Neutrophil extracellular traps in bronchial aspirates: a quantitative analysis

Shigeto Hamaguchi[1,6], Tomoya Hirose[2,6], Naoya Matsumoto[2], Yukihiro Akeda[3], Taro Irisawa[2], Masafumi Seki[1], Hideo Hosotsubo[2], Kouji Yamamoto[4], Osamu Tasaki[5], Kazunori Oishi[3], Takeshi Shimazu[2] and Kazunori Tomono[1]

Affiliations: [1]Division of Infection Control and Prevention, Osaka University Graduate School of Medicine, Osaka, [2]Dept of Traumatology and Acute Critical Medicine, Osaka University Graduate School of Medicine, Osaka, [3]International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, [4]Dept of Medical Innovation, Osaka University Hospital, Osaka, [5]Dept of Emergency Medicine, and Unit of Clinical Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. [6]Both authors contributed equally.

Correspondence: K. Tomono, Division of Infection Control and Prevention, Osaka University Graduate School of Medicine, 2-15 Yamadaoka, Suita City, Osaka 565-0871, Japan. E-mail: tomono@hp-infect.med.osaka-u.ac.jp

ABSTRACT Neutrophil extracellular traps (NETs) are structures composed of DNA and granular proteins, which rapidly trap and kill pathogens. The formation of NETs has been detected during infection in animal experiments, but their role in humans is unclear. The purposes of this study were to quantitatively evaluate the production of NETs during acute respiratory infection and to study the relationship between the NET length and various inflammatory mediators.

We examined bronchial aspirates collected from nine intubated patients in an intensive care unit. Samples were collected at the onset of acute respiratory infection (day 0) and on days 1, 3–5, and 6–8. The NET length was visualised by immunohistochemistry and quantified using computer tracing software.

The NET length was measured and compared at each time point. The length differed significantly between time points ($p<0.001$). NETs were significantly longer on day 1 than on day 0 ($p<0.001$). Neutrophils released NETs abundantly in response to respiratory infection and regression analysis showed that NET length correlated with six clinical parameters (white blood cells, platelets, lactate, CXC ligand-2, interleukin-8, and procalcitonin) as the explanatory variables.

NETs in bronchial aspirates may reflect disease progression of respiratory infections. Quantification of NETs in bronchial aspirates may provide a new indicator of inflammation.

@ERSpublications
NET length increases with respiratory inflammation which correlates with progression of infections
http://ow.ly/sPbEX

This article has supplementary material available from www.erj.ersjournals.com

Received: Aug 11 2013 | Accepted after revision: Dec 29 2013 | First published online: March 6 2014

Support statement: This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (No. 21390163 and No. 25293366) and ZENKYOREN (National Mutual Insurance Federation of Agricultural Cooperatives).

Conflict of interest: None declared.

Introduction

A patient admitted to an intensive care unit (ICU) sometimes acquires a critical infectious disease such as ventilator-associated pneumonia or sepsis [1]. To date, the therapeutic strategies for treating respiratory infection focus on early diagnosis [2]. To detect respiratory infection before the revelation of lung infiltration on a chest radiograph, Gram staining is performed at the bedside. In Gram staining of sputum, in addition to bacteria and neutrophils, numerous fibrous structures in the background are frequently observed. These structures are thought to be composed of fibrin. However, we reported that these structures might be neutrophil extracellular traps (NETs) [3].

Neutrophils provide a first line of defence in innate immunity [4]. The mechanisms used by neutrophils to eliminate microbes have historically been known to include phagocytosis, generation of reactive oxygen species (ROS), and degranulation [5]. In 2004, BRINKMANN *et al.* [6] reported a newly identified neutrophil activity, which they called NETs [6]. NETs are produced by activated neutrophils in response to a variety of pro-inflammatory stimuli including lipopolysaccharide, interleukin (IL)-8, tumour necrosis factor (TNF), and various bacteria and fungi [7–9]. High mobility group box 1 (HMGB-1) was also recently reported to promote the formation of NETs [10]. The main components of NETs are decondensed chromatin, histones, and antimicrobial proteins such as neutrophil elastase, myeloperoxidase, and LL37 [11]. Citrullinated histone H3 (Cit H3) is also a characteristic feature involved in NET formation *in vitro*. Citrullination of histone H3 by peptidylarginine deiminase 4 plays a pivotal role in chromatin decondensation during "NETosis" [7, 12]. Inhibition of peptidylarginine deiminase 4 prevents NET formation [13].

Recently, this novel phenomenon, NETosis, has been researched extensively. However, the significance of NET formation *in vivo* in clinical conditions is not understood fully. We published a preliminary report showing that bronchial aspirates from patients with acute respiratory infections contain abundant NETs [14].

The purposes of this study were to confirm that the fibrous structures in Gram-stained bronchial aspirates are composed of NETs, to measure changes in the length of NETs with time, to evaluate the citrullination of histone H3, and to determine if there is a relationship between the NET length and various inflammatory mediators in acute respiratory infection.

Methods

Sample collection

The outline of this study is described in the online supplementary material.

During the study period, from April 2011 to June 2011, bronchial aspirates samples were collected at the onset of acute respiratory infection (day 0), and then on days 1, 3–5, and 6–8. The collection of bronchial aspirates was performed by using an Argyle Suction Catheter with Mucus Trap (Covidien, Mansfield, MA, USA) over the endotracheal tube for deeper-lying sampling. The sample was promptly and gently applied to a glass slide. After drying, one sample immediately underwent Gram staining and the other samples were stored at -80°C until the immunohistochemical analysis could be performed.

Acute respiratory infection was diagnosed based on the new onset of purulent endotracheal secretion and the existence of bacterial phagocytosis revealed on a Gram stain of the bronchial aspirate.

Evaluation of clinical background and severity of illness

Age, sex, body temperature, acute physiological and chronic health evaluation (APACHE II) score, and sequential organ failure assessment (SOFA) score were recorded at the time of admission. Blood samples were collected at each time point when the bronchial aspirates samples were collected and were analysed for laboratory data. The following parameters were measured: white blood cell (WBC) count, platelet count, the concentration of C-reactive protein (CRP), D-dimer, lactate, procalcitonin (PCT), TNF-α, IL-6, IL-8, CXC ligand (CXCL)2, HMGB-1, and E-selectin (refer to online supplementary methods).

Identification of NETs

To identify NETs, we used immunostaining to visualise the major NET components: DNA, neutrophil elastase, and histone H3. Cit H3 was also evaluated as a predictor of NET formation by immunohistochemistry (refer to online supplementary methods). Gram staining was conducted using a standard method.

Quantification of NETs

NET length was quantified using Neurolucida (MBF Bioscience, Williston, VT, USA), software for neuron mapping. NETs were depicted graphically as white lines (fig. 1b). The neutrophil cell bodies (yellow, fig. 1b) were excluded from the calculation of NET length. NET length was defined as the mean length of

 DOI: 10.1183/09031936.00139813

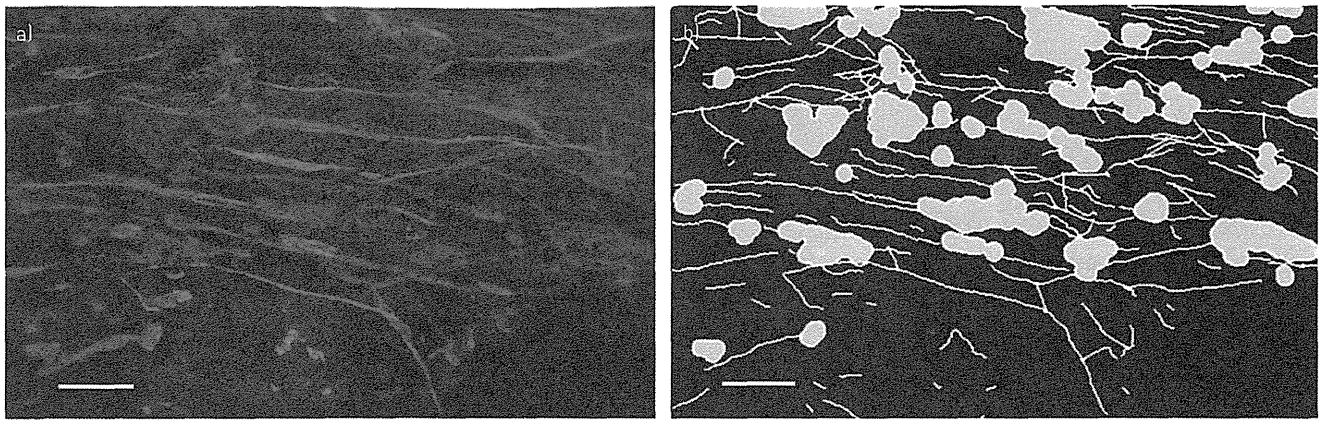

FIGURE 1 Representative adapted images of neutrophil extracellular traps (NETs). a) Analysed using microscopy or b) acquired using Neurolucida (MBF Bioscience, Williston, VT, USA), computer software for tracing neural networks. To calculate the length of NETs, all components merged in a fluorescent picture were traced by the software and each aggregate was excluded from calculation as an actual cell body. Yellow circle: cell body; white line: NETs. Scale bars=30 μm.

NETs calculated by dividing the total length of the white line in the graphical depiction by the total number of white lines.

Statistical analysis

Continuous variables are presented as median and interquartile range (IQR). Fisher's exact test and t-test were used to identify differences between time points in the study group. Single- and multiple-regression analyses were used to identify associations between NET length and biological parameters. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA) for regression analyses and Prism software (version 5.02; GraphPad Software, San Diego, CA, USA).

Results

Identification of NETs in Gram-stained bronchial aspirates

To clarify whether the fibrous structures in bronchial aspirates are indeed NETs, we evaluated purulent bronchial aspirates using both Gram stain and immunostain on the same slide. As shown in figure 2, almost all fibres detected in Gram staining also stained simultaneously with 4,6-diamidino-2-phenylindole (DAPI) and neutrophil elastase. This result indicates that most of these fibrous structures observed using Gram stain were NETs.

Characteristics of the study participants

In this study, we evaluated the production of NETs in bronchial aspirates at each time point based on our protocol. During the study period, 263 patients were admitted to the ICU, and 49 of the 263 patients were intubated. 40 patients were excluded according to the exclusion criteria, details of which are given in the online supplementary material. The final number of patients included in the study was nine.

The characteristics of the patients are shown in table 1. The study group comprised of six males and three females with a median age of 63.0 years (IQR, 52.0–75.0 years). The major diagnoses for hospitalisation were trauma (n=2), resuscitated cardiopulmonary arrest (n=3), peritonitis (n=1), gas gangrene (n=1), bacterial meningitis (n=1), and pneumonia (n=1). At the time of admission, the median body temperature was 37.6°C (IQR 36.8–38.2°C), the median SOFA score was 7.0 (IQR 4.0–10.0), and the median APACHE II score was 21.0 (IQR 21.0–25.0). Seven out of the nine patients survived; two patients died during their ICU stay.

Variation in the appearance of NETs over time

To detect the NETs in the bronchial aspirates, DNA and histone H3 were visualised simultaneously using immunostaining. NETs were recognised as the merged fibrous structures using these components (fig. 3a).

Previously, we reported that the appearance of NETs in bronchial aspirates increased gradually with the duration and severity of infection and decreased with improvement in the patient's condition [14]. Likewise, the immunohistochemical images of a representative case are shown in figure 3. Only a few NETs were identified in the sample on day 0, the onset of acute respiratory infection. On day 1, we observed infiltration of neutrophils and abundant expanse of NETs. After treatment with appropriate antibiotics, the

DOI: 10.1183/09031936.00139813

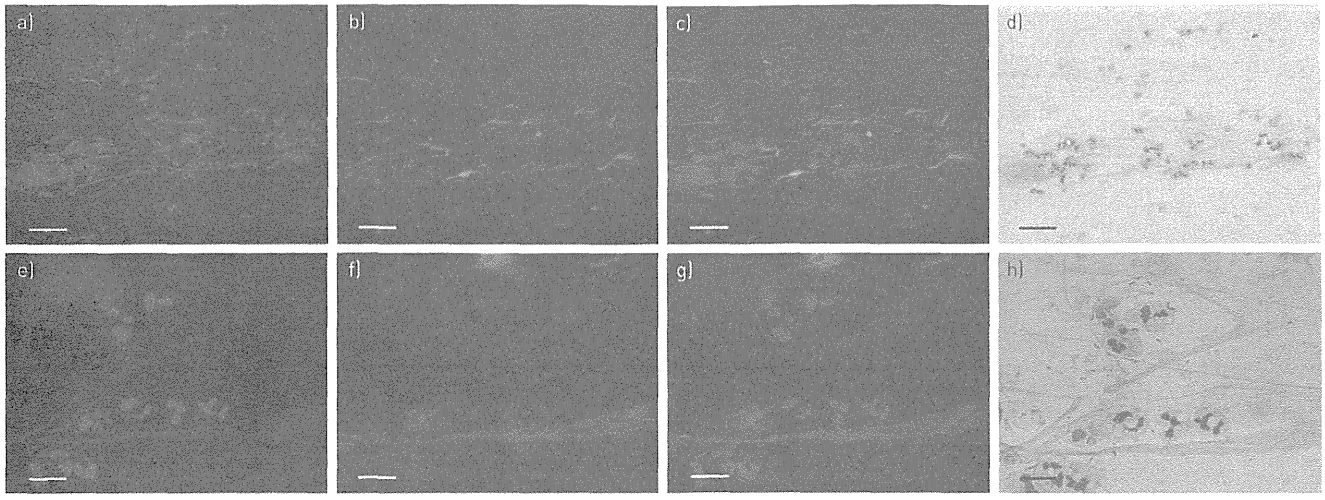

FIGURE 2 Representative images of immunostaining (a–c and e–g) and Gram staining (red, d and h) of bronchial aspirates in the same sample. Neutrophil extracellular traps were detected as co-localised fibre with 4,6-diamidino-2-phenylindole (blue, a and e) and neutrophil elastase (green, b and f) by immunohistochemistry; they are identical to the fibrous structures delineated using the Gram stain. Images c) and g) are merged images of a) and b), and e) and f), respectively. a–d) Scale bars=50 μm and e–f) scale bars=10 μm.

respiratory infection improved gradually. In association with the recovery, the NET length shortened, and the numbers of NETs decreased gradually by days 5 and 7.

Citrullination of histone H3 before NET formation

We observed Cit H3 simultaneously to identify the preliminary phase of NET formation. A representative case is shown in figure 3. Cit H3 was detected in the nucleus of a subset of neutrophils on day 0 even though these neutrophils did not extrude fibrous NETs (white arrowhead in fig. 3). On day 1, the detected area of Cit H3 spread extensively inside the nucleus in some cells, and Cit H3 was detected in parts of the released NETs (white arrow in fig. 3). In general, neutrophils and NETs containing Cit H3 were rarely observed on day 5 or later (fig. 3).

Quantification of NETs by length

The NET length was measured using the Neurolucida software program to quantify the change in the NET length. The mean NET lengths at each time point (days 0, 1, 3–5, and 6–8) were compared. Significant changes were identified during the clinical course by analysis of variance for repeated measures ($p<0.001$; fig. 4). Tukey's *post hoc* multiple comparison test showed that the NET length was significantly longer on day 1 than on day 0 ($p<0.001$) and that the NET length was significantly shorter on days 6–8 than on day 1 ($p=0.0132$).

To confirm the identification of the filamentous structure as NETs, we further analysed how the length of this structure changed over time and how it degrades with DNase I in live samples with SYTOX orange (Invitrogen, Carlsbad, CA, USA), a cell membrane impermeant nucleic acid stain, by time-lapse imaging

TABLE 1 Characteristics of study participants

Patient	Age years	Sex	Condition	Body temperature °C	SOFA score	APACHE II score	Survived
1	63	Male	Trauma	38.2	2	6	Yes
2	82	Male	Cardiac arrest	37	7	21	Yes
3	52	Female	Trauma	38.9	9	21	Yes
4	41	Female	Meningitis	38.1	4	6	Yes
5	10	Male	Cardiac arrest	36.3	11	28	Yes
6	78	Female	Pneumonia	36.4	4	22	Yes
7	73	Male	Gas gangrene	36.8	10	25	Yes
8	75	Male	Peritonitis	38.3	5	27	No
9	56	Male	Cardiac arrest	37.6	10	21	No

SOFA: sequential organ failure assessment; APACHE II: acute physiological and chronic health evaluation.

DOI: 10.1183/09031936.00139813

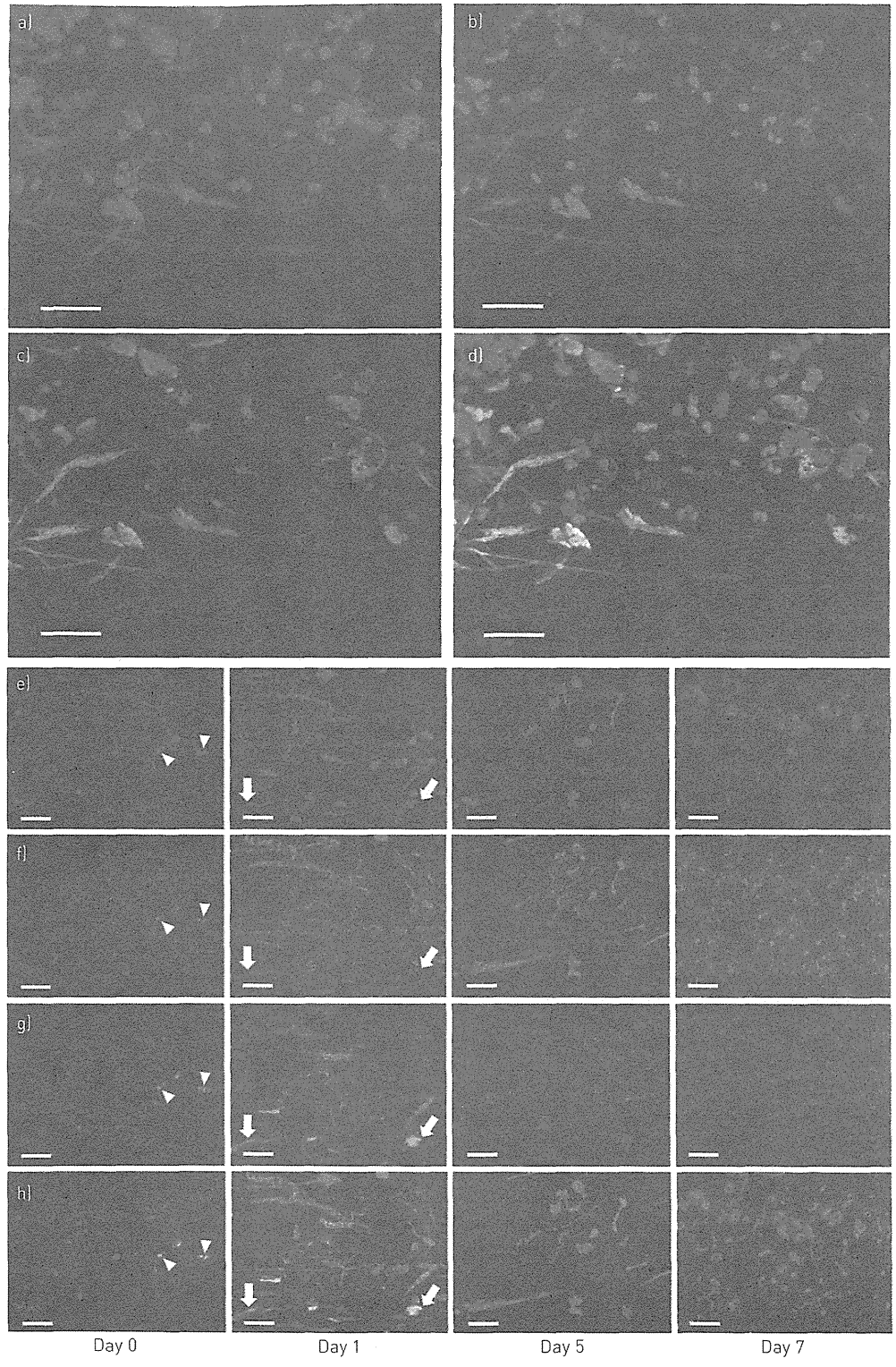

FIGURE 3 Representative time course images for the immunohistochemical staining of bronchial aspirates in the same patient. Single stain images for a) 4,6-diamidino-2-phenylindole (DAPI), b) histone H3, c) citrullinated histone H3 (Cit H3), and d) their merged image. Neutrophil extracellular traps (NETs) are recognised as fibrous structures with these components. e–h) Representative images of time-dependent changes using e) DAPI f) histone H3, and g) Cit H3 to show appearance of NETs related to disease progression of respiratory infection from day 0 to day 7. h) The merged image of e–g for each day. White arrowhead: Cit H3 detected in the nucleus; white arrow: Cit H3 on NETs. Scale bars=30 μm.

DOI: 10.1183/09031936.00139813

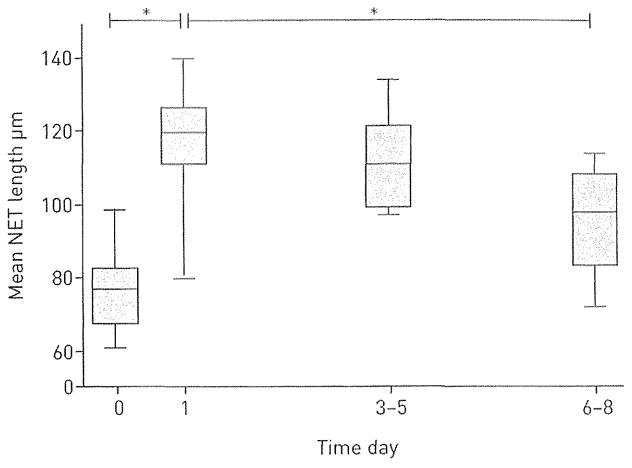

FIGURE 4 Time-dependent changes in mean neutrophil extracellular trap (NET) length in bronchial aspirates evaluated by Neurolucida software (MBF Bioscience, Williston, VT, USA). The changes in mean NET length during the clinical course of infection between day 0 and day 1, and between day 1 and day 6–8 were significant. *: $p<0.05$, ANOVA for repeated measure was $p<0.001$.

over a 0.5-h period. As a result, filamentous NETs were gradually stretched along with the stream of bronchial aspirate (online supplementary video 1, arrow head). Furthermore, compared with control (online supplementary video 2), treatment of bronchial aspirates with DNase I results in the gradual shortening and melting of the filamentous NETs (online supplementary video 3).

The relationships between NET length and clinical parameters

To evaluate the associations between NET length and systemic inflammation, we analysed the relationships between the mean NET length and clinical parameters and inflammatory cytokine levels in serum. The data for each time point are shown in table 2. The relationships between NET length and the following parameters were assessed: body temperature, SOFA score, APACHE II score, WBC and platelet count, blood concentration of lactate, and serum concentration of C-reactive protein, D-dimer, PCT, tumour necrosis factor-α, IL-6, IL-8, CXCL-2, HMGB-1, and E-selectin.

We investigated these associations by multiple regression analysis to confirm whether NET length could be predicted by present or previous clinical parameters. A multiple regression model was constructed with the NET length as the dependent variable and clinical data as independent variables. The multiple regression analysis demonstrated that the regression model with dependent and independent variables on day 1 was only meaningful because the F-tests for the model rejected the hypothesis of joint nonsignificance of the independent variables. Scatter plots of all clinical parameters and NET length on day 1 are shown (fig. 5). In

TABLE 2 Clinical parameters, serum inflammatory cytokine concentrations, and neutrophil extracellular traps (NETs) length in bronchial aspirates at each time point

Parameter	Day			
	0	1	3–5	6–8
WBC per μL	13050 (9980–17880)	10560 (8060–21130)	8630 (7590–10450)	10340 (7490–11900)
PLT $\times 10^3$ per μL	147 (123–191)	137 (97–149)	142 (95–219)	211 (136–218)
CRP mg·dL^{-1}	1.8 (0.4–21.6)	8.6 (4.7–22.3)	4 (2.8–12.6)	6.3 (2.3–7.2)
D-dimer ng·mL^{-1}	4 (2.2–22.7)	3.7 (1.4–15.0)	8.2 (4.3–13.6)	6.4 (2.8–8.6)
Lactate mg·dL^{-1}	39 (17–57)	21 (9–26)	11 (9–15)	13.5 (7.8–18.5)
PCT ng·mL^{-1}	0.7 (0.4–9.2)	3.6 (1.8–16.4)	2.2 (0.7–3.0)	0.8 (0.1–1.1)
TNF-α pg·mL^{-1}	17.3 (9.2–28.0)	9.4 (7.4–17.9)	10.9 (8.5–16.3)	10 (7.7–14.8)
IL-6 pg·mL^{-1}	184 (85–910)	82.8 (34.9–710.5)	113.3 (22.0–288.3)	82.7 (11.1–203.0)
IL-8 pg·mL^{-1}	118 (51.7–229.0)	41.6 (24.5–151.8)	47.6 (32.5–81.4)	43.3 (22.4–172.7)
CXCL-2 pg·mL^{-1}	25.8 (11.0–36.1)	19.2 (10.7–47.3)	13.9 (10.1–30.3)	12.7 (10.5–28.6)
HMGB-1 ng·mL^{-1}	10.1 (7.3–11.5)	7.1 (4.6–8.5)	7.1 (6.0–8.7)	7.7 (5.7–10.5)
E-selectin ng·mL^{-1}	105 (57–286)	148 (88.6–267.8)	124 (80.4–217.3)	96.9 (86.3–106.5)
NETs mean length μm	76.5 (70.9–78.1)	119.3 (113.8–122.6)	111.2 (98.5–119.3)	97.8 (86.3–106.5)

Data are presented as median (interquartile range). WBC: white blood cells; PLT: platelet; CRP: C-reactive protein; PCT: procalcitonin; TNF: tumour necrosis factor; IL: interleukin; CXCL: CXC ligand; HMBG-1: high-mobility group box-1.

DOI: 10.1183/09031936.00139813

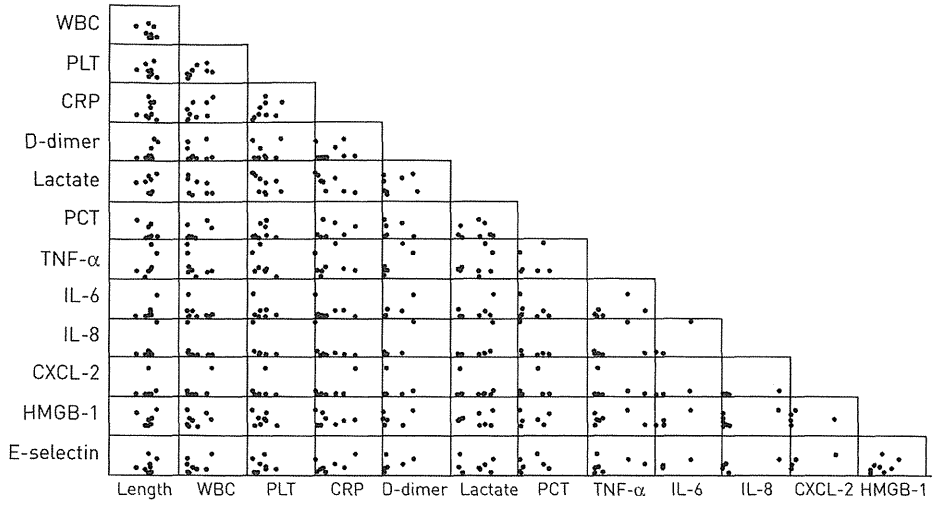

FIGURE 5 Scatter plots of each clinical parameter and mean neutrophil extracellular trap (NET) length as independent variables on day 1. None of these independent variables showed a significant difference. WBC: white blood cells; PLT: platelet; CRP: C-reactive protein; PCT: procalcitonin; TNF: tumour necrosis factor; IL; interleukin; CXCL: CXC ligand; HMBG-1: high-mobility group box-1.

the regression model, six parameters (WBC and platelet counts, and concentrations of CXCL-2, IL-8, lactate, and PCT) were selected using a stepwise method (table 3). NET length on day 1 correlated with these six inflammatory markers on day 1. The estimated regression model with these six parameters was as follows: mean NET length = $-1.23\times$WBC count $+ 0.88\times$CXCL-2 $+ 0.44\times$IL-8 $- 0.29\times$lactate $+ 0.18\times$platelet count $- 0.06\times$PCT.

Discussion

The Gram stain is widely used in clinical-infection diagnostics because of its simplicity. In the ICU of our hospital, Gram staining is performed on a routine basis at bedside. Through this experience, we noticed the presence of numerous fibrous structures in the bronchial aspirate samples from patients with acute respiratory infection and that the amount of fibres fluctuated over time. We previously demonstrated for the first time that NETs could be detected immunohistochemically even after Gram staining in the same smear sample [3]. In this study, we used the same method to demonstrate definitively that these fibrous structures seen in Gram staining are composed of NETs (fig. 2). This observation suggests that NETs can be identified using Gram staining alone without the need for immunostaining. This finding will be of great value to the clinician because it means that, along with the identification of type and propagation status of bacteria, evaluation of NET appearance on Gram-stained samples alone can provide information about the biological response to infection without the need for time-consuming procedures.

The quantitative analysis of NET length showed that NET length in bronchial aspirates changed dynamically during the course of respiratory infection. Neutrophils released abundant NETs into bronchial aspirates in response to acute respiratory infections, after which the amount of NETs decreased and the fibres became fragmented as the infection subsided (fig. 3).

Although the mechanism of varying NET length is uncertain, it is known that DNase I exists and has some function in sputum [15]. Our data suggested that exposure to DNase I mainly results in shortening of NETs (online supplementary video 3). It is possible that nascent production of NETs from neutrophils mainly results in extension of existing NETs. A flow of bronchial secretion in the airway might play a part in increasing NET length (online supplementary video 1), as evidenced by some reports showing that DNA was stretched by the traction force [16, 17]. Thus, it is conceivable that decreasing NET length indicates termination of bronchial infection because of a lack of new production of NETs in bronchi resulting from the resolution of infection.

Researchers have tried to quantify NETs *in vivo*. However, quantification of NETs *in vivo* is more difficult than expected because NETs are an extracellular fibrous component, which is difficult to measure precisely by commonly used experimental techniques such as Western blotting, ELISA, PCR, or flow cytometry. Some studies have reported on using the quantity of circulating free DNA to estimate the quantity of NETs in serum [18, 19]. However, it is uncertain whether circulating free DNA reflects the amount of NETs directly. The quantification method we used here is considered to be reliable because the NETs are

DOI: 10.1183/09031936.00139813

TABLE 3 Results of the multiple regression analysis of variables related to neutrophil extracellular trap length

	Standardised regression coefficient	p-value
Intercept	0	0.004
White blood cells	-1.23	0.016
CXC ligand-2	0.88	0.016
Interleukin-8	0.44	0.029
Lactate	-0.29	0.041
Platelet	0.18	0.075
Procalcitonin	-0.06	0.199

The value of adjusted R2=0.999.

visualised morphologically and the length is measured directly on the slides. A disadvantage is that it takes a relatively long time to obtain the result. Therefore, a simpler method to quantify NET appearance in human samples is needed for more practical clinical application.

In the current study, Cit H3 was detected inside the nucleus in a subpopulation of neutrophils on day 0, and many neutrophils expressing Cit H3 appeared at the time of maximum NET production on day 1. Citrullination of histones is thought to be the first step of nuclear decondensation [12]. In this research, polymorphonuclear leukocytes containing Cit H3 were predominant in the early infection phase, but were observed rarely in the convalescent period. This observation supports the idea that citrullination of histone H3 is mandatory for the process of NETosis before nuclear decondensation. A previous report suggested that Cit H3 is released into the circulation during the early stage of lipopolysaccharide-induced shock and it is associated with the severity of shock in a mouse model [20]. Our data suggests that the quantity of Cit H3 has potential as a clinical biomarker of the severity of infection, independent from other well-known inflammatory biomarkers such as C-reactive protein and PCT.

The dynamic change in NET length described above correlated with six inflammatory parameters (table 3): WBC and platelet counts, blood concentration of lactate, and serum concentrations of CXCL-2, IL-8, and PCT. These are well-known markers that reflect the biological response to infection. WBC count correlated negatively and most strongly with NET length. In general, WBC count is elevated in the early stages of inflammation. By contrast, we found that the peak of the NET length was 1 day after the onset of infection. We speculate that this gap and the negative correlation between WBC count and NET length reflect the shift of WBCs from circulation to the respiratory tract.

After WBC count, CXCL-2 levels had the second strongest correlation with NET length. Also called MIP-2 or growth-regulated protein (GRO)-beta, CXCL-2 is secreted by monocytes and macrophages, and promotes chemotaxis of neutrophils [21]. The third most strongly correlated parameter was IL-8 concentration, which also belongs to the CXC chemokine family. IL-8 is a pro-inflammatory cytokine produced by macrophages and other cells, such as epithelial cells, and its level increased during community-acquired pneumonia and ventilator-associated pneumonia [22]. It has been reported that IL-8 can trigger the release of NETs *in vitro* [6]. Both the accumulation of polymorphonuclear leukocytes and the induction of NETs in the respiratory tract might be induced partly by CXCL-2 or IL-8 produced by resident macrophages in the lung [23], although confirmation is needed.

An increase in blood lactate level reflects peripheral hypoperfusion and is a predictor of patient mortality under septic conditions in the ICU [23]. In this study, lactate level correlated negatively with NET length. ROS species are an essential substance needed for production of NETs [24]. Volume resuscitation and improved oxygenation by initiation of ventilator management in hypoxic conditions should increase the generation of ROS because of abrupt oxidative stress [25]. One possible explanation of the negative correlation between lactate level and NET production is that the amount of ROS might be insufficient to induce NET production during the phase when lactate level increases. The relationship between lactate level and NETs should be explored to elucidate the biological function of NETs in critical illnesses such as circulatory insufficiency and hypoxia.

Platelet count and PCT level also correlated significantly with NET length in the regression analysis. Platelet–neutrophil interactions play a pivotal role in NETosis [26, 27]. PCT is commonly used as an inflammatory marker and is useful for predicting the prognosis and deciding whether to use antibiotics in pneumonia [28, 29].

DOI: 10.1183/09031936.00139813

Nosocomial tracheobronchitis is common in mechanically ventilated ICU patients [30]. A recent paper reported that approximately one third of ventilator-associated tracheobronchitis (VAT) patients later developed ventilator-associated pneumonia (VAP) [31]. Our method could potentially be utilised to predict the progression to VAT, which could result in decreased numbers of VAP patients. Our study also suggests that Gram staining can be used to evaluate NETs as a surrogate for immunohistochemical detection.

Limitations of this study are the small number of patients enrolled in this study and that the patient population was heterogeneous. Simple correlations could not been detected between any single inflammatory parameter and the mean NET length, even though there were significant differences in the NET length. Identification of biological parameters related to NET formation will need larger studies with more participants. Additionally, in this study, we did not investigate the relationship between NET length and bacterial number in bronchial aspirates. Elucidating this relationship may promote further understanding of the biological relevance of NET length. Other candidate molecules involved in NET production or clearance should be examined in the blood as well as bronchial aspirates. Further studies will help clarify the biological significance of NETs in bronchial aspirates in various clinical conditions.

In conclusion, our findings suggest that NET length in bronchial aspirates reflects the immunological and inflammatory states associated with disease progression in respiratory infections. NET length in bronchial aspirates might provide a new inflammatory biomarker that could be applied when making decisions about the initiation and cessation of antibiotics or immunomodulatory therapy.

References

1 Richards MJ, Edwards JR, Culver DH, *et al.* Nosocomial infections in medical intensive care units in the United States. *Crit Care Med* 1999; 27: 887–892.
2 Matsushima A, Tasaki O, Shimizu K, *et al.* Preemptive antibiotic treatment based on Gram staining reduced the incidence of ARDS in mechanically ventilated patients. *J Trauma* 2008; 65: 309–315.
3 Hamaguchi S, Seki M, Yamamoto N, *et al.* Case of invasive nontypable *Haemophilus influenzae* respiratory tract infection with a large quantity of neutrophil extracellular traps in sputum. *J Inflamm Res* 2012; 5: 137–140.
4 Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. *N Engl J Med* 2000; 343: 1703–1714.
5 Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 2006; 6: 173–182.
6 Brinkmann V, Reichard U, Goosmann C, *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004; 303: 1532–1535.
7 Remijsen Q, Kuijpers TW, Wirawan E, *et al.* Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ* 2011; 18: 581–588.
8 Fuchs TA, Abed U, Goosmann C, *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007; 176: 231–241.
9 Pilsczek FH, Salina D, Poon KK, *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010; 185: 7413–7425.
10 Tadie JM, Bae HB, Jiang S, *et al.* HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. *Am J Physiol Lung Cell Mol Physiol* 2013; 304: L342–L349.
11 Metzler K, Fuchs T, Nauseef W, *et al.* Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 2011; 117: 953–959.
12 Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* 2008; 180: 1895–1902.
13 Wang Y, Li M, Stadler S, *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* 2009; 184: 205–213.
14 Hirose T, Hamaguchi S, Matsumoto N, *et al.* Dynamic changes in the expression of neutrophil extracellular traps in acute respiratory infections. *Am J Respir Crit Care Med* 2012; 185: 1130–1131.
15 Cantin AM. DNase I acutely increases cystic fibrosis sputum elastase activity and its potential to induce lung hemorrhage in mice. *Am J Respir Crit Care Med* 1998; 157: 464–469.
16 Gore J, Bryant Z, Nöllmann M, *et al.* DNA overwinds when stretched. *Nature* 2006; 442: 836–839.
17 van Mameren J, Gross P, Farge G, *et al.* Unraveling the structure of DNA during overstretching by using multicolor, single-molecule fluorescence imaging. *Proc Natl Acad Sci USA* 2009; 106: 18231–18236.
18 Margraf S, Logters T, Reipen J, *et al.* Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. *Shock* 2008; 30: 352–358.
19 Altrichter J, Zedler S, Kraft R, *et al.* Neutrophil-derived circulating free DNA (cf-DNA/NETs), a potential prognostic marker for mortality in patients with severe burn injury. *Eur J Trauma Emerg Surg* 2010; 36: 551–557.
20 Li Y, Liu B, Fukudome EY, *et al.* Identification of citrullinated histone H3 as a potential serum protein biomarker in a lethal model of lipopolysaccharide-induced shock. *Surgery* 2011; 150: 442–451.
21 Iida N, Grotendorst GR. Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. *Mol Cell Biol* 1990; 10: 5596–5599.
22 Bonten MJ, Froon AH, Gaillard CA, *et al.* The systemic inflammatory response in the development of ventilator-associated pneumonia. *Am J Respir Crit Care Med* 1997; 156: 1105–1113.
23 Trzeciak S, Dellinger RP, Chansky ME, *et al.* Serum lactate as a predictor of mortality in patients with infection. *Intensive Care Med* 2007; 33: 970–977.
24 Keshari RS, Verma A, Barthwal MK, *et al.* Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA- induced NETs release from human neutrophils. *J Cell Biochem*, 2013: 532–540.
25 Kavazis AN, Talbert EE, Smuder AJ, *et al.* Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production. *Free Radic Biol Med* 2009; 46: 842–850.

DOI: 10.1183/09031936.00139813

26 Clark SR, Ma AC, Tavener SA, *et al.* Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 2007; 13: 463–469.
27 Fuchs T, Brill A, Durschmied D, *et al.* Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA* 2010; 107: 15880–15885.
28 Kruger S, Ewig S, Marre R, *et al.* Procalcitonin predicts patients at low risk of death from community-acquired pneumonia across all CRB-65 classes. *Eur Respir J* 2008; 31: 349–355.
29 Christ-Crain M, Stolz D, Bingisser R, *et al.* Procalcitonin guidance of antibiotic therapy in community-acquired pneumonia a Randomized trial. *Am J Respir Crit Care Med* 2006; 174: 84–93.
30 Nseir S, Di Pompeo C, Pronnier P, *et al.* Nosocomial tracheobronchitis in mechanically ventilated patients: incidence, aetiology and outcome. *Eur Respir J* 2002; 20: 1483–1489.
31 Craven DE, Lei Y, Ruthazer R, *et al.* Incidence and outcomes of ventilator-associated tracheobronchitis and pneumonia. *Am J Med* 2013; 126: 542–549.

DOI: 10.1183/09031936.00139813

Correlation between Outbreaks of Multidrug-Resistant *Pseudomonas Aeruginosa* Infection and Use of Bronchoscopes Suggested by Epidemiological Analysis

Hisako Machida,[a,b,#] Masafumi Seki,[*,a,#] Nori Yoshioka,[a] Kaori Yabuno,[a,b] Koji Miyawaki,[a,b] Hisao Yoshida,[a] Norihisa Yamamoto,[a] Shigeto Hamaguchi,[a] and Kazunori Tomono[a]

[a] *Division of Infection Control and Prevention, Osaka University Hospital; and* [b] *Department of Pharmacy, Osaka University Hospital; Suita, Osaka 565–0871, Japan.*
Received June 13, 2013; accepted October 17, 2013

An outbreak of Multi-Drug Resistance *Pseudomonas aeruginosa* (MDRP) infections occurred in intensive care unit (ICU) and emergency room (ER) between June and August 2007. Five patients who isolated MDRP in the outbreak of 2007 were all used bronchoscopes, thus, we suspected contamination of the bronchoscopes as the cause of outbreak. Although we did not detect MDRP from any bronchoscopes, the outbreak finally ended after all the bronchoscopes had been disinfected appropriately with the reexamination of washing process in 2008 and 2009. We retrospectively reviewed eleven patients who isolated MDRP in 2006 and 2007, and the fact was revealed that bronchoscopes were used in most patients in ICU and ER. Bronchoscopes were significantly used during 2006–2007 period, compared with 2008–2009 period in ICU and ER, and the case-control analysis among all *Pseudomonas aeruginosa* isolated patients identified that bronchoscopes [risk ratio (RR) 8.25, 95% confidence interval (CI) 1.328–51.26] was one of the most important risk factors for MDRP isolation. Duration from admission to MDRP isolation was significant longer in MDRP-isolated cases (19.82±12.77 d), compared with in non MDRP-isolated controls (11.76±11.69 d: $p=0.0453$). Our epidemiological analysis suggested the significant risk factors for an MDRP outbreak, and could contribute the estimation of the focus and prevention of future outbreaks.

Key words *Pseudomonas aeruginosa*; bronchoscope; hospital infection; centralized sterilization

The importance of *Pseudomonas aeruginosa* (*P. aeruginosa*) as a cause of sporadic cases of nosocomial infection, particularly pneumonia, and outbreaks in intensive care units (ICUs) and emergency rooms (ERs) are well established.[1] Among these, special vigilance is required when nosocomial outbreaks arise due to multidrug-resistant *P. aeruginosa* (MDRP).[2]

Several nosocomial cross-infections of *P. aeruginosa* due to medical devices have been reported,[3,4] and bronchoscopes have been thought as one of the most important instruments related to outbreaks in the ICU and ER. Bronchoscopes are semicritical instruments that come into contact with mucous membranes and require high-level disinfection.

Between June and August 2007, MDRP was isolated from several patients in the ICU and ER of our hospital, and all of whom had recently undergone flexible bronchoscope examinations. Active epidemiological surveillance was commenced and additional cases were detected in 2006 and 2007.

The present epidemiological investigation was conducted to verify the primary hypothesis of a relationship between infections and bronchoscopes, to identify other potential risk factors for infection, and to implement infection control measures to terminate the outbreak.

MATERIALS AND METHODS

Hospital and Patients Setting Our hospital is a 1076-bed university hospital located in Osaka. A surveillance of hospital-acquired infections using 1-year data from April 2003 to March 2004 found that the mean number of MDRP strains isolated in this hospital is 1.08±0.67 per month. As soon as the hospital's infection control team recognized that an outbreak of MDRP had occurred in the 8-bed surgical ICU and 20-bed ER, epidemiological information was collected and analyzed in 2007.

The clinical investigation comprised a medical chart review, patient examination, and discussion with nurses and physicians. Cases were defined as patients admitted to the ICU and ER between April 2006 and August 2009 from whose sputum MDRP was isolated.

Case-Control Study Isolated cases were defined as patients who had stayed in the ICU or ER more than 2 d and from whom MDRP was isolated. Non-isolated controls were defined as patients from whom non-multidrug-resistant *P. aeruginosa* was isolated, but showed similar histories and backgrounds, such as age and sex, and stayed in the ICU or ER in same period as isolated cases.

Microbiological Studies *P. aeruginosa* identification and susceptibility tests were performed using a MicroScan WalkAway systems (Siemens, Munich, Germany).[5] *P. aeruginosa* was considered multidrug-resistant if the bacteria proved resistant to three or more families of antimicrobial agents.[2,6] In this study, MDRP was defined as *P. aeruginosa* resistant to carbapenems (such as imipenem or meropenem), fluoroquinolones, and amikacin.[7]

Statistical Analysis The chi-squared test and Fisher's exact test were used to compare categorical variables, and Student's *t*-test was employed to compare continuous variables.

Risk ratios (RRs) were calculated to estimate the magnitude of associations between each exposure and outcome using logistic regression analyses. All tests of significance were two-tailed, and values of $p<0.05$ were considered statistically

The authors declare no conflict of interest.

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: seki@hp-infect.med.osaka-u.ac.jp

significant.

RESULTS

Outbreak Characterization From June to August 2007, five cases with detection of MDRP were identified among patients in the ICU and ER (Table 1). The number of MDRP isolation at that time were much more than that of the previous three months of 2007 ($p<0.0001$) (Fig. 1). An outbreak investigation was therefore initiated.

While examining potential failures in contact isolation precautions, mechanical ventilators and breathing circuits, we discovered that all five cases had recently undergone flexible bronchoscope (Olympus BF-PE2; Olympus, Tokyo, Japan) examination in the ER or ICU.

Bronchoscope Cleaning Procedures and Improvement When we became aware of the potential association between infection and bronchoscope exposure, we performed tests to detect MDRP from these bronchoscopes. Unfortunately, we could not detect any MDRP from bronchoscopes, but a careful review of disinfection protocols and maintenance of the bronchoscope revealed major deviations from hospital policies.

According to our manual, bronchoscopes were usually cleaned just after use. They were manually cleaned by wiping the outer surface and brushing the inner channel and suction ports. The suction button was removed and cleaned. Bronchoscopes were then disinfected in an automated endoscope reprocessor (AER) (Olympus, Tokyo, Japan). We always ensured that bronchoscopes were immersed in germicide and that all channel connectors were attached to the reprocessor, in accordance with the instructions from the manufacturer. After disinfection, the bronchoscopes were rinsed and the channels flushed with sterile water to remove the disinfectant. Channels were then flushed with 70% alcohol. Finally, bronchoscopes are air-dried and stored in a vertical position.

All the steps for cleaning and disinfection of endoscope equipment were checked. We had an endoscope center and most endoscopes in the hospital were disinfected or sterilized there according to our manual. However, bronchoscopes used in the ICU and ER were disinfected separately from other scopes. Unfortunately, compliance with the guidelines and recommendations for the cleaning of those bronchoscopes appeared poor. This cleaning step was sometimes skipped before the outbreaks in the ICU and ER.

We therefore started washing and disinfection in the material management section to centralize the disinfection of endoscopes. Washing spaces were separated depending on each kind of endoscope, and flow lines of endoscopes delivery were simplified. Staff numbers were increased and detailed records of washing and disinfection processes were also renewed. In addition, high-level disinfection was reexamined, and *ortho*-phthalaldehyde (Cidex OPA; Johnson & Johnson, Tokyo, Japan) was used as the liquid germicide.

Recommended changes included improvements in cleaning,

Table 1. Clinical Data of MDRP Isolated Patients in ICU and ER in the Outbreak of June and August of 2007

Male/Female	Underlying disease	Sample	Age	Ward
Male	Post-heart transplantation	Throat swab	45	ICU
Male	Trauma	Sputum	45	ER
Female	Post livertransplantation	Urine	35	ICU
Male	Esophageal cancer	Sputum	55	ICU
Female	Esophageal cancer	Sputum	62	ICU

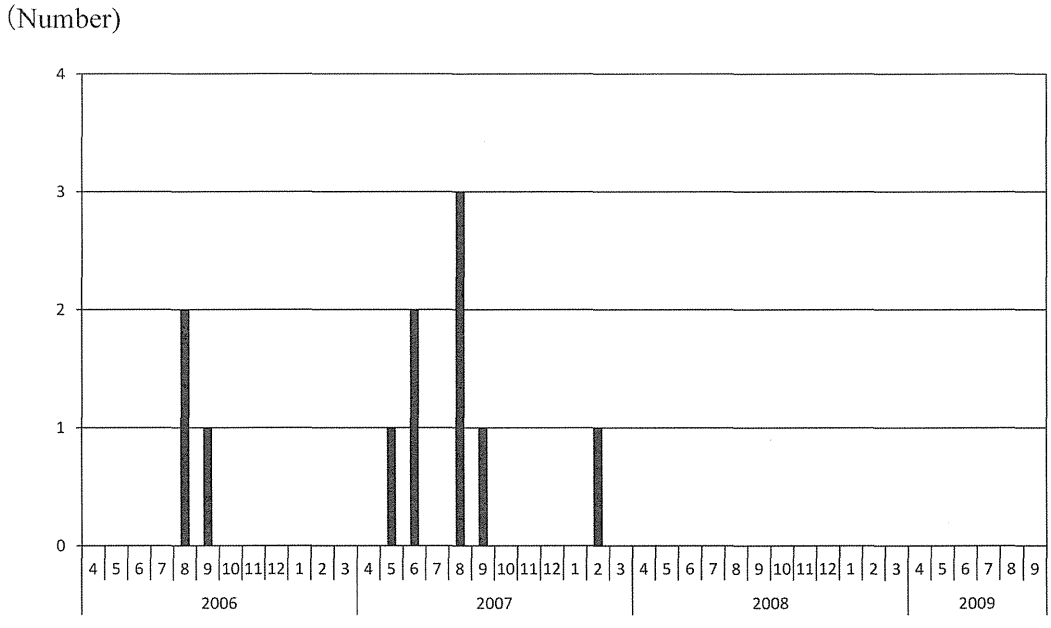

Fig. 1. The Number of the Patients from Whom Multidrug-Resistant *Pseudomonas aeruginosa* (MDRP) Were Isolated from April 2006 to August 2009 in the ER and ICU of the Hospital

disinfection and maintenance of the bronchoscope. Since then, the number of patients from whom MDRP was isolated in our hospital were decreased (Fig. 1), and no additional patients undergoing bronchoscope examinations have shown isolation of MDRP in 2008 and 2009, compared with 2006 (3 cases) and 2007 (8 cases) (p=0.0076).

Characteristic Changes of Admitted Patients in the ICU and ER during 2006–2007 and 2008–2009 We suspected that potential outbreaks have occurred in the whole period of 2006 to 2007 in the ICU and ER, and therefore, retrospectively reviewed all patients in the ICU and ER from whom MDRP was isolated in 2006 and 2007. Overall, 11 patients were identified with MDRP, including isolation from the respiratory tract (sputum in 5 cases, throat swab in 3 cases) and urinary tract (3 cases).

We then retrospectively compared patients admitted to the ICU or ER from April 2006 to March 2008 (n=2607) and from April 2008 to September 2009 (n=2281) (Table 2), and also found that bronchoscope examinations were performed more frequently during the former period than in the latter period (p=0.0062). Duration of hospitalization (p=0.0216), surgery (p=0.045), use of a urinary catheter (p=0.0001), and use of an upper intestine scope (p=0.0418) also differed significantly between 2006–2007 and 2008–2009.

However, the doses (AUD) of anti-*Pseudomonas* antibiotics used, including 4th generation cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones, did not differ significantly between the two above-mentioned periods.

Risk Factors for *P. aeruginosa* Infection Next, we performed a case-non-case study based on comparative data between 2006–2007 and 2008–2009 to detect risk factors for MDRP isolation in the ICU and ER. Multivariate analysis identified bronchoscopes as the one of the most important risk factors for isolation of MDRP (p=0.0238; RR, 8.25; 95% confidence interval (CI), 1.328–51.26) (Table 3).

In addition, duration from admission to *P. aeruginosa* isolation was significantly longer in MDRP-isolated cases (19.82± 12.77 d) than in non-MDRP-isolated controls (11.76±11.69 d; P=0.0453).

No significant differences were found in use of other devices, including gastrointestinal scopes and urinary catheters, or administration of antibiotics such as carbapenems and fluoroquinolones.

DISCUSSION

P. aeruginosa has been implicated in three reported hospital outbreaks involving flexible bronchoscopes.[3,4,8,9] The present report studied outbreaks of MDRP isolation suggested to be attributable to use of a flexible bronchoscope, revealing how deficits in decontamination can contribute to the transmission of *P. aeruginosa*. Recent exposure to bronchoscopes among the first identified cases suggested that use of flexible bronchoscopes could have been associated with MDRP transmission. Important failures in processing and storage of flexible bronchoscopes were discovered, and cross-infection might also represent a significant risk factor in the development of infections, probably *via* the hands of healthcare workers.[10,11]

Many reports can be found on nosocomial infections associated with endoscopes, including our previous report about an outbreak associated with transesophageal echocardiography (TOE) in 2004.[7] Proper disinfection and sterilization is essential if we are to avoid transmitting infectious pathogens to patients *via* endoscopes, including bronchoscopes. *P. aeruginosa* is one of the most important organisms related to bronchoscope-associated nosocomial infections. Failure to comply with hygiene guidelines has led to numerous outbreaks of infection.[12] Flaws related to bronchoscopes; such as a loose biopsy port cap, have also caused large nosocomial outbreaks.[12,13] In these reports of nosocomial outbreaks, bronchoscopes had been used for bronchoscopic observation, for collecting bronchoalveolar lavage fluid samples, for therapy for medical disorders, and as an aid in medical procedures. In addition, bronchoscopes are also used during anesthesia procedures. The one-lung technique is widely used to facilitate thoracic surgical visualization.[4,14]

Bacteriological investigations, including genetic analysis of MDRP by pulse field gel electrophoresis (PFGE), of the bronchoscopes unfortunately could not confirm its transmission, because we did not detect any bacteria from bronchoscopes used in the ICU and ER, or the detergent tank of the AER, although we could detected the MDRP from TOE and performed PFGE in the outbreak of 2004.[7] However, the epidemiological study strongly suggested that bronchoscopes were associated with transmission of MDRP in these sections. We therefore changed the recommendations, including improvements in bronchoscope cleaning, disinfection and maintenance. Subsequently, no additional patients undergoing

Table 2. Comparison of Admitted Patients in ICU and ER during 2006–2007 and 2008–2009

	2006–2007 (N=2607)	2008–2009 (N=2281)	p-Value
Male (%)	1641 (63.0)	1451 (63.6)	0.6344
Age (years), mean∓S.D.	54.9±23.6	53.6± 24.9	0.3865
Duration of hospitalization (d), mean±S.D.	47.6±104.3	40.7±74.8	0.0216
Surgery (%)	1285 (49.3)	1058 (46.4)	0.045
Anti-cancer chemotherapy (%)	4 (0.2)	8 (0.4)	0.2462
Immuno-suppresiive drugs (%)	323 (12.4)	311 (13.6)	0.2007
Intravenous hyperalimentation (%)	1328 (50.9)	1169 (51.2)	0.8634
Urinary catheter (%)	2041 (78.3)	1678 (73.6)	0.0001
Mechanical ventilation (%)	1291 (49.2)	1115 (48.9)	0.6672
Bronchoscope (%)	369 (14.2)	262 (11.5)	0.0062
Upper intestine scope (%)	89 (3.4)	55 (2.4)	0.0418
Lower intestine scope (%)	16 (0.6)	20 (0.9)	0.3165

Table 3. Risk Factors for MDRP among 28 Patients

	Case (n=11)	Non cases (n=17)	p-Value	Risk ratio (95%CI)
Age (years, mean±S.D.)	51.2±12.1	55.9±12.4	0.3001	
Duration from admission to *P. aeruginosa* isolation (d, mean±S.D.)	19.8±12.8	11.8±11.7	0.0453	
Surgery (%)	8 (72.7)	15 (88.2)	0.3531	0.3556 (0.04888–2.586)
Anti-cancer chemotherapy (%)	0 (0)	0 (0)	NA	NA
Immuno-suppresiive drugs (%)	5 (45.5)	5 (29.4)	0.4443	2 (0.4119–9.712)
Urinary catheter (%)	11 (100)	13 (81.3)	0.2579	NA
Mechanical ventilation (%)	11 (100)	15 (88.2)	0.5053	NA
Bronchoscope (%)	9 (81.8)	6 (35.3)	0.0238	8.25 (1.328–51.26)
Upper intestine scope (%)	3 (27.3)	1 (5.9)	0.2694	6 (0.5351–67.28)
Lower intestine scope (%)	0 (0)	1 (5.9)	1	0
Central venous catheter (%)	11 (100)	15 (88.2)	0.5053	NA
Administration of carbapenem (%)	4 (36.4)	3 (17.7)	0.3809	2.667 (0.4632–15.35)
Administration of 4th generation cephalosporin (%)	1 (9.1)	1 (5.9)	1	1.6 (0.08962–28.57)
Administration of aminoglycoside (%)	2 (18.2)	1 (5.9)	0.5433	3.556 (0.2817–44.88)
Administration of fluoroquinolone (%)	3 (27.3)	0 (0)	0.0504	NA

bronchoscope examinations showed isolation MDRP in 2008 and 2009.

Several reports have indicated that cleaning alone reduces microbial contaminants, with a reduction rate of 99.99%.[12,15] Without the removal of protein, disinfectant becomes useless for killing bacteria.[12,16] Cleaning should be performed promptly after each use of an endoscope, to prevent the drying of secretions. Once secretions have dried, thick biofilms may form, reducing the effectiveness of detergents and disinfectants.

In our case, we could not detect any *P. aeruginosa* from bronchoscopes, but outbreak of MDRP were suspended by renewal of process of disinfection of bronchoscopes and its related devices. Therefore, we could not neither determine the only bronchoscopes as the focus of the outbreak and nor there was the possibility that the other roots/focus of transmission of MDRP, however, it became clear that the disinfection of devices, reexamination of the process, and education of the medical stuffs were also very critical although the items other than bronchoscopes exist as the focus of the outbreak.

Furthermore, we retrospectively compared patients admitted the ICU or ER from the April 2006–2007 period and from 2008–2009, and also found that more bronchoscope examinations were performed during the former period than in the latter period. Conversely, used doses (AUD) of anti-*Pseudomonas* antibiotics did not differ between the two periods. These findings suggest that use of antibiotics might be unrelated to this outbreak, and MDRP isolates were not a result of drug pressures in patients, but rather transferred from other patients.

A case-control study was then performed and revealed bronchoscopes and duration from admission to MDRP isolation as significant factors in patients with MDRP. Bou *et al.* reported an outbreak of *P. aeruginosa* infection in a 27-bed ICU, and performed epidemiological analysis.[3] Their logistic regression analyses demonstrated that cases were more likely than non-controls to have had a longer stay in the ICU, and to have undergone mechanical ventilation and antimicrobial treatment. Multivariate analysis yielded results similar to our own, identifying recent bronchoscope examinations and exposure to an infected patient as independent risk factors. Kanemitsu *et al.* also reported the usefulness of case-control studies in contributing to the detection and confirmation of **TOE** as the causative factor in an *Enterobacter cloaca* outbreak in a cardiovascular ward.[17] Epidemiological analysis might strongly contribute to the detection and confirmation of causative factors in outbreaks.

In conclusion, analytical epidemiological methods contributed to the identification of significant risk factors for an MDRP outbreak in our hospital. We could not detect the pathogen from any bronchoscopes, but the outbreak ended after the washing process was properly established. A case/non-control study might be one of the strong tools to detect and confirm causative factors in the outbreak.

REFERENCES

1) American Thoracic Society/Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am. J. Respir. Crit. Care Med.*, **171**, 388–416 (2005).
2) Mikura S, Wada H, Okazaki M, Nakamura M, Honda K, Yasutake T, Higaki M, Ishii H, Watanabe T, Tsunoda T, Goto H. Risk factors for bacteraemia attributable to *Pseudomonas aeruginosa* resistant to imipenem, levofloxacin, or gentamicin. *J. Hosp. Infect.*, **79**, 267–268 (2011).
3) Bou R, Aguilar A, Perpiñán J, Ramos P, Peris M, Lorente L, Zúñiga A. Nosocomial outbreak of *Pseudomonas aeruginosa* infections related to a flexible bronchoscope. *J. Hosp. Infect.*, **64**, 129–135 (2006).
4) Shimono N, Takuma T, Tsuchimochi N, Shiose A, Murata M, Kanamoto Y, Uchida Y, Morita S, Matsumoto H, Hayashi J. An outbreak of *Pseudomonas aeruginosa* infections following thoracic surgeries occurring *via* the contamination of bronchoscopes and an automatic endoscope reprocessor. *J. Infect. Chemother.*, **14**, 418–423 (2008).
5) Snyder JW, Munier G, Johnson CL. Direct comparison of the BD phoenix system with the MicroScan WalkAway system for identification and antimicrobial susceptibility testing of Enterobacteriaceae and nonfermentative Gram-negative organisms. *J. Clin. Microbiol.*, **46**, 2327–2333 (2008).
6) Harris A, Torres-Viera C, Venkataraman L, DeGirolami P, Samore M, Carmeli Y. Epidemiology and clinical outcomes of patients with multiresistant *Pseudomonas aeruginosa*. *Clin. Infect. Dis.*, **28**, 1128–1133 (1999).

7) Seki M, Machida N, Yamagishi Y, Yoshida H, Tomono K. Nosocomial outbreak of multidrug-resistant *Pseudomonas aeruginosa* caused by damaged transesophageal echocardiogram probe used in cardiovascular surgical operations. *J. Infect. Chemother.*, **19**, 677–681 (2013).
8) Cêtre JC, Nicolle MC, Salord H, Pérol M, Tigaud S, David G, Bourjault M, Vanhems P. Outbreaks of contaminated broncho-alveolar lavage related to intrinsically defective bronchoscopes. *J. Hosp. Infect.*, **61**, 39–45 (2005).
9) Kirschke DL, Jones TF, Craig AS, Chu PS, Mayernick GG, Patel JA, Schaffner W. *Pseudomonas aeruginosa* and *Serratia marcescens* contamination associated with a manufacturing defect in bronchoscopes. *N. Engl. J. Med.*, **348**, 214–220 (2003).
10) McNeil SA, Nordstrom-Lerner L, Malani PN, Zervos M, Kauffman CA. Outbreak of sternal surgical site infections due to *Pseudomonas aeruginosa* traced to a scrub nurse with onychomycosis. *Clin. Infect. Dis.*, **33**, 317–323 (2001).
11) Zawacki A, O'Rourke E, Potter-Bynoe G, Macone A, Harbarth S, Goldmann D. An outbreak of *Pseudomonas aeruginosa* pneumonia and bloodstream infection associated with intermittent otitis externa in a healthcare worker. *Infect. Control Hosp. Epidemiol.*, **25**, 1083–1089 (2004).
12) Alvarado CJ, Reichelderfer M, Association for Professionals in Infection Control. APIC guideline for infection prevention and control in flexible endoscopy. Association for Professionals in Infection Control. *Am. J. Infect. Control*, **28**, 138–155 (2000).
13) Rello J, Diaz E. Pneumonia in the intensive care unit. *Crit. Care Med.*, **31**, 2544–2551 (2003).
14) Ovassapian A. The flexible bronchoscope. A tool for anesthesiologists. *Clin. Chest Med.*, **22**, 281–299 (2001).
15) Rutala WA, Weber DJ. Reprocessing endoscopes: United States perspective. *J. Hosp. Infect.*, **56** (Suppl. 2), S27–S39 (2004).
16) Gorman SP, Scott EM, Hutchinson EP. Interaction of the *Bacillus subtilis* spore protoplast, cortex, ion-exchange and coatless forms with glutaraldehyde. *J. Appl. Bacteriol.*, **56**, 95–102 (1984).
17) Kanemitsu K, Endo S, Oda K, Saito K, Kunishima H, Hatta M, Inden K, Kaku M. An increased incidence of *Enterobacter cloacae* in a cardiovascular ward. *J. Hosp. Infect.*, **66**, 130–134 (2007).

私の推奨する呼吸器診断法

肺炎の迅速原因菌検査 グラム染色

大阪大学医学部附属病院感染制御部
朝野 和典

Ⅰ. はじめに

肺炎の細菌学的診断に用いる検体としては，喀出痰，気管内吸引痰，経皮針吸引検体，経気管吸引(trans tracheal aspiration：TTA)および気管支鏡を用いた侵襲的方法による局所採痰検体などがある。痰以外では，血液培養，肺炎球菌，レジオネラなどの特定の細菌の尿中抗原検出，あるいは抗体価の測定などが行われる。

喀出痰や吸引痰などの検体は培養と同時にグラム染色，抗酸菌染色など塗抹標本の顕微鏡による観察も行われる。これらの検査を組み合わせて，肺炎の原因菌は推定，同定される。

ここでは迅速診断法である痰の塗抹検鏡のうちでもグラム染色の有用性とその解釈および限界について解説する。

Ⅱ. 塗抹検査の解釈

1. 品質評価

TTA などの侵襲的な検査は殆ど行われないため，肺炎の診断に通常用いられる喀出痰は，口腔内の常在菌の汚染を避けることができない。そのため，まず痰の品質評価が行われる。即ち，その喀出痰を培養した結果の信頼性を確認するための評価である。一般には Geckler 分類が用いられる(**表 1**)[1]。Geckler 分類では，喀出痰の塗抹標本の低倍率観察(100 倍)で，1 つの視野に，口腔から由来している上皮細胞がどの程度存在するかを確認する。10 視野程度を観察し，最も膿性度の高い視野を評価する。上皮細胞の数が 25 個/視野以下のグループ 6，5 および 4 ならば，TTA によって得られた培養結果とよく相関することから培養結果に信頼性のある良質の検体と判断される。好中球の存在は炎症の程度を示すもので，何個以上なければならないということではない。Geckler の原著では，グループ 1～3 は培養しても有用な情報が得られないため，培養しないで再提出を求める判断の基準となっている。

2. 原因菌の推定

良質の喀出痰はさらに高倍率(1,000 倍)で観察し，そこにいる細菌の種類を確認する。肺炎の原因となる細菌の多くは，グラム染色で鑑別可能である(**図 1**)。ただし，少数の特徴のよく似た細菌が数視野の観察ごとに観察されたとしても，病原的な意義は不明である。1 視野にびまん性にかつ膿性部分のすべての視野に同一形態の細菌が認められた場合，原因菌である確率が高くなる。このような状況下で肺炎球菌とインフルエンザ菌は特異性高く診断できる[2]。

好中球による貪食像の確認も，炎症局所に存在する細菌の種類を示すものである。

嫌気性菌による肺炎は，喀出痰の培養では検査ができない。口腔内には嫌気性菌が常在し，喀出痰では嫌気性菌の汚染が避けられないからである。そのため，嫌気性菌による肺炎の細菌学的診断は喀出痰では不可能である。このような培養の弱点をグラム染色は補完する意義も有している。嫌気性菌による肺炎の場合には，好中球が多種類の細菌を高率に貪食している所見が特徴的である(**図 2**)。

Ⅲ. 肺炎の原因菌診断の限界

以上に述べた如く Geckler 分類を基準とし，高倍率視野で存在する細菌の種類を推測することで，初期治療における抗菌薬の選択の根拠とすることができる。一方，グラ

Rapid examination for causative pathogens of pneumonia ; gram stain
Kazunori Tomono
Division of Infection Control and Prevention, Osaka University Medical Hospital, Osaka 565-0871, Japan

表1　Geckler 分類

グループ	細胞数/×100 視野	
	頬粘膜扁平上皮細胞	白血球
6	＜25	＜25
5	＜10	＞25
4	10～25	＞25
3	＞25	＞25
2	＞25	10～25
1	＞25	＜10

(Geckler RW, *et al*[1]. *J Clin Microbiol* 6:1977 より引用, 改変)

菌名	グラム染色の形態	特　徴
肺炎球菌		透明な莢膜を伴うグラム陽性二連球菌（双球菌）。正円ではなく楕円形（ランセット型）の球菌である。
インフルエンザ菌		小型のグラム陰性球桿菌。球菌のように見えるが必ず桿菌が存在する。数も多く、砂粒のようにびまん性にみられる。
黄色ブドウ球菌		大きめの正円のグラム陽性球菌。レンサ球菌とは明らかに大きさが異なるため、鑑別しやすい。
クレブシエラ		大型のグラム陰性桿菌。周囲に透明の広い莢膜を伴う。
緑膿菌		小型のグラム陰性桿菌。クレブシエラや大腸菌に比べてスリムな菌体が特徴である。ときに着色した莢膜を伴う。

図1　呼吸器原因菌のグラム染色所見

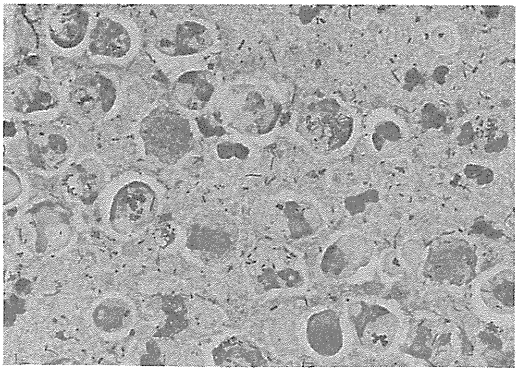

図2　誤嚥性肺炎の症例の吸引痰のグラム染色所見（×1,000）：多種類の細菌を好中球が貪食している。

ム染色所見からは下気道由来の検体であることは判断できるが，肺炎の局所から由来するのか，気道の炎症の部位から由来するのか鑑別ができない。気道に炎症のない人の市中肺炎の場合は，下気道由来と判断される痰の炎症部分に特徴的な細菌がびまん性に確認されれば，診断的な価値は高い。しかし，院内肺炎あるいは人工呼吸器関連肺炎では，良質の痰であっても下気道の定着菌なのか肺炎の原因菌なのかの鑑別が困難である（図3）。

したがって，市中肺炎では品質のよい痰を採取することが最も重要であり，その場合には特異度高く原因菌を推測することができる。一方，院内肺炎では，MRSA や緑膿菌などの薬剤耐性菌を推測する細菌が下気道由来検体から観察されても原因細菌であるか否かの判断は，さらに貪食像などを加えて総合的に判断することが必要である。

このように，呼吸器感染症の診断に有用な情報を得ることができるグラム染色の有用性と限界を理解して臨床に用いることが重要である。

文　献

1）Geckler RW, Gremillion DH, McAllister CK, *et al.* Microscopic and bacteriological comparison of paired sputa and transtracheal aspirates. *J Clin Microbiol* 6：396—399, 1977
2）Anevlavis S, Petroglou N, Tzavaras A, Maltezos E, *et al.* A prospective study of the diagnostic utility of sputum Gram stain in pneumonia. *J Infect* 59：83—89, 2009

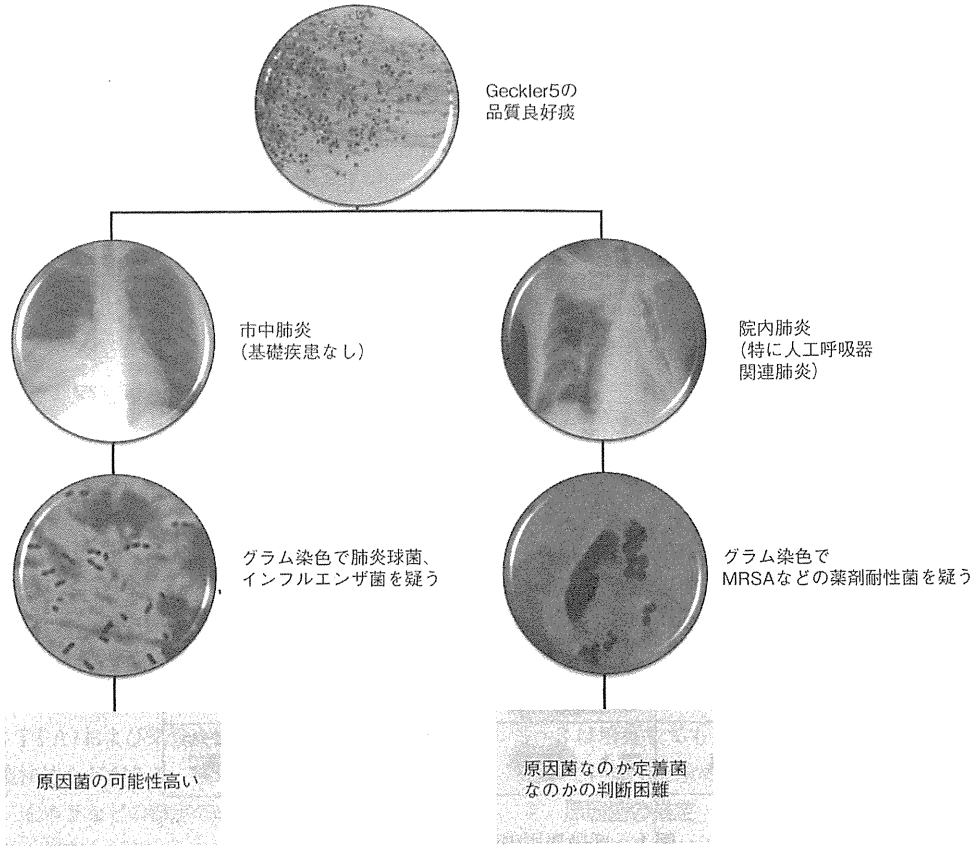

図3　良質痰でも市中肺炎と院内肺炎では解釈が異なる。

10. ストップ肺炎キャンペーン

朝野和典*

Keywords●高齢化社会，健康寿命，肺炎，誤嚥性肺炎，ワクチン／an aging society, healthy life years, pneumonia, aspiration pneumonia vaccine

要旨●肺炎は，日本人の死亡原因の第3位となり，そのほとんどが高齢者によって占められている。肺炎による死亡者数を減らすことはできないが，肺炎に罹患しない健康寿命の延長をめざすことが，これからの高齢化社会で最も必要なことである。そのため，日本呼吸器学会はストップ肺炎キャンペーンを開始した。

1 ストップ肺炎キャンペーンの経緯

日本呼吸器学会呼吸器感染症ガイドライン作成委員会の「医療・介護関連肺炎（nursing and healthcare-associated pneumonia：NHCAP）ガイドライン」[1]が2012年に出された。NHCAPガイドラインは，主に介護を受ける高齢者や継続的に医療を受けている患者の肺炎を対象としている。

肺炎で亡くなる人の95%以上が65歳以上の高齢者である。若年成人が肺炎で死ぬ確率は極めて低く，高齢者の1/1000以下である。したがって，肺炎は高齢者の病気であり，高齢者は繰り返し肺炎となり，肺炎で亡くなったり，あるいは他疾患の療養末期に肺炎を併発して亡くなっていく。「肺炎は老人の友である」と言われる所以である。

2012年，肺炎による死亡者の数は，脳血管障害を抜き，悪性新生物，心疾患に次いで日本人の死亡原因の第3位になった。一方，各年齢層における肺炎の死亡率は変化していない。各年齢層の死亡率は変化せず，総数が増加しているということは，日本人の人口に占める肺炎で亡くなる率の高い高齢者の割合が増えているにすぎない。したがって，肺炎死亡は社会の高齢化とともにますます増加していくであろう。

そこで，日本呼吸器学会，感染症結核部会として，肺炎の死亡者数が死亡原因の第3位になったことを踏まえ，より広くガイドラインを利用してもらうことを目的として「ストップ肺炎キャンペーン」[2]を行うこととなった。

2 「健康寿命の延長」がキャンペーンの目指す目的

日本人の死亡原因の第3位となり，今後も増え続けていく肺炎による死亡に対し，「肺炎で亡くなる人の数を減らすことが重要な課題か？」と言うと，その答えは否である。人間はいつかは死ぬ。もし肺炎で亡くならなかったとしても，ほかの原因で人間は亡くなる。あるいは，今回の肺炎で亡くならなかったとしても次の肺炎のエピソードで亡くなるかもしれない。

Stop Pneumonia Campaign
Kazunori TOMONO*
* *Department of Infection Control and Prevention, Osaka University Hospital, Suita*
* 大阪大学医学部感染制御部（〒565-0871　大阪府吹田市山田丘2-2）

表 1　ストップ肺炎・一般用冊子の内容目次

感染症の一般的な予防
　かぜ，インフルエンザにならないために，また周りに拡げないために
肺炎の予防
　インフルエンザワクチン
　肺炎球菌ワクチン
　インフルエンザワクチンと肺炎球菌ワクチンを両方，接種しましょう
　肺炎を起こさせない栄養管理：栄養不良（低栄養）を防ぎましょう
　摂食への配慮と肺炎予防
　肺炎とタバコの関係
　口腔ケアと肺炎予防
肺炎を見つけるために
　肺炎をみつけるために

表 2　ストップ肺炎・医療従事者用冊子の内容目次

肺炎の予防
　ワクチンによる予防
　　1．インフルエンザワクチン
　　2．23 価肺炎球菌ワクチン
　　3．インフルエンザワクチンと 23 価肺炎球菌ワクチンを両方，接種しましょう
　栄養管理と摂食への配慮
　　栄養管理
　　食事の工夫
　　口腔ケアと肺炎予防
肺炎の診断と治療
　肺炎をみつけるために
　　1．症状・所見から肺炎を予測する
　　2．胸部 X 線写真を撮影
　　3．高齢者の肺炎に注意
　肺炎の治療
　　【外来治療】
　　【入院治療】

NHCAP ガイドラインの作成過程で問題となったのは，日本人に多い誤嚥性肺炎であった。介護保険の対象となる高齢者の肺炎の多くは誤嚥が原因であり，繰り返し肺炎を起こしていること。それらの患者は薬剤耐性菌の分離される頻度が高くなること，そのため，市中肺炎ではなく院内肺炎と類似した診療が求められることが特徴として挙げられ，その特徴に応じた診療をガイドラインの作成方針が提言された。そこで最初に議論されたことは，繰り返す肺炎を重症肺炎として広域抗菌薬の選択や集中治療の適応となるかという問題であった。その結果，呼吸器感染症のガイドラインでは初めて，生命倫理の考え方を導入し，その柱に「個人の意思の尊重」を取り入れた。

同時期に，日本老年医学会からは，高齢者ケアの意思決定プロセスに関するガイドライン[3]のなかで「高齢者のケアの意思決定に本人の予後を見通して，全体として延命が QOL 保持と両立しない場合には，医学的介入は延命ではなく QOL を優先する」という指針が出された。このような背景から NHCAP ガイドラインでは，肺炎の診療方針決定の最初の段階で，患者自身の QOL を考慮して，人工呼吸器管理を含めた集中治療を行うか否か，選択する「治療区分」という概念を設けている。

こうした肺炎医療を取り巻く背景因子から，肺炎の罹患は単なる感染症ではなく，社会的に，患者の QOL を低下させ，医療費もかかり，かつ抗