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# An Assessment by *In Situ* Hybridization Method for Pathogens of Severe Respiratory Infection

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It has been shown that the *in situ* hybridization (ISH) method for the detection of phagocyted bacteria in polymorphonuclear neutrophils is more sensitive than conventional blood culture in patients with sepsis. The present study was designed to further evaluate the clinical utility of the ISH method, using a commercially available kit (Hybrizep®), for detecting pathogens in patients with severe respiratory infections. Peripheral blood was taken from patients with severe respiratory infections for both routine blood culture and the ISH method, respectively. In addition, focal samples including sputum, bronchoalveolar lavage, central and thoracic catheter, etc. were simultaneously examined for bacterial culture. A total number of 22 specimens was examined. The positive cases by ISH were 50.0 % in the respiratory infections, which were significantly higher than those in blood cultures (9.1 %). However, identical pathogens in the ISH method were not isolated from blood and/or other sources in respiratory sites.

These findings suggest a possibility of high frequency of bacteremia or multifactorious pathogens in severe respiratory infections. The ISH method may provide additional information on serious respiratory infections for the detection of bacteremia. However, the clinical utility by the ISH method for patients with severe respiratory infection remains undetermined. *Shinshu Med J 59 : 223—228, 2011*

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## I Introduction

Recently, several biological techniques have been developed for the detection of bacterial pathogens. Neutrophils ingest bacteria at an early stage of bacteremia and retain some surviving bacteria in the intracellular sites[1)2)]. Matsuhisa et al,[2)] developed a method of identifying causative pathogens in neutrophil blood smear by *in-situ* hybridization (ISH) using biotinylated DNA probes which was specific for genomic DNA of pathogens. Subsequently, Shimada et al,[3)] reported that the ISH analysis of genes of pathogens surviving in neutrophil smears increased the accuracy of bacterial identification compared with that by conventional blood cultures in patients with sepsis. Several clinical studies using ISH were conducted and showed that the sensitivity of ISH in the diagnosis of bacteremia was approximately four times higher than that of conventional blood culture in sepsis[3)4)].

The ISH probes can detect five microorganisms' DNA, but do not include a specific probe for *Streptococcus pneumoniae*. *Streptococcus pneumoniae* is a common pathogen in respiratory infection, espe-

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cially in community-acquired pneumonia[5)-7)]. Theoretically, it is speculated that the ISH method is less useful for etiologic diagnosis in respiratory infections. However, numerous studies have emphasized polymicrobial infections in the etiology of hospital-acquired pneumonia or severe respiratory infections[6)8)-10)]. It has been suggested that blood culture is an optional tool to isolate an etiologic pathogen in pulmonary infection[11)12)], but, the positive rate for blood culture is less than 20 % in subjects with respiratory infections in several studies[8)-16)].

Our hypothesis was that bacteremia might be more frequent than suggested by the clinical evidence in respiratory infection, especially in patients with hospital-acquired pneumonia. Thus, the study was conducted to evaluate the performance of the ISH method in patients with respiratory infections and its substantial role in defining the etiology. We prospectively analyzed the results obtained with the ISH method in patients with severe respiratory infection and compared them with results concomitantly obtained by the conventional techniques including blood and other cultures from pulmonary sites.

## II Method and Subjects

The present study was performed from December 2003 to March 2005 at Shinshu University Hospital. Patients who required hospital admission because of community-acquired pneumonia or who developed pneumonia during hospitalization in the critical care unit or respiratory center in Shinshu University were eligible for this study. The diagnosis of respiratory infection was based on the detection of a new, persistent pulmonary infiltrate and on the presence of at least two of the following clinical criteria : 1) fever or hypothermia (temperature $>38$ or $<35.5$ ℃) ; 2) leukopenia or leukocytosis (white blood cells $\leq 4{,}000/\mu$l or $\geq 12{,}000/\mu$l) ; or 3) purulent respiratory secretions. The clinical diagnosis of hospital-acquired pneumonia was made based on the criteria of the Japanese Respiratory Society Guidelines[17)]. The diagnosis of pleuritis or pyothorax was made by the presence of pleural effusion and the analysis. Written informed consent was obtained from each patient prior to the study. Samples for ISH and blood cultures were taken simultaneously in each patient. The blood samples were obtained by arterial or venous puncture for routine blood culture and ISH methods. The samples for general culture were incubated in aerobic and anaerobic culture bottles, respectively, at 37 ℃ and then checked daily. The other samples from parts suspected of harboring infected foci including sputum, bronchial alveolar fluid (BAL), pleural effusion, catheter, etc. were collected on the same day. Thoracocentesis for the sampling of pleural fluid was also performed when applicable. Its results were compared with each of the specimens.

Intracellular bacteria from blood samples were tested by the ISH method using a commercially available kit (Hybrisep kit®, Fuso Pharmaceuticals Industries, Osaka, Japan). The ISH method was performed based on the manufacturer's protocol, which had been reported previously[2),3)]. Briefly, heparinized blood samples (5 ml) were fixed, permeabilized and incubated with digoxygenin (Dig)-labeled DNA hybridization probes. The process involves the extraction of the DNAs of the targeted bacterium from the white blood corpuscles in the blood and fixing them on slide glass. The process was performed at Shinshu University Hospital. Next, the second treatment-stage consists of hybridization with a specific DNA probe to target the bacterial genome, using color, and observation under the microscope. After washing, anti-Dig antibody labeled with alkaline phosphatase (ALP) was used to detect the Dig-labelled hybridized signals. To visualize the signals, color development was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate for ALP. The identification of color reaction by intracellular signals was done by light microscopic examination ($\times 1{,}000$). The detection and/or identification of the bacterial genes were carried out at Miroku Medical Laboratory. The ISH probes in Hybrisep were specific for microorganisms such as *Staphylococcus aureus, (SA) Sta-*

*phylococcus epidermidis (SE), Pseudomonas aeruginosa (PA), Enterococcus faecalis (EF),* and a group of *colibacillus (EK) (Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae).*

Statistics

The continuous variables were compared using the Mann-Whitney U-test and categorical variables were contrasted by the Chi-squared test or Fisher's exact test. The level of significance was set at less than 5 %.

## Ⅲ Results

A total of 26 subjects were enrolled in the present study. Among them, 4 patients were excluded because of subsequent definite diagnosis of pneumonia due to influenza viral infection and diffuse pulmonary hemorrhage. The characteristics of the patients and the positive rate in blood culture and ISH are summarized in Table 1. The mean age of the enrolled patients was 60.0 years and 8 patients were pre-treated with antibiotics at the study. Mean peripheral blood white cell counts and C-reactive protein (CRP) in patients both pre-treated and untreated with antibiotics are shown in Table 1. There were no significant differences in these values between the groups. Two patients were positive for blood culture, while half of the enrolled patients were positive by the ISH method. Thus, the positive rate by ISH was significantly higher than that in blood cultures (9.1 %). In addition, the positive rate by the ISH method was identical in patients both pre-treated and untreated with antibiotics, indicating independence of the use of antibiotics.

The corresponding findings based on ISH and subculture tests for each case are shown in Table 2. Case numbers 1 to 7 were community-acquired pneumonia who required admission, and the others were hospital-acquired pneumonia. In cases of community-acquired pneumonia, possible causative pathogens were obtained in sputum cultures in 5 cases, but were not detectable in the blood cultures of all patients. However, ISH revealed bacteremia in four cases of community-acquired pneumonia and the detectable pathogens were different from those observed in sputum. In cases of hospital-acquired pneumonia, there was a patient who reported positive for both blood culture and ISH signal, but the detectable pathogens were different (case 8). In addition, among the cases positive for ISH signals, the same pathogens were not detected in other sources including sputum, BAL, pleural effusion, or catheters. Thus, identical pathogens in the ISH methods were not isolated from blood and/or other

Table 1 Patient characteristics and positive rates of ISH and blood culture

| | Patients | | |
|---|---|---|---|
| Antibiotics | (+) n=8 | (−) n=14 | Total n=22 |
| Mean Age yr (range) | 55.9 (37−77) | 67.1 (19−87) | 60.0 (19−87) |
| WBC $\mu$l mean ± SD (range) | 17,200±9,800 (6040−35,790) | 8,940±4,200 (3,000−20,470) | 11,630±7,300 (3,000−35,790) |
| CRP mg/dl mean ± SD (range) | 13.6±9.3 (4.1−30.4) | 10.3±7.9 (2.2−24.9) | 12.0±8.8 (2.2−30.4) |
| Blood culture Positive case (%) | 1 (12.5 %) | 1 (7.1 %) | 2 (9.1 %) |
| ISH Positive case (%) | 4 (50.0 %) | 7 (50.0 %) | 11 (50.0 %)* |

* $<0.05$ vs blood culture

Patient characteristics and positive rates in blood culture and the ISH method.
ISH ; *In situ* hybridization.

Table 2 Positive pathogen results for each patient with respiratory infection

| case | Underlying disease or complications | In situ Hybridization | | | | | Blood culture | Sputum culture | Others cultures | Antibiotics |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SA | SE | PA | EF | EK | | | | |
| 1 | | − | + | − | − | − | (−) | SA | N.D | (−) |
| 2 | Bronchial asthma, Heart failure | − | − | − | − | + | (−) | Streptococcus pneumoniae | N.D | (−) |
| 3 | | + | − | − | − | − | (−) | (−) | N.D | (−) |
| 4 | Pleurisy | − | − | − | − | + | (−) | N.D | pleural effusion (−) | (−) |
| 5 | | − | − | − | − | − | (−) | KP | N.D | (−) |
| 6 | | − | − | − | − | − | (−) | SA | N.D | (−) |
| 7 | | − | − | − | − | − | (−) | HI | N.D | (−) |
| 8 | Lung cancer | − | − | − | − | + | SE | N.D | N.D | (+) |
| 9 | ARDS | − | − | − | − | − | SE | (−) | bronchoalveolar lavage : (−) | (−) |
| 10 | Hodgkin's disease, ARDS | − | − | − | − | + | (−) | SA, Candida albicans | N.D | (+) |
| 11 | MDS myelofibrosis | − | − | − | − | + | (−) | SE | N.D | (−) |
| 12 | COPD | − | − | − | − | + | (−) | (−) | pharynx : Streptococcus Group G | (−) |
| 13 | Lung cancer | − | − | − | − | + | (−) | N.D | | (+) |
| 14 | Pneumothorax | − | − | − | − | + | (−) | N.D | thoracostomy tube : MRSA, pleural effusion : SA | (−) |
| 15 | Overlap syndrome, interstitial pneumonia | − | − | − | − | + | (−) | (−) | N.D | (+) |
| 16 | Diffuse panbronchiolitis, Pulmonary hypertension | − | − | − | − | − | (−) | SA, PA | N.D | (+) |
| 17 | Interstitial pneumonia, Dermatomyositis | − | − | − | − | − | (−) | KP | bronchoalveolar lavage : (−) | (−) |
| 18 | COPD | − | − | − | − | − | (−) | MRSA | N.D | (−) |
| 19 | Diffuse panbronchiolitis | − | − | − | − | − | (−) | PA | N.D | (+) |
| 20 | Lung cancer, Cancerous pleurisy | − | − | − | − | − | (−) | N.D | pleural effusion (−) | (−) |
| 21 | Interstitial pneumonia | − | − | − | − | − | (−) | (−) | N.D | (+) |
| 22 | Aspiration pneumonia, DIC, Acute renal failure | − | − | − | − | − | (−) | (−) | CV catheter (−) | (+) |

Abbreviations ; ARDS ; acute respiratory distress syndrome, DIC ; disseminated intravascular coagulation, SA ; *staphylococcus aureus,* SE ; *staphylococcus epidermidis,* PA ; *pseudomonas aeruginosa,* EF ; *entercoccus faecalis,* EK ; a group of *colibacillus* (*Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae (KP)*). MRSA ; methicillin resistant *Staphylococcus aureus,* EA ; *Enterobacter aerogenes,* HI ; *Haemophilus influenzae,* CV catheter ; central venous catheter, N.D ; not done.

sources in respiratory sites.

## Ⅳ Discussion

Shimada et al,[3] analyzed 292 cases with sepsis and found positive ISH signals in 123 cases (42.0 %), while only 32 cases (11 %) were positive by blood culture. Subsequently, Kubo et al, reported a 41.7 % positive ISH signal, compared with 11.7 % in blood culture in 60 patients with the criteria for sepsis[4]. Thus, the sensitivity of ISH in the diagnosis of bacteremia was approximately four times higher than that of conventional blood culture in these studies. In the present study, we applied the ISH method in patients with severe respiratory infections. We found an almost similar positive rate by the ISH method as those previously reported in sepsis[3][4]. The present study thus suggests a frequent coexistence of bacteremia in patients with hospital-

acquired pneumonia or community-acquired pneumonia requiring hospitalization. In addition, it has been shown that the ISH method is unrelated to the prior use of antibiotics, which is consistent with the present study. This is a remarkable advantage of the ISH method, compared with conventional blood culture.

Blood culture is an optional tool to isolate an etiologic pathogen in pulmonary infection[11)12)]. In general, the positive rates of isolating bacteremia by conventional blood cultures were 8-20 % in hospital-acquired pneumonia[9)13)-15)] and 6.6-13 % in community-acquired pneumonia admitted to the hospital[6)-8)16)]. Because of these low positive rates, the clinical usefulness of blood cultures for isolating the pathogen in both community- and hospital-acquired pneumonia remains questionable[6)-16)]. However, it has been suggested that positive blood culture is a predictive factor for the prognosis and severity in pulmonary infection[11)12)]. Thus, our data indicate that the ISH method, can at least detect a relatively higher incidence of bacteremia in patients with severe respiratory infection than that reported for conventional blood cultures.

However, it is still unclear whether the pathogen identified by the ISH method can be considered an exact etiologic or diagnostic indicator in patients with respiratory infections. We found that neither the identical pathogen in blood culture nor in other sites was detected among patients positive for ISH signals. Thus, the isolation of a microorganism using the ISH method does not always confirm the microorganism as the pathogen causing the respiratory infection. In other words, the multiple pathogens detected with the ISH method and other sites in patients with respiratory infections suggested the coexistence of polymicrobial pathogens in the enrolled subjects. On the whole, our data based on the ISH method indicate that bacteremia can be detected more frequently than by the routine clinical examinations and that multi-factorial infection can exist in patients with severe respiratory infections.

In the present study, *S. epidermidis* was isolated by blood culture in two patients. However, the isolation of *S. epidermidis* in blood culture may be a result of contamination, rather than infection[13)]. Indeed, the identical pathogen was not detected in the ISH method and other sources in respiratory sites. Thus, whether the pathogen is responsible for bacteremia or respiratory infection remains undetermined in these cases. Compared with conventional culture methods, the ISH method is considered less susceptible to contamination in the handling process[2)-4)]. In terms of sensitivity, the ISH method is a useful tool for detecting bacteremia.

Although the present result is preliminary, concomitant testing using the ISH method with subcultured samples from pulmonary sites might indicate the severity of the respiratory infection and/or alternative therapy. The sample size of the present study was too small to evaluate the clinical utility of the ISH method. Further case control studies focusing on the clinical outcomes including alternation of therapy are needed. In addition, bacterial isolation by ISH can methodologically be obtained in 8 hours, which is another superior merit as opposed to the conventional culture technique[2)-4)]. The clinical usefulness in the time reduction to the diagnosis was unable to be evaluated in the present study, because our results for ISH were obtained by two separated steps. However, we believe that the rapid and simultaneous identification of pathogenic bacteria in blood samples by the ISH method may improve the diagnosis and clinical management in certain types of respiratory infection, such as ventilator-associated pneumonia.

In summary, it can be stated that *in-situ* hybridization (Hybrisep®) is a highly reliable non-cultural method for the detection of bacteremia and that it can provide an alternative to the conventional culture techniques. However, the clinical utility of the findings obtained with the ISH method remains uncertain. Further clinical studies using the *in-situ* hybridization method might be warranted for the identification of etiologic pathogens and clinical outcomes in pulmonary infections.

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