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RESEARCH ARTICLE

Can Gram-Stained Sputum Be an Effective Therapeutic Marker of Effectiveness of Antimicrobial Agents in Bacterial Pneumonia and Bronchitis?

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ABSTRACT

Gram staining is one of the most crucial staining techniques in microbiology.

The use of Gram stain facilitates rapid use of appropriate antimicrobial agents. In bacteria pneumonia, the most useful sample which reflects the status of inflammation in the lung is supposed to be sputum from the infectious areas in the lung. The changing patterns of Gram-stained sputa can be used as the therapeutic marker of effectiveness of antimicrobial agents. When the first administered antimicrobial agent is effective against the target pathogen; *S. pneumoniae*, *M. catarrhalis* or *Haemophilus influenzae*, a decrease in number of the pathogen in the sputa was clear and almost no or little pathogen were seen in the sputum obtained several-hours after the first administration of antimicrobial agent or before the second administration, which is mostly administered 8-12 hours-after the first one, showing that Gram-stained sputum is a definite effective marker of the effectiveness of the agent. In pneumococcal pneumonia, a loss of gram-positive-staining of pneumococci was another early marker of the effectiveness of the agent. We can expect the effectiveness at least 1 h after completion of the first administration of the agent, when a loss of gram-positive-staining of pneumococci with a decrease in the number of cocci is found in the sputa. The reason for the loss of staining is supposed to be by reduction in peptidoglycan synthesis induced by antimicrobial agents distributed in the sputa. To find effective marker showing the effectiveness of administered antimicrobial agent in bacterial pneumonia or bronchitis, we compared the white blood cell count (WBC), serum C-reactive protein (CRP) level, and the decrease in the bacterial density in gram-stained sputa in which the administered antimicrobial agents were effective. The data showed that at least 2 to 4 days were needed to evaluate the effectiveness when the WBC or CRP level was used as a therapeutic marker, but the median duration needed to determine the effectiveness of the agent was 6.5 hours (range, 1 to 12 hours) in Gram-stained sputa, which showed that Gram-stained sputum after the first administration of antimicrobial agents can be used as the quickest therapeutic marker in treating bacterial respiratory infections. We showed that Gram staining of sputum is a useful and effective tool to check the effectiveness of administered antimicrobial agents in bacterial pneumonia and bronchitis.

Keywords: bacterial pneumonia, gram staining, therapeutic marker

Introduction

Pneumonia is the leading infectious cause of mortality worldwide and one of the most common lower respiratory tract infections that is contributing significantly to the burden of antibiotic consumption. Due to the complexity of its pathophysiology, it is widely accepted that clinical diagnosis and prognosis are inadequate for the accurate assessment of the severity of the disease.[1] Pneumococcal disease is a major cause of clinical and economic burden worldwide.[2] Recently, shortening the duration of antibiotic therapy for patients admitted to hospital with community-acquired pneumonia (CAP) is tried, which leads to reduced antibiotic consumption and thus bacterial resistance, adverse events, and related costs. Discontinuing β-lactam treatment after 3 days was non-inferior to 8 days of treatment.[3] In terms of sensitivity, the high prevalence of resistance to penicillin by Streptococcus pneumoniae is considered as a great concern, particularly in Asian countries.[4] In bacteria pneumonia, the most useful sample which reflects the inflammation in the lung is supposed to be sputum where the infectious battle is going between bacteria and white blood cells in the alveolar cavities in the host. Serial examinations of the sputum reveal several changes in the bacteria in course of the repeated antimicrobial administrations. Gram staining is one of the most crucial staining techniques in microbiology. It gets its name from the Danish bacteriologist Hans Christian Gram who first introduced it in 1882, mainly to identify organisms causing pneumonia. Gram stain is often the initial diagnostic test for the evaluation of infections. Gram staining is indicated whenever a bacterial infection is suspected for easy and early diagnosis. It aids in the diagnosis of a disease or a pathologic condition. The use of Gram stain facilitates the rapid use of appropriate antibiotics.[5] One of attractivity contained in Gram staining[6] is its ability for visualizing inflammatory changes in tissues directory and promptly with ease and inexpensively. Gram staining is one of useful tools which visualize the course of the battle in bacterial pneumonia. We have shown in patients admitted to hospital with *S. pneumoniae* and *M. catarrhalis* pneumonia (CAP) that gram-stained sputum obtained just after or one hour after two grams of ampicillin (ABPC) administration present useful information as a reliable and the earliest therapeutic marker of the effectiveness of the agent.[7] The changing patterns of Gram staining can also be used as the quickest therapeutic marker of the effectiveness of administered antimicrobial agent in outpatient with bacterial respiratory infections.[8] Although Gram staining has been classified as a tool for diagnosis, it should also be put in the tools which can evaluate the effectiveness of the administered antimicrobial agents. In respiratory infections we need to utilize sputa more frequently and completely as the direct letter from the infected respiratory tract and lung. In this article, we try to describe its usefulness more closely.

Materials and Methods

To evaluate the effectiveness of antimicrobial agents first administered, we checked the sputa obtained from the patients with bacterial pneumonia or bronchitis on admission in department of internal medicine of Teikyo-University hospital just after the completion of the first antimicrobial agent administration or 1 to 2 hours after the completion of the administration to check morphological changes of the pathogenic bacteria by using Gram staining, in which a crystal violet dye as the first step, iodine as fixing the dye, ethanol as decolorizer and safranin as a counterstain were used. We confirmed the effectiveness of the agents by checking Gram-stained sputa obtained several-hours after the first administration of antimicrobial agents or before the second administration which was mostly administered 8 to12 hours after the first one.

Results

1, Bacterial density in the sputum obtained several-hours after or before second administration of antimicrobial agents as a definitive marker of their effectiveness

Most definite sample deciding the effectiveness of the first administered antimicrobial agent was the sputum obtained several hours-after or before the second administration of the agent, which was mostly administered 8-12 h after the first one. The density of the bacteria in the sputum shows the effectiveness of the first administered agents. When the first administered antimicrobial agent is effective against the pathological bacteria, a decrease in thenumber of the bacteria is clear. Figure 1 shows the sputa obtained from pneumococcal pneumonia cases (CAP) before the antimicrobial treatment (Fig.1a; case1 of 53-year-old man, from whom *S.pneumoniae* (penicillin-resistance *S.pneumoniae*; PRSP) 3+ and *Haemophilus.influenzae* (β-lactamase negative ampicillin resistance;BLNAR) 1+ were cultured, Fig.1b; case2 of 72-year-old woman, from whom *S.pneumoniae* (penicillin-sensitive *S.pneumoniae*; PSSP) 3+ and *Haemophilus.influenzae* (non-BLNAR) 1+ were cultured, Fig.1c; case3 of 59-year-old woman, from whom *S.pneumoniae* (PRSP) 3+ and *Haemophilus.influenzae* (non-BLNAR) 1+ were cultured, Fig. 1d; case4 of 67-year-old man, from whom *S.pneumoniae* (PRSP) 3+ was cultured).

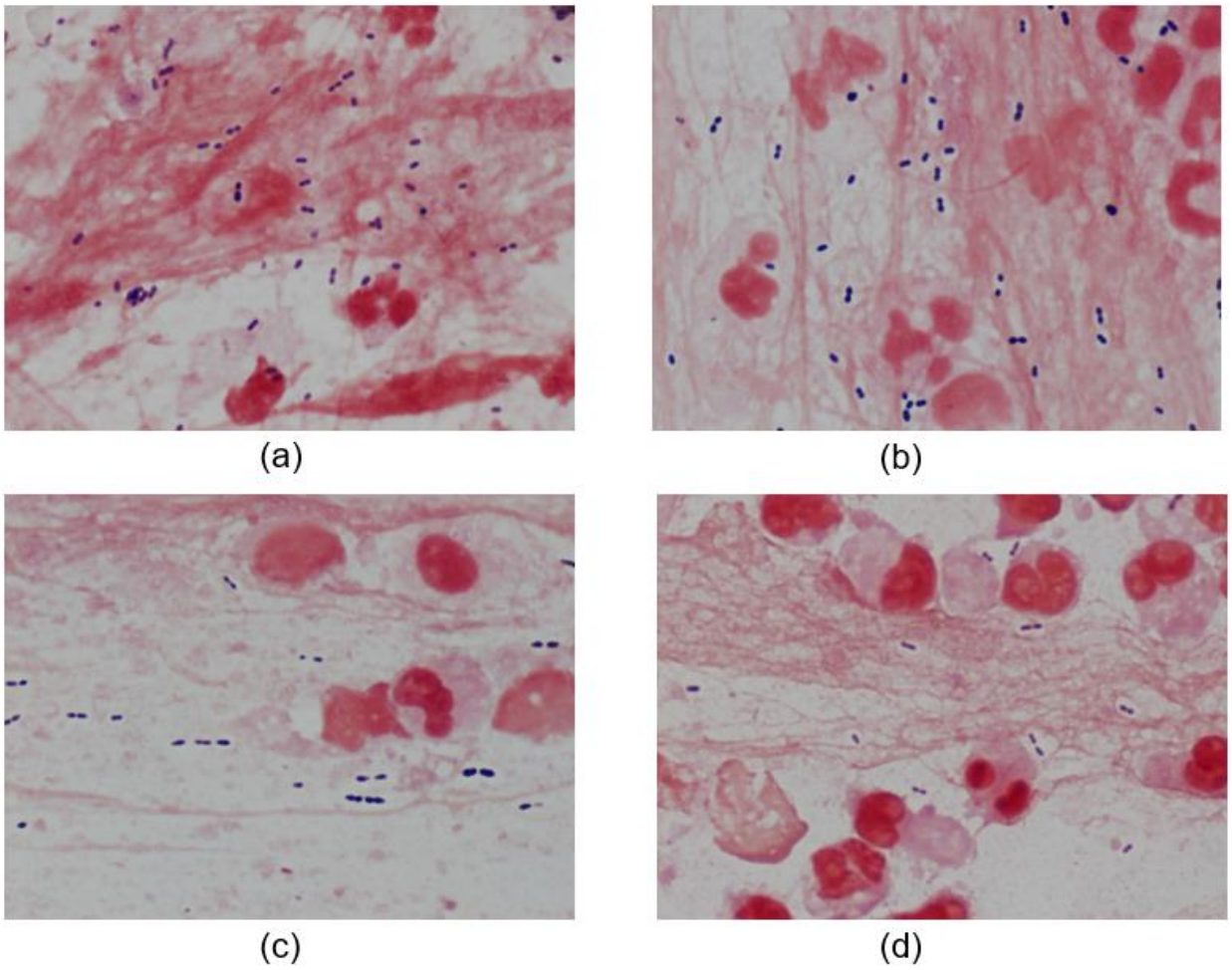

Figure 1. Gram staining of sputum samples (X 1000) before the first administration of antimicrobial agents
The sputum sample showed a lot of gram-positive diplococci and a little gram-negative rod. (a) is obtained from case1,(b) from case2, (c) from case3 and (d) from case4. *S.pneumoniae* (3+) were cultured from case 1,2,3,4. *H.influenzae* (1+) were cultured from case 1,2,3.

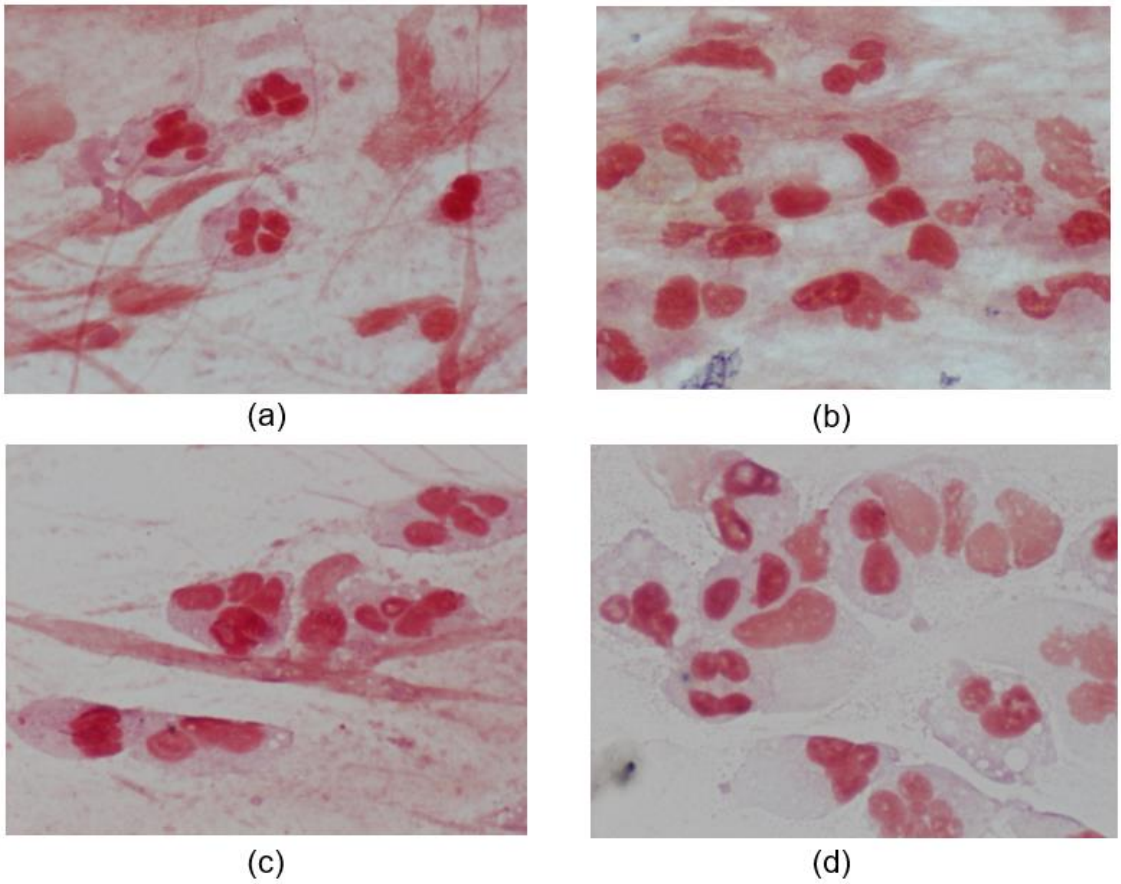

Figure 2. Gram staining of sputum samples (X 1000) obtained 1 to12 h after the completion of first administration of antimicrobial agents; Case 1 (a), Case 2 (b), Case 3 (c), Case4 (d). Gram staining of sputum samples revealed no or little microorganisms.

Figure 2 shows the sputum obtained several hours after the first administration or just before the second administration of the agent. Figure 2a (case 1) shows sputum obtained 12 hours after the first administration of 2 g of ABPC over a period of 1 hour and before the second administration. Figure 2b (case 2) shows gram-stained sputum obtained 1 h after the first administration of 2 g of ABPC over a period of 1 h. Figure 2c (case 3) shows sputum obtained 12 h after the first administration of 1 g of piperacillin over a period of 1 h and before the second administration. Figure 2d (case 4) shows gram-stained sputum obtained 6 h after the first administration of 2 g of cefotaxime (CTX) over a period of 1 h. Figure 3 shows the sputum obtained from pneumonia (CAP) and bronchitis cases of *Moraxella catarrhalis*. Figure 3a (case 5 of 58-year-old woman) shows sputum before antimicrobial therapy and Fig.3b that obtained 2 h after the completion of first administration of 1 g of CTX over a period of 1 h. Figure 3c (case 6 of woman) shows gram-stained sputum obtained before therapy and Fig.3d that obtained 6 h after the completion of 1g of flomoxef over a period of 1 h. When the first administered antimicrobial agents were effective, almost no or little cocci were seen in the following gram-stained sputum. Bacterial density in the sputum several-hours after the first administration or before the second administration of antimicrobial agents is a definitive marker showing the effectiveness of the agents.

2. A loss of gram-positive-staining of pneumococci as an early marker of the effectiveness of administered agents.

Although the loss of bacterial density is a definitive marker for the effectiveness, it can be followed only in patients who stay several to twelve hours in the hospital. We need a more rapid tentative marker for checking the effectiveness of the first administered antimicrobial agent including out-patients and want to know the effectiveness, if possible, at once.

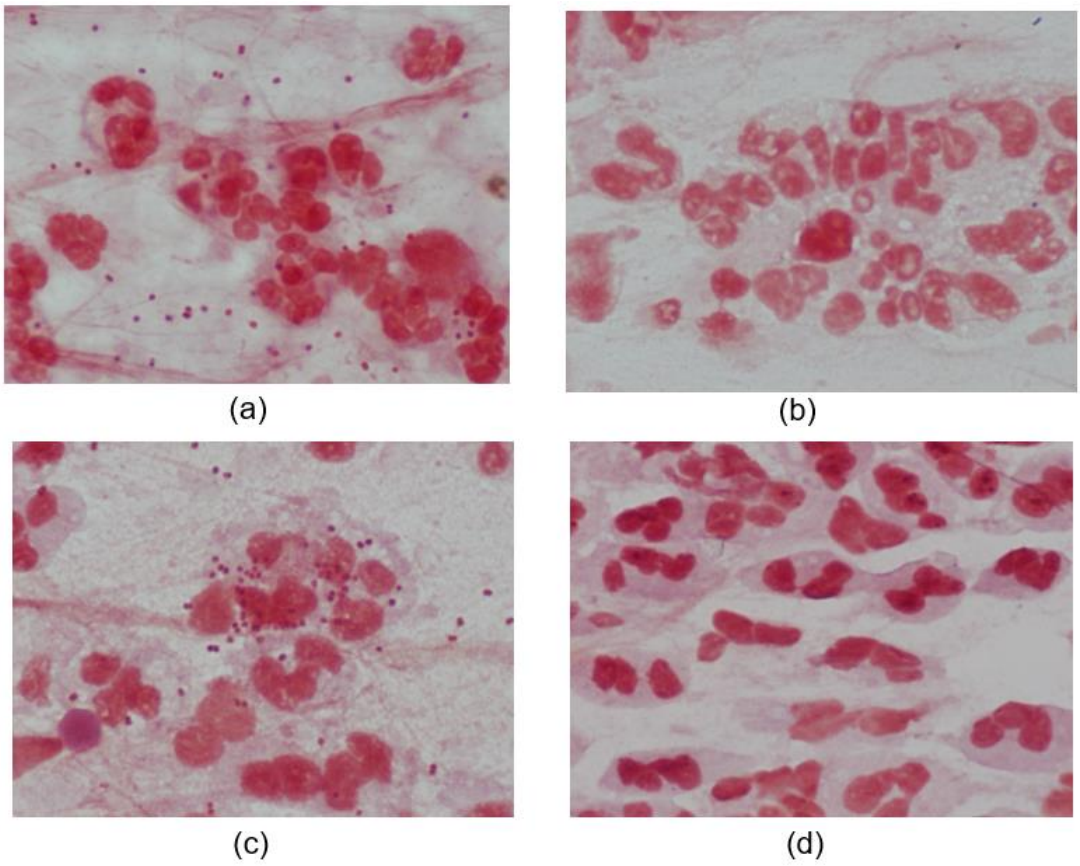

Figure 3. Gram staining of sputum samples (X 1000) before and after the first administration of antimicrobial agents. The sputum sample showed a lot of gram-negative diplococci, identified as *Moraxella catarrhalis* in case 5 (a) and in case 6 (c). Gram staining of sputum samples obtained 2 to 6 h after the completion of the first administration of antimicrobial agents in Case 5 (b) and in Case 6 (d), at a glance with no microorganisms.

A loss of gram-positive-staining of pneumococci was seen when the antimicrobial agent was effective. Some cocci were stained weekly gram positive and some stained weakly gram negative. Figure 4 shows mixture of gram positive and gram-negative diplococci in the sputum. Figure 4 shows the sputum obtained at the completion of 1 h administration of 2 g of ABPC in case1. A loss of gram-positive-staining of pneumococci was obvious compared with the sputum before the therapy (Fig.4f). Figure 4c and 4d show the sputum obtained 1 h after the completion, in which proportion of gram- positive pneumococci was decreased in the sputum. Reduced number of pneumococci was also clearer in the sputum 1 h after than that obtained at the completion. The decrease in the number of pneumococci and the increase in the proportion of gram- negative pneumococci seen in the sputum indicate the effectiveness of the agent.

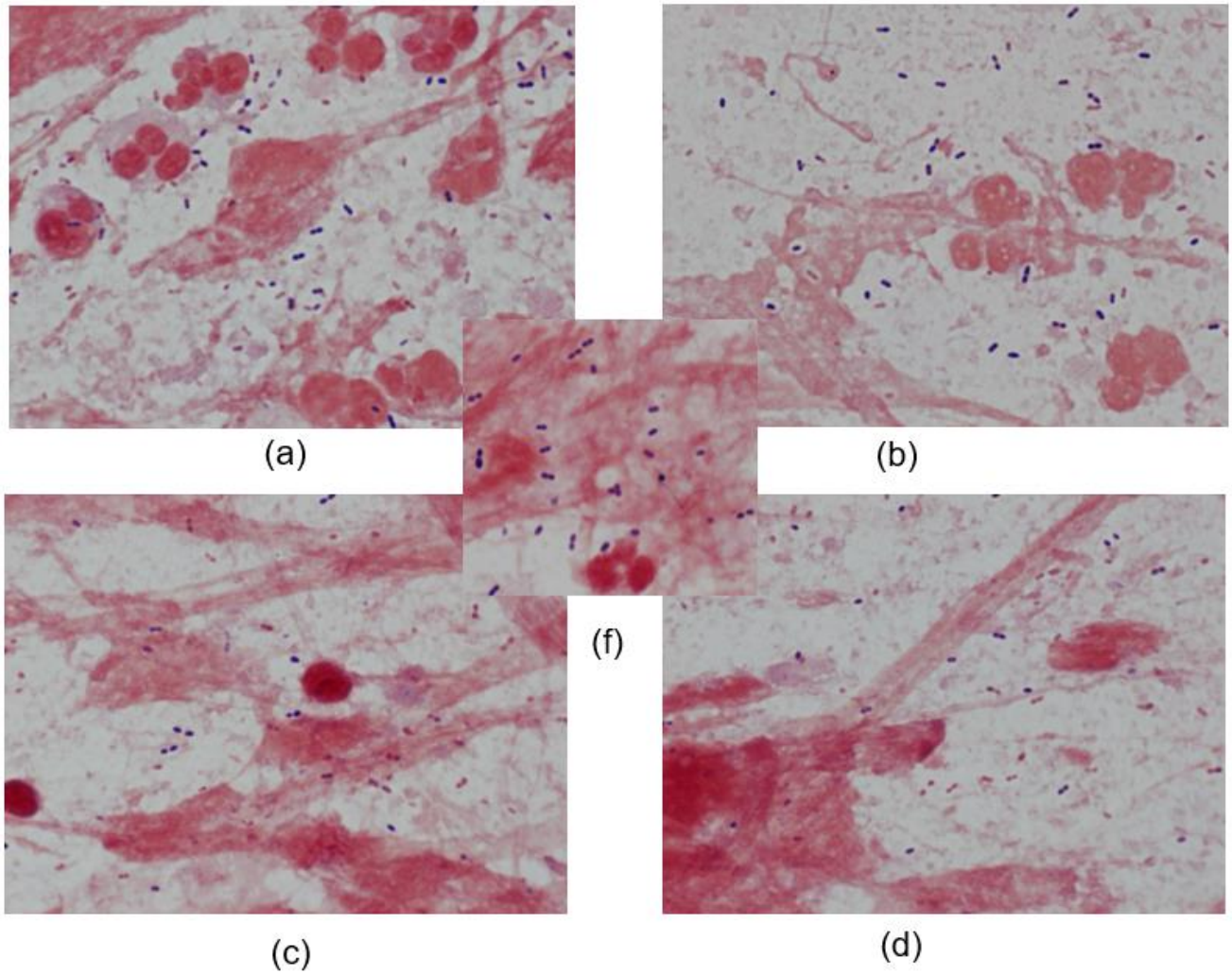

Figure 4. Gram staining of sputum samples (X 1000) obtained at the completion of administration of 2 g of ampicillin (a,b), and one hour after the completion (c,d) in Case1. A loss of gram-positive-staining of pneumococci was seen and mixture of gram-positive and gram-negative diplococci is obvious. Some cocci were stained weakly gram positive and some stained gram negative. The proportion of gram- positive pneumococci was decreased in the sputum obtained 1 h after the completion of administration (c,d). Decrease in the number of pneumococci was also clearer in the sputum obtained 1 h after (c,d) than that obtained at the completion(a,b). Gram stain of sputum before therapy is shown in the center (f).

Figure 5 shows the several changes in the sputum with strong magnification to find the difference more clearly. Figure 5a, b and c show the same sputum as Fig 4a and b. Figure 5d and e show the same sputum as Fig 4c and d. A loss of gram-positive appearance was detected as gram-negative diplococci. Some cocci were stained weakly gram positive and some stained gram negative. Sometimes, in one pair of pneumococci, one coccus was stained violet and the other was stained pink (Fig.5c; →). The size of some pneumococci 1 h after the completion of 2 g of ABPC administration has become smaller than those seen before the therapy in case 2 (Fig.5f). In pneumococcal pneumonia, when 2 g of ABPC is used as empiric therapy, we can check the effectiveness at least after 2 h after completion of the first administration. The gram-stained sputa obtained about 1 h after the first administration of 2 g of CTX in case4 showed a slightly elongated cocci (Fig.5g), which may reflect the difference in antimicrobial mechanism how to act against penicillin-binding proteins (PBPs), which are responsible for building the bacterial cell wall and the differences in the spectrum activity of β-lactam antibiotics are due to their affinity for different PBPs.[9] There were some cases in which the changes of the loss of staining and decrease in the number of pneumococci were not so remarkable as ABPC in sputa obtained several hours after the completion of piperacillin or ceftriaxone (data not shown).

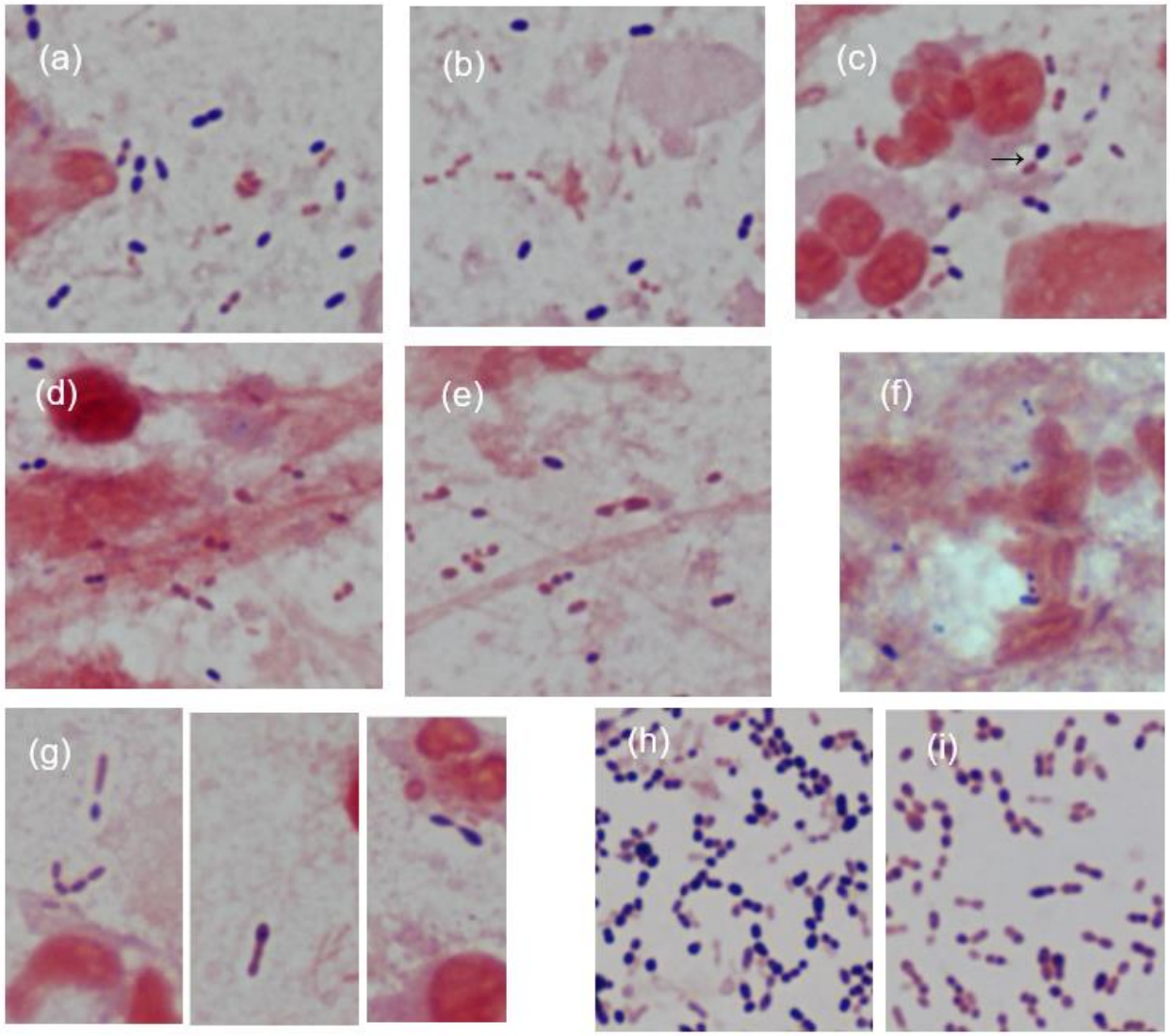

Figure 5. Gram staining of sputum samples (X 1000) with strong magnification.
A loss of gram-positive-staining of pneumococci in case 1 was seen(a-e). Figure 5a, b and c show the same sputum as Fig 4a and 4b. Figure 5d and e show the same sputum as Fig 4c and 4c. Some diplococci stained gram positive and some stained gram negative (a,b,c) at the completion of the first 1 h administration of 2 g of ABPC. Sometimes, in one pair of pneumococci, one coccus was stained violet and the other was stained pink (c; →). The proportion of gram-negative pneumococci in number was increased 1 h after the completion (d,e). Some pneumococci became smaller in size1 after the completion of 2 g of ABPC administration than those seen before the therapy in case 2 (f). When CTX was administered a little elongated cocci was seen in case 5 (g). *S. pneumoniae*-strain (the MIC against PCG of the strain was 0.06μg/mL) cultured in the presence of 4 μg/ml of ABPC for 4 hours were stained as gram-negative (i) compared with one cultured without the agent (h).
→; arrow shows one violet-stained coccus and one pink-stained coccus

We investigated whether the change in staining patterns of *S. pneumoniae* was related to the presence of beta-lactam antimicrobial agents. Clinically isolated *S. pneumoniae*-strain (the MIC of benzylpenicillin (PCG) against the strain was 0.06μg/mL) was cultured in the absence (Fig. 5h) of or in the presence of 4 μg/ml of ABPC (Fig. 5i) for 4 hours and the morphology was compared. In the presence of 4μg/ml of ABPC, most of the bacteria were stained weakly gram positive or gram negative (Fig. 5i), suggesting that the change in staining patterns was due to the influence of antimicrobial activity. When clinically isolated PRSP strain (the MIC of PCG against the strains was 2μg/ml) cultured in the presence of 4 μg/ml of ABPC for 4 hours was used, most of the bacteria were also stained weekly gram positive or gram negative (data not shown).

In gram staining the first step is the use of crystal

violet dye and the next step involves using iodine to form crystal violet- iodine complex to prevent easy removal of dye. Subsequently, a decolorizer, often solvent of ethanol and acetone, is used to remove the dye. The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. Gram-positive microorganisms have bacterial cell wall higher peptidoglycan content, whereas gram-negative organisms have higher lipid content.[1] Peptidoglycan or murein is a vital constituent of the bacterial cell wall that provides mechanical stability to it. It is an extremely conserved constituent of both the gram- positive and gram-negative envelopes. Nevertheless, peptidoglycan is a thick structure in gram-positive bacteria (≥10 layers), while it is thin (one or two layers) in gram-negative ones. The beta-lactam antibiotics inhibit the last step in peptidoglycan synthesis by acylating the transpeptidase involved in cross-linking peptides to form peptidoglycan.[10] These morphological changes of pneumococci were considered to reflect the effectiveness of ABPC. The reason for the loss of staining ability in the sputum is supposed to be by reducing in peptidoglycan synthesis induced by distributed antimicrobial agents in the sputa, which would lead to a thinner cell wall, may make the bacteria more susceptible to be decolorized by alcohol, which will also make the bacteria more susceptible to attack of action of host defense-system, including neutrophil phagocytosis.

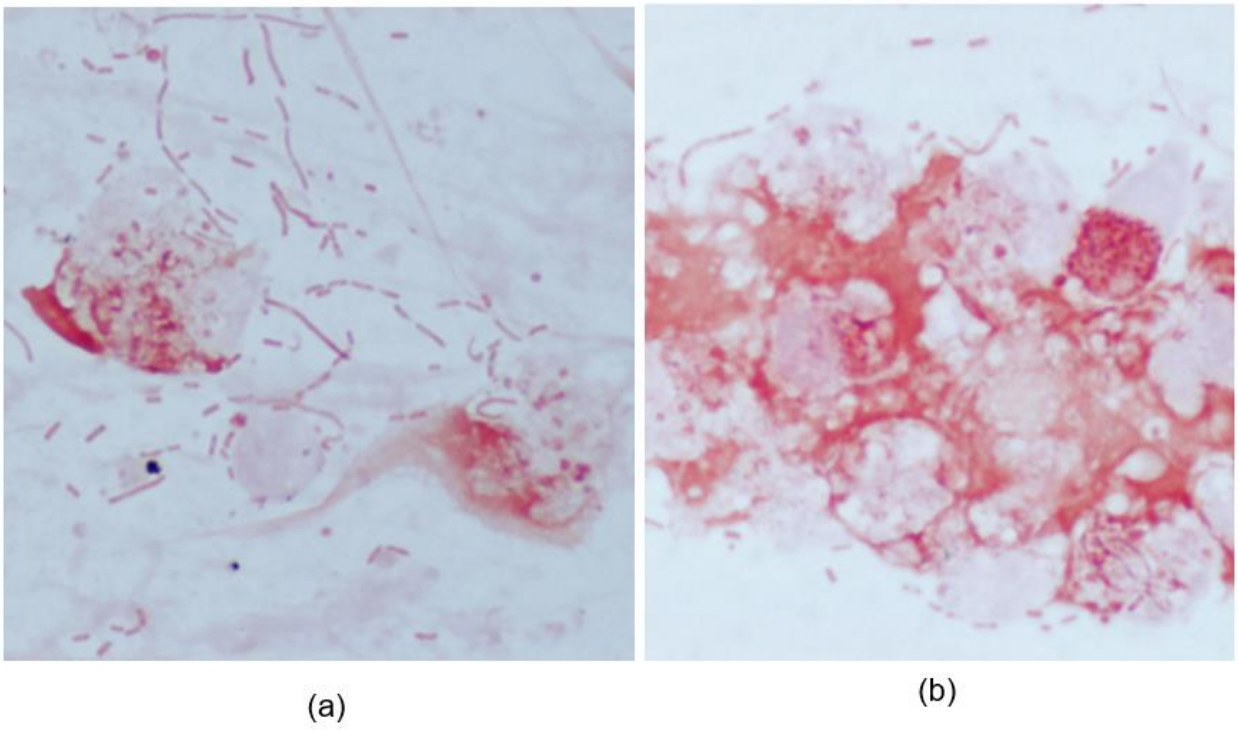

Figure 6. Gram staining of sputum samples (X 1000).
The sputum an old sister from the Philippine who was transferred to our hospital after ineffective CTX administration. The elongated gram-negative bacilli were seen(a). The neutrophils were severely destroyed(b). She was administered 0.5μg of imipenem/cilastatin sodium, but 10 h later she died. *Klebsiella pneumoniae* was cultured from her sputum.

3.A marker for effectiveness of antimicrobial agent in bacterial pneumonia

As the effective marker of administered antimicrobial agent in bacterial pneumonia or bronchitis we prospectively compared the WBC and CRP levels and the decrease in the bacterial density in gram-stained sputa in which the administered antimicrobial agents were effective. We checked ten people with respiratory infection admitted to the hospital of Teikyo-University School of Medicine (eight pneumonia patients and two bronchitis patients; from31 to 95-year-old (median age was 69)). Causative bacterial organisms cultured from the sputa were six strains of *Streptococcus pneumoniae* (five strains were PRSP, one strain was PSSP), five strains of *Haemophilus influenzae* (one was BLNAR) and three strains of *Moraxella catarrhalis*. The administered antimicrobial agents included ABPC (n=2), PIPC (n=2), CTX (n=2), cefepim+gentamicin (n=2), meropenem (n=2).

The median duration for the WBC to decrease to within the normal range (n=7) was 96 hours (range, 24 to 96 hours). The median duration for the maximal level of CRP to decrease to half of the prior maximal level (n=10) was 96 hours (range, 48

to 144 hours). These data showed that at least 2 to 4 days were needed to evaluate the effectiveness of the antimicrobial agent when the WBC or CRP level was used as a therapeutic parameter. In gram staining of sputa (n=10), we determined the antimicrobial agents being effective when a significant decrease in the bacterial density or almost no bacteria were seen in about ten fields of microscope (X1000) (Fig.2). The median duration needed to determine the effectiveness of the agent was 6.5 h (range, 1 to 12 hours), which showed that a prompt decrease in the number of microorganisms in Gram-stained sputum after the first administration of antimicrobial agents can be used as the quickest therapeutic marker in treating bacterial respiratory infections.

Conclusions and future perspectives

In managing respiratory tract bacterial infections, we need not to wait several days or till the next medical examination in checking the effectiveness of the first administered antimicrobial agents. Decrease in bacterial density in the sputum obtained several-hours after or before the second administration of antimicrobial agents is a definitive marker showing the effectiveness of the antimicrobial agents. In pneumococcal pneumonia, we can expect the effectiveness at least 1 h after completion of the first administration of the agent, when a loss of gram-positive-staining of pneumococci with a decrease in the number of extracellular cocci is found. The changing patterns of Gram-stained sputa can be used as the quickest therapeutic indicator of the effectiveness of antimicrobial agents in bacterial respiratory infections earlier than WBC and CRP levels.

As for markers of CAP, some scores, including the Pneumonia Severity Index (PSI) and CURB-65, are useful predictors of treatment failure and clinical stability in patients with CAP, but they don't include biomarker surrogates of host immune response which has been demonstrated that immune dysregulation correlates with a poorer prognosis.[11] Procalcitonin [12,13] and CRP remain the most widely used biomarkers. However, none of them appear to be ideal, and the search for novel biomarkers that will most sufficiently predict the severity and treatment response in pneumonia has lately intensified.[1] Savvateeva EN et.al. showed, because CAP is a rapidly developing disease, that dynamic observation of the changes in the biomarker levels is of particular interest. The biomarker signature is only a fixed representation, a photograph, of the captured state; the results of subsequent analyses, even throughout a single day, can vary substantially. Clearly, the dynamic monitoring of changes in biomarker levels can be a useful auxiliary tool for the prompt selection of individual therapies for CAP.[13] Zhou B et.al. established the biomarker function of serum metabolites for the diagnosis and evaluation of young patients with CAP and suggested that global metabolomics provides potential circulatory markers for diagnosing, evaluating and treating young adults with CAP.[14] Sputum has various information in respiratory track.[15,16] Nagaoka successfully tried to uncover some physicochemical properties of sputum in Japan.[17] Merit gained from using Gram-stained sputum as a therapeutic marker is that it can be gained easily and used at any time for subsequent analyses. We should pay more attention not only to pathogens but also to neutrophils and biomarkers in the sputum.

Figure 6 showed the sputum from an old sister from the Philippines who was transferred to our hospital from another clinic after ineffective CTX administration (data not shown). The elongated gram-negative bacilli were seen. The neutrophils were severely destroyed. She was administered 0.5g of imipenem/cilastatin sodium, but 10 h later she died. *Klebsiella pneumoniae* was cultured from her sputum. The destroyed neutrophils showed the severity of the CAP. Morphological patterns of neutrophils in sputum may show the extent of severity of the inflammation in CAP. Sputum biochemical analysis will open new aspects in understanding of bacterial pneumonia. In the future, analysis of biomarkers in the sputum and its comparison with serum biomarkers by dynamically monitoring changes in the biomarker levels will bring us useful information to successfully control in CAP.

We had been made a limitation for the ability of Gram staining only to the initial diagnostic test for the evaluation of bacterial infections. However, we have shown by using the focus chaining technique that Gram staining is useful as an initial diagnostic clue for tuberculosis[18-20] And now, we have shown that Gram-staining of sputum is a useful effective tool in checking the effectiveness of antimicrobial agents in bacterial pneumonia and bronchitis. Further studies are needed to confirm whether gram staining is available as a monitoring tool in following other bacterial pneumonias and when other antimicrobial agents are used.

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