1000) under ordinary focus in case 1. The purulent samples contained abundant of neutrophils without any causative bacteria. The small right upper figure shows left hand catching dial at ordinary style.

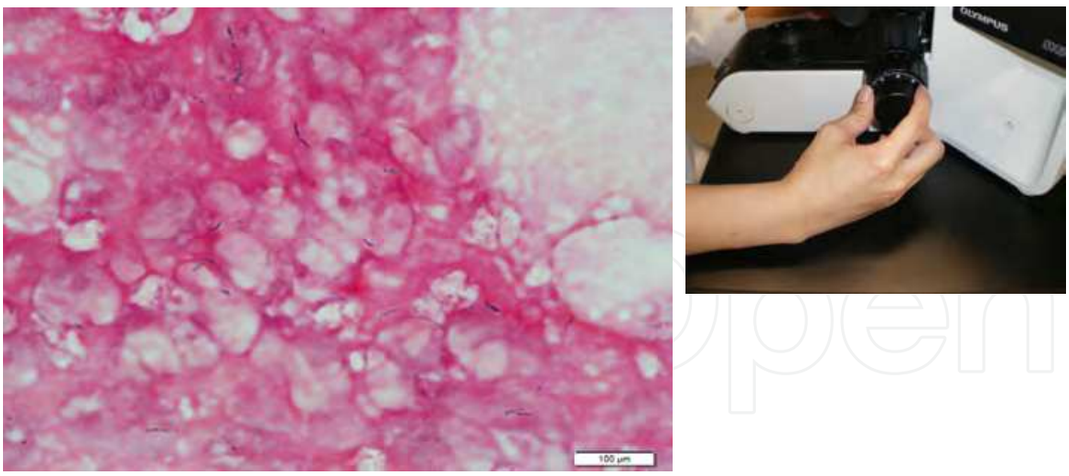


Fig. 3. Gram-positive cord-like bacilli in Gram stain in case 1. Turning the dial to adopt a slightly longer focus distance clearly showed gram-positive cord-like bacilli. The small right upper figure of hand catching dial showed position of the hand making longer focus.

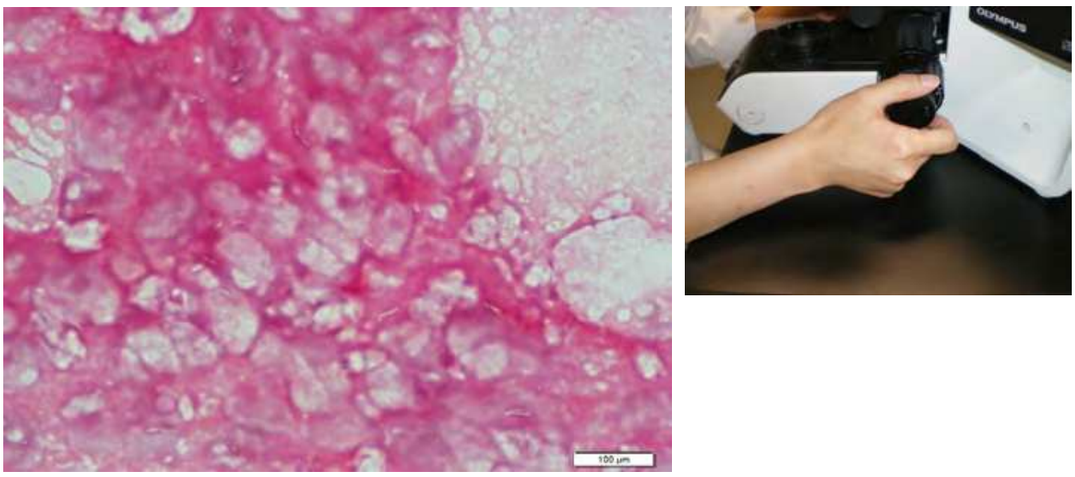
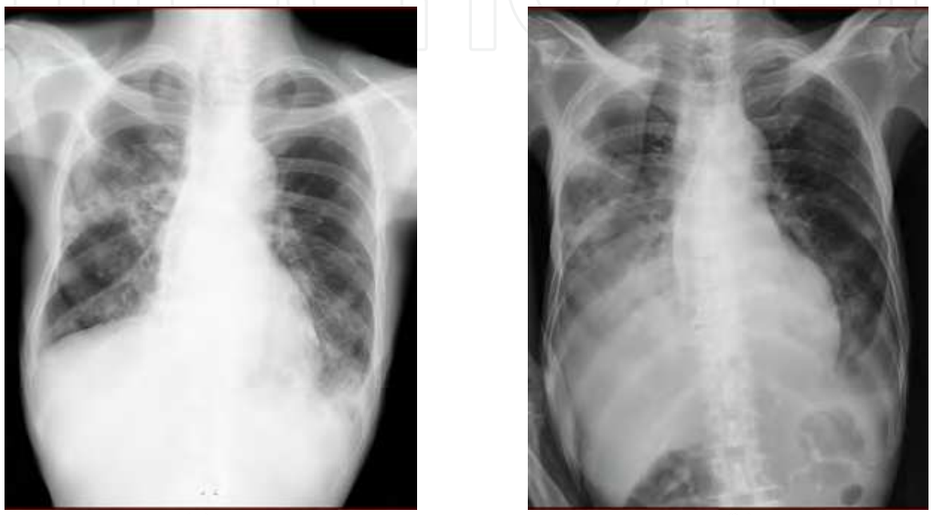


Fig. 4. Brightened rods and colorless bacilli in Gram stain in case 1. A slightly shorter focus distance revealed brightened rods and colorless bacilli in Gram stain. The small right upper figures of hand catching dial showed position of the hand making shorter focus.

Case 2

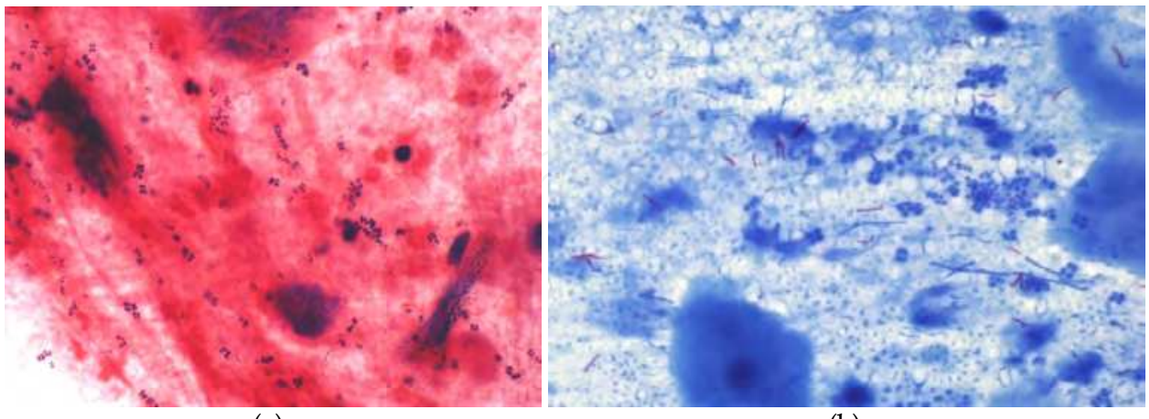
A 62-year-old man with type II diabetes mellitus presented to our hospital because of fatigue and loss of appetite. Five months earlier, he had undergone surgery for cancer at the base of the oral cavity (T4N0M0). Fifty days prior to the current admission, he noticed general malaise, loss of appetite, and fever. He was seen in our hospital, at which time the serum sodium level was 115 mEq/l. His temperature was 38.1°C. He had hypoxemia and hypoalbuminemia. Antimicrobial therapy was started. On the fourth hospital day, bilateral pleural effusion was detected, and pneumonia and congestive heart failure were suspected. On the fifth hospital day, he gradually lost consciousness. Gram staining of his sputum revealed many gram-positive cocci and gram-negative bacilli, with large numbers of neutrophils and oral epithelial cells. With small changes in focus under the microscope,

some bacilli showed a change in staining pattern from gram-positive to unstained neutral. The sample contained 3+ bacilli on Z-N staining, and the presence of *M. tuberculosis* was confirmed by PCR and culture(Fig. 5,6,7). Electrocardiogram and laboratory data suggested the existence of ischemic heart disease, and he died after a decrease in blood pressure. However, the exact cause of death was unclear. Autopsy revealed the existence of miliary tuberculosis with no myocardial infarction.



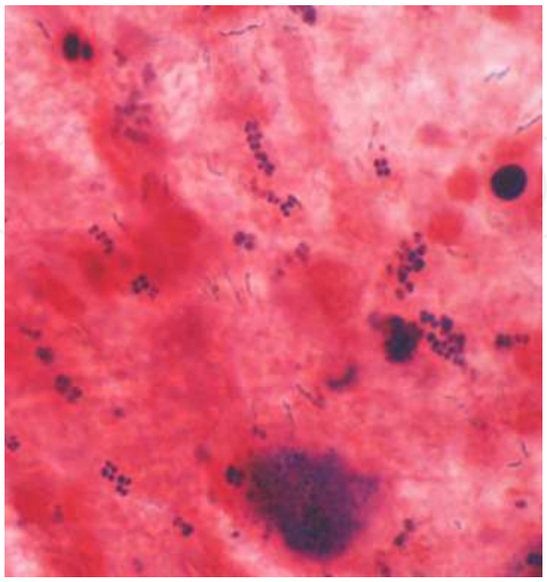
(a) (b)

Fig. 5. Chest X-rays in case 2. On admission consolidation was detected in the right lung (a). Consolidation shadows and pleural effusion were detected in the right lung on the sixth hospital days (spine position) (b).

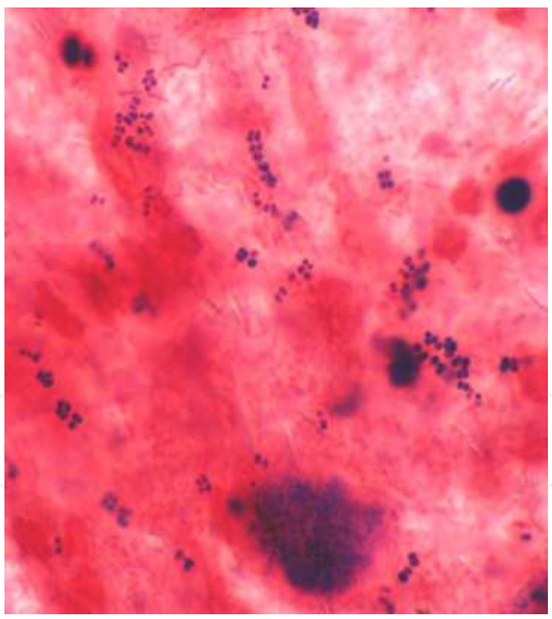


(a) (b)

Fig. 6. Gram stain and Ziehl-Neelsen staining of the sputa in case 2. Gram staining of his sputum revealed many gram-positive cocci and gram-negative bacilli, with large numbers of neutrophils and oral epithelial cells (X 1000) (a). The sample contained 3+ bacilli on Ziehl-Neelsen staining(X 1000) (b), and the presence of *M. tuberculosis* was confirmed by PCR and culture.



(a)

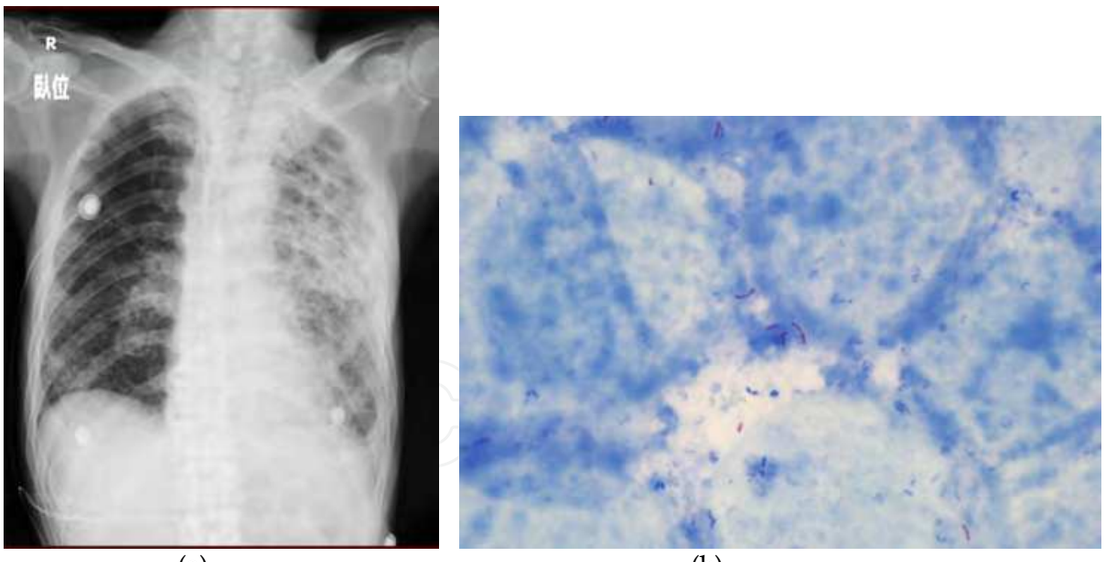


(b)

Fig. 7. Changing focus examination of sputa in case 2. Gram staining of pus in case 2 (X 1000). Turning the dial to adopt a slightly longer focus distance clearly showed gram-positive cord-like bacilli (a) and to adopt a slightly shorter focus distance revealed brightened rods and colorless bacilli (b).

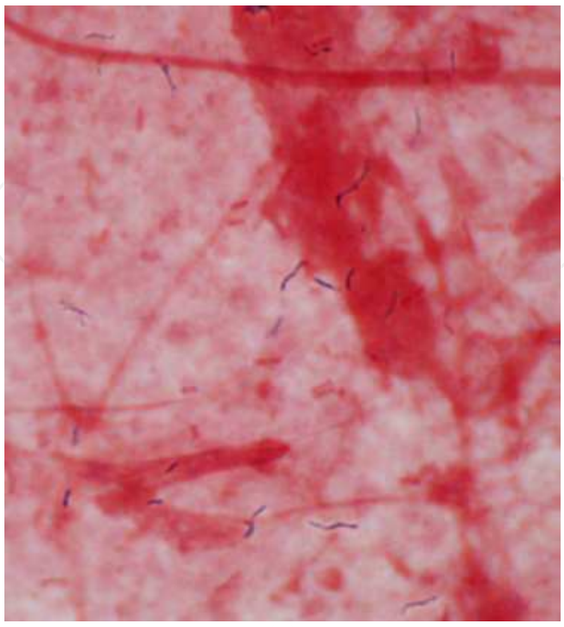
Case 3

A 75-year-old man was transferred to our hospital to receive additional therapy for pneumonia that was treated unsuccessfully at another hospital. He had been treated with home oxygen therapy for chronic obstructive pulmonary disease for the past 19 years. Thirteen days earlier, he was admitted to another hospital because of pneumonia in the left lung. Several antimicrobial agents (meropenem, ciprofloxacin, imipenem/cilastatin, and vancomycin) were successively administered, but were ineffective. After being transferred to our hospital, he was administered biapenem in addition to minocycline and micafungin. Z-N staining of his sputum, done only once on admission, was negative. His inflammatory laboratory data improved slightly, but thirteen days later, the data showed abnormal levels again and hypoxemia emerged. Biapenem was changed to tazobactam/piperacillin. Gram staining of the sputum showed a large number of gram-negative rods with many neutrophils; the bacteria were confirmed to be *Burkholderia cepacia*. However, the sputum contained gram-positive granular rods, which were observed as brightened rods with a change in focus, suggesting the presence of *M. tuberculosis*. The bacilli were recognized to be positive (2+) for Z-N staining(Fig. 8,9). The presence of *M. tuberculosis* in the sputum was later confirmed by PCR.

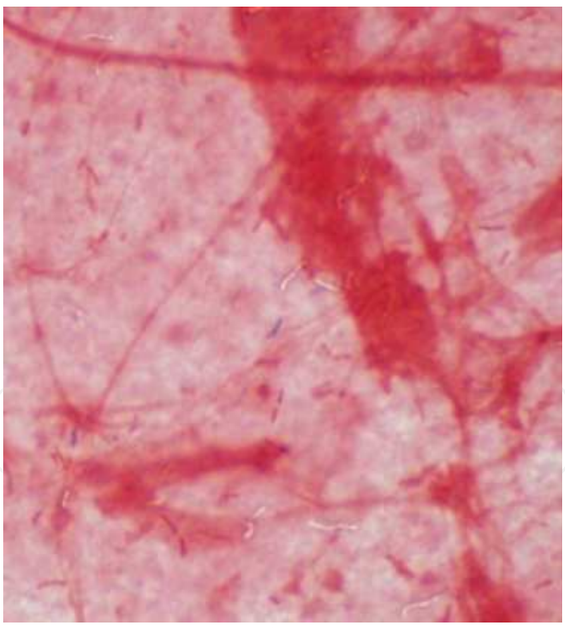


(a) (b)

Fig. 8. Chest X-rays and Ziehl-Neelsen staining of the sputa in case 3. On the seventh hospital days the part of consolidation in the upper of the left lung with emphysematous lung was persisted after successively and ineffectively administration of broad spectrum antimicrobial agents (a). Ziehl-Neelsen staining of the sputum were recognized to be positive (2+). The presence of *M. tuberculosis* was later confirmed by PCR.



(a)



(b)

Fig. 9. Changing focus examination of sputa in case 3. Gram staining of pus in case 3 (X 1000). Turning the dial to adopt a slightly longer focus distance clearly showed gram-positive cord-like bacilli (a) and to adopt a slightly shorter focus distance revealed brightened rods and colorless bacilli (b).

2. Discussion

Gram staining is a useful technique for detecting bacteria in infectious diseases. But when infections with tuberculosis is probable, special staining, such as Ziehl-Neelsen staining is essential in detecting of *Mycobacterium tuberculosis* .

Hinson (Hinson et al., 1981) and Trifiro et al. (Trifiro et al. ,1990) observed tubercle bacilli as ghost mycobacteria in Gram staining of clinical samples.

In some textbooks one and/or two patterns of gram-stained tubercle bacilli have been described. *M. tuberculosis* often shows neutral staining (Raviglione & O'Brien,2008) , often appears as beaded gram-positive bacilli or fails to stain at all (Inderlied, 2004), or shows weak gram-positive staining and appears as colorless rods or "ghosts" (Fitzgerald,2005).

We presented a technique of Gram staining for detecting infective tuberculosis in clinical samples, the focus chaining technique (Atsukawa et al., 2011). It is a very useful procedure as an easy and rapid initial diagnostic tool to recognize highly infective tuberculosis because it can be directly applied to clinical specimens, such as sputum and pus.

Recently, we experienced a case with tuberculosis meningitis, in which Gram staining of cerebrospinal fluid with predominantly neutrocytic pleocytosis was useful as an initial adjunct to the diagnosis of tuberculous meningitis(Kawakami et al., 2012).

There are two types of samples for which the changing focus procedure on Gram staining is useful. One is purulent samples including pus without any causative bacteria. In the samples search for tubercle bacilli should be made. In an usual fixed focus, we might miss the ghost bacilli (Figure 2). The changing focus procedure is done in three steps as follows. 1) Firstly, in the ordinary focus, weakly stained gram-positive long bacilli or no conspicuous bacilli are found in samples(Fig. 2,6a), 2) with a slightly longer focus distance the gram-positive thin cord-like bacilli can be clearly observed(Fig. 3,7a,9a), and 3) with shorter focus distance the gram-positive bacilli have changed into the brightened, colorless or ghost bacilli(6)(Fig. 4,7b,9b).

The other is the samples with various amounts of gram-negative and/or gram-positive organism. Especially in purulent sputum, the existence of abundant of organisms with neutrophils usually leads a diagnosis of bacterial pneumonia. In the case2, the Gram staining showed many gram-positive cocci (Fig. 6a) and in the case3, it showed many gram-negative rods, which were confirmed to be *Burkholderia cepacia* by culture. The diagnosis of these cases by the clinicians were bacterial pneumonia and antimicrobial agents were administered.

In the ordinary procedure of Gram staining of purulent sputa, neutrophils firstly are brought into focus to check the adequacy of the safranin-staining and after once setting a focus, there is no need to change it. In the focus gram-negative and/or -positive organisms usually being also brought into focus and only we have to do is to investigate phagocytosed bacteria to catch causative organisms. However, in the focus, tubercle bacilli are weakly stained as unclear thin cord-like positive rods or sometimes inconspicuous neutral crystal-like fragments among abundant of gram-negative and/or gram-positive organism. In addition, the number of tubercle bacilli is far less than the other gram-negative and/or -positive organisms. These facts may explain why tubercle

bacilli are most likely to be missed so far in Gram stained purulent sputum with various organisms and the staining have been recognized as useless one in detecting pulmonary tuberculosis. In pulmonary tuberculosis with atypical features as in case 2 and 3, sometimes the delay of Z-N staining of sputum, which is sometimes done after the first antimicrobial agent's therapy has proven unsuccessful, lead to the delay in diagnosis of pulmonary tuberculosis. The delay of Z-N staining is partly due to the complexity of the staining and the need for trained staff. However, with the repeated changing focus procedure, in a slightly longer focus distance, the weekly stained gram-positive cord-like rod had changed into clear conspicuous gram positive thin bacilli though the other organism were out of focus. And with shorter focus distance brightened and colorless bacilli, "gram-neutral" or "gram-ghost", were revealed.

Our experience has shown that, even when the sample contains various amounts of organisms, repeated changing the focus of the microscope slightly longer and shorter during the examination of the slide is indispensable in searching for tubercle bacilli. The staining characteristics of the tubercle bacilli in Gram stain , biphasic stain patterns as conspicuous thin long gram-positive bacilli changing into gram-neutral, is only noticeable by changing the focus.

The patients in case1and 2 had previous surgery for cancers. The patient in case 1 also had been administered prednisolone and cyclophosphamide and the patient in case 2 had type II diabetes mellitus. The patient in case 3 had been successively and ineffectively administered many antimicrobial agents with the broad spectrum. The patients in the case 1 and 2 dead. If their tuberculosis had been diagnosed earlier, a more rapid start of anti-tubercle therapy might save their lives.

Especially, when patients have predisposing factors to active tuberculosis, such as diabetes mellitus, liver cirrhosis, hemo-dialysis, and administration of immune-suppressive drugs, careful examination on gram staining is needed along with Z-N staining. The present study showed that gram staining is an effective initial test to check for infective tuberculosis. Considering that the clinical diagnosis of tuberculosis begins with a high index of suspicion , we should always check samples of Gram staining with the focus changing procedure.

There are certain types of images to which attention must be paid to avoid misidentification on Gram staining. Crystal-like fragments are sometimes visualized as thin, brightened neutral rods. However, when changing the focus, the brightened neutral rods never change into long gram-positive bacilli as tubercle bacilli.

The tuberculosis epidemic is far from over and is aggravated by multi-drug resistant tubercle bacilli and the even more dangerous form, extensively drug-resistant tubercle bacilli.

This study showed that gram staining represents an easy and rapid procedure for recognizing highly infective *M. tuberculosis*. The ease of the procedure and the rapidity of staining will contribute greatly to initial testing for tuberculosis, not only in developing countries but also developed countries where the number of immune-suppressed patients have increased in hospitals. Further studies are needed to clarify the usefulness of gram staining in finding infectious tuberculosis in various clinical fields or situations.

3. References

Atsukawa,Y., Kawakami,S., Asahara, M., Ishigaki, S.,Tanaka, T., Ono,Y., Hishiya, H., Fujisaki, R., Koga, I., Ota, Y.& Miyazawa, Y. (2011). The usefulness of changing focus during examination using Gram staining as initial diagnostic clue for infective tuberculosis. J Infec Chemother Vol 17: 571-574.

Fitzgerald, D. & Haas, D.(2005) Mycobacterium tuberculosis, in chapter 248 of Mandell, Bennett & Dolin (ed),*Principles and Practice of Infectious Disease 6th ed* , Elsevir Churchill Livingstone, Philadelphia, Pennsylvania, pp.2853.

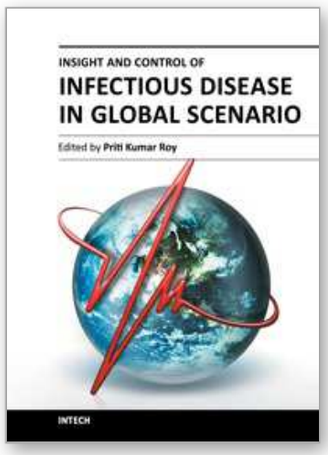
Hinson, M., Bradsher,W. & Bodner, J. (1981). Gram-stain neutrality of Mycobacterium tuberculosis. Am Rev Respir Dis Vol 123: 365-366.

Inderlied, B.(2004). Mycobacteria, in chapter 233 of Cohen & Powderly (ed), *Infectious Diseases 2ed ed*. Mosby Elsevir Limitted, Edinburgh London ,pp.2297.

Kawakami, S., Kawamura, Y., Nishiyama, K., Hatanaka, H., Fujisaki, R., Ono, Y., Miyazawa, Y. & Nishiya, H. (2012). Case of *Mycobacterium tuberculosis* meningitis: Gram staining as a useful initial diagnostic clue for tuberculous meningitis. J Infec Chemother (accepted)

Raviglione MC, O`Brien RJ. Tuberculosis (chapter 158) In; Harrison`s Principal of Internal Medicine 17th ed. New York; The McGraw-Hill Companies, Inc.: 2008.p.1006

Trifiro, S., Bourgault, M. & Lebel, F. (1990). Ghost mycobacteria on Gram stain. J Clin Microb Vol 28:146-147.



Insight and Control of Infectious Disease in Global Scenario
Edited by Dr. Roy Priti

ISBN 978-953-51-0319-6
Hard cover, 442 pages
Publisher InTech
Published online 21, March, 2012
Published in print edition March, 2012

This book is projected as a preliminary manuscript in Infectious Disease. It is undertaken to cover the foremost basic features of the articles. Infectious Disease and analogous phenomenon have been one of the main imperative postwar accomplishments in the world. The book expects to provide its reader, who does not make believe to be a proficient mathematician, an extensive preamble to the field of infectious disease. It may immeasurably assist the Scientists and Research Scholars for continuing their investigate workings on this discipline. Numerous productive and precise illustrated descriptions with a number of analyses have been included. The book offers a smooth and continuing evolution from the principally disease oriented lessons to a logical advance, providing the researchers with a compact groundwork for upcoming studies in this subject.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Yoshiko Atsukawa, Sayoko Kawakami, Yasuo Ono, Ryuichi Fujisaki, Yukihisa Miyazawa and Hajime Nishiya (2012). Three Cases of Mycobacterium tuberculosis Infection Initially Recognized by Focus Changing Examination in Gram Staining, Insight and Control of Infectious Disease in Global Scenario, Dr. Roy Priti (Ed.), ISBN: 978-953-51-0319-6, InTech, Available from: http://www.intechopen.com/books/insight-and-control-of-infectious-disease-in-global-scenario/gram-staining-as-initial-diagnostic-clue-for-infective-tuberculosis

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen