アメーバに対しては共生関係となる性質が、ヒトを含め他の生物に対しては同様であるかどうか、今後の議論になると思われる。その意味でもアメーバ共生体研究は原因不明疾患例の原因解明の一助になること、さらには新興感染症への備えとなることが期待される。

E.結　論

環境中に生息する *Acanthamoeba* の共生体として、ヒトの呼吸器疾患との関連性が知られる *Parachlamydia* 科の共生体を含む多様な共生体を検出した。共生アメーバは安定した共生関係を維持し、アメーバに対する病原性は極めて低いものと想定されたが、ヒトへの健康に及ぼす影響は *Parachlamydia* 科の共生体を始めとして、エビデンスの蓄積が重要と考えられた。

F. 参考文献

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G. 健康危惧情報

なし

H. 研究発表

なし

I. 知的財産権の出願・登録状況

なし

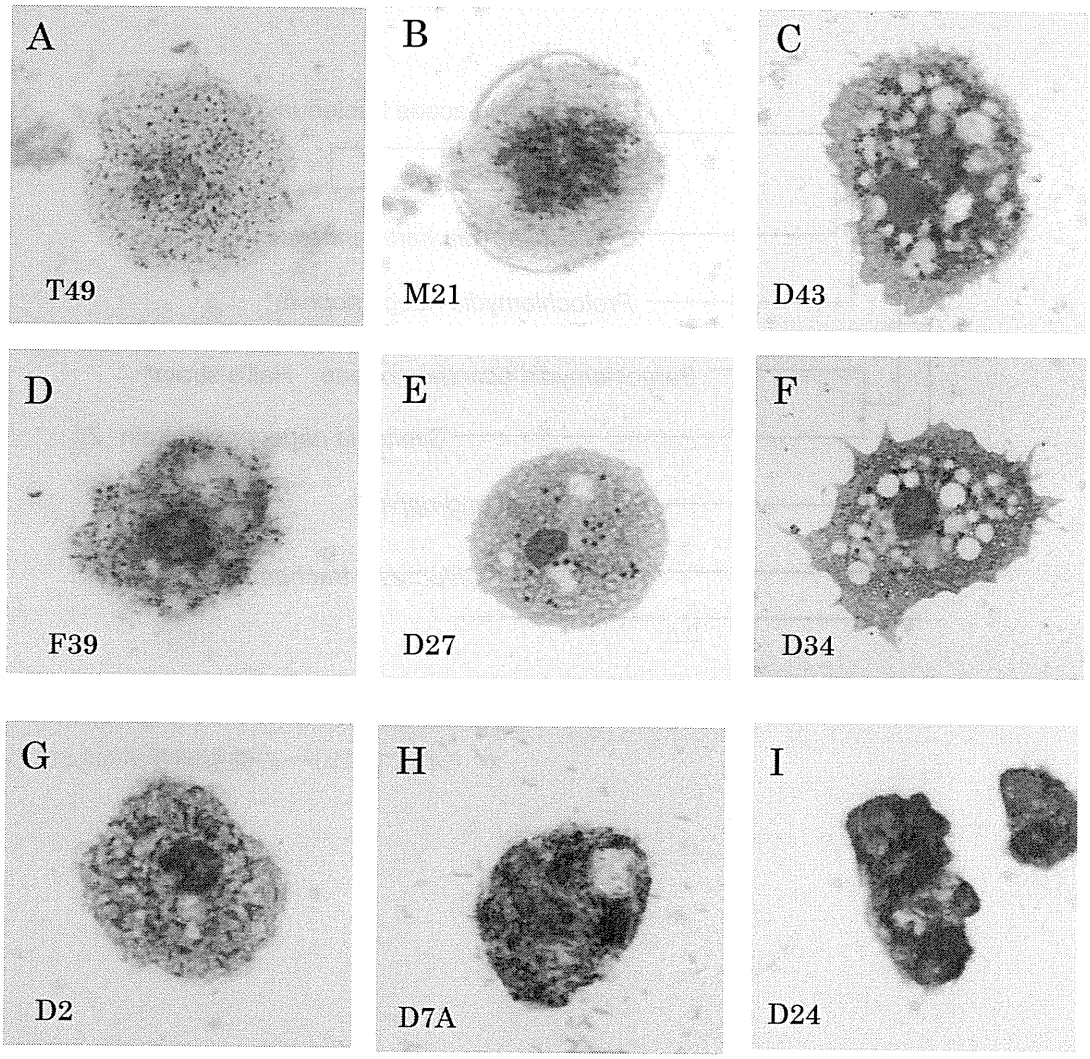

図-1、検出された*Acanthamoeba*共生体のギムザ染色像

顕微鏡倍率 1,000 倍

表-1、アメーバの分離環境と検出された共生体の BLAST 検索結果

環境	BLAST で共生体と高い相同性を示した微生物(本研究で調べた共生体)
温水 (温泉等)	Parachlamydiaceae bacterium CRIB38 (MZ7,MZ21,MZ23,T49,YM2) *Criblamydia sequanensis* (G5,F39,M21,Z32)
ハウスダスト	*Propionibacterium acnes* (D2,D5,D10,D42,D50) *Sinorickettsia chlamys* (D7A,D53) *Methylophilus* sp. (D24) Endosymbiont of *Acanthamoeba* sp. R18 (D27,D29,D33) Endosymbiont of *Acanthamoeba* sp. UWC22 (D34A) *Neochlamydia* sp. CRIB37 (D43)

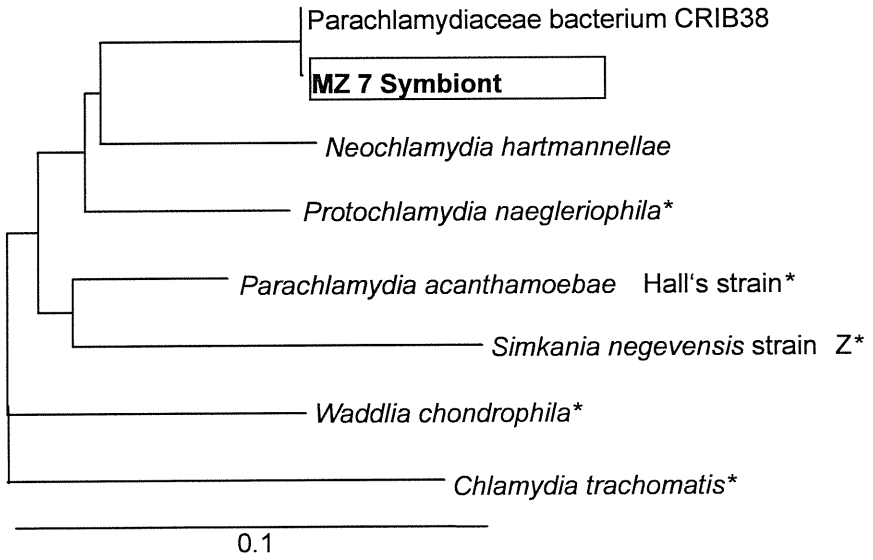

図-2、16SrRNA 遺伝子解析に基づくクラミジア関連微生物の系統関係

＊ これまでにヒトへの健康影響が知られるもの

III.　研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Taguri T, Oda Y., Sugiyama K., Nishikawa T, Endo T, Izumiyama S, Yamasaki M., and Kura F	A rapid detection method using flow cytometry to monitor the risk of legionellosis in bath water.	J. Microbiol. Methods	in press		2011
Matsui M, Fujii S, Shiroiwa R, Amemura-Maekawa J, Chang B, Kura F, Yamauchi K	Isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila.*	J. Med. Microbiol.	59(10)	1242-1246	2010
Amemura-Maekawa J, Kura F, Helbig JH, Chang B, Kaneko A, Watanabe Y, Isobe J, Nukina M, Nakajima H, Kawano K, Tada Y, Watanabe H, and Working Group for *Legionella* in Japan	Characterization of *Legionella pneumophila* isolates from patients in Japan according to serogroups, monoclonal antibody subgroups and sequence types.	J. Med. Microbiol.	59(6)	653-659	:2010
前川純子、倉　文明	レジオネラ感染の分子機構と診断法の進歩	呼吸	30(2)	124-128	2011
倉　文明、常　彬、前川純子	レジオネラの環境中での生態とその迅速検出	化学療法の領域	26(12)	2385-94	2010
杉山寛治、小坂浩司、泉山信司、縣　邦雄、遠藤卓郎	モノクロラミン消毒による浴槽レジオネラ属菌の衛生対策	保健医療科学	59(2)	109-115	2010

IV.　研究成果の刊行物・別刷

Journal of Medical Microbiology (2010), 59, 1242–1246 DOI 10.1099/jmm.0.016089-0

Case Report

Isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila*

Mikio Matsui,[1] Shin-ichiro Fujii,[2] Rieko Shiroiwa,[3] Junko Amemura-Maekawa,[4] Bin Chang,[4] Fumiaki Kura[4] and Kohei Yamauchi[5]

Correspondence
Kohei Yamauchi
kyamauch@iwate-med.ac.jp

[1]Medical Corporation Matsui Internal Clinic, Iwate, Japan

[2]Iwate Prefecture Research Center for Environmental Health, Iwate, Japan

[3]Iwate Prefecture Ninohe Health Center, Iwate, Japan

[4]Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan

[5]Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka 020-8505, Japan

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We report what we believe to be the first clinical isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila*. *L. rubrilucens* strains were found in both a patient's sputum and the water of a hot spring in which the patient bathed, and DNA analysis by PFGE showed that they were indistinguishable.

Introduction

Since Brenner *et al.* (1979) isolated the bacterium that caused pneumonia in patients with Legionnaires' disease and named it *Legionella pneumophila*, more than 20 species of *Legionella* have been implicated in human diseases (Stout *et al.*, 2003). In 1980, an environmental strain of *Legionella rubrilucens* was isolated from tap water by G. W. Gorman (Brenner *et al.*, 1985). However, to date, isolation of *L. rubrilucens* has not been described from patients co-infected with *L. pneumophila*.

Here we report that *L. rubrilucens* strains were found in both a patient's sputum and the water of a hot spring in which the patient bathed, and DNA analysis performed by PFGE (Amemura-Maekawa *et al.*, 2005) showed that they were indistinguishable. To our knowledge, this is the first report suggesting that *L. rubrilucens* can co-infect humans infected with *L. pneumophila*. Since the progress of *Legionella* pneumonia is very rapid in general, it can be fatal without early diagnosis and treatment. In this study, due to the early diagnosis, *Legionella* pneumonia was treated successfully.

Case report

A 54-year-old Japanese man, height 165 cm, weight 72 kg, presented with a high fever, a feeling of weariness and pain in the joints. He had smoked 20 cigarettes a day for 34 years and had been drinking 350 ml beer and about 360 ml shouchu (Japanese liquor, alcohol concentration 25–30 %) daily for the past 10 years. He had been working as a caretaker for the elderly.

After continuous high fever for 2 days, he was admitted for treatment and examination of the cause of the fever. He still complained of weariness throughout the body without signs of pains in the joints, cough or sputum. His vital signs were stable with blood pressure 120/70 mmHg, pulse rate 120 beats min^{-1} and body temperature 39.2 °C. No rale was audible in either of the lungs. The Influenza A and B Antigen test was negative. Laboratory data showed a white blood cell count of 17 100 cells μl^{-1} (neutrophils 87.7 %) and a C-reactive protein value of 15.86 mg dl^{-1} in the serum. Urine analysis revealed that both a protein test and occult blood test were strongly positive (3+), assumingly due to an inflammatory response of the urinary tract from a bacterial infection. On the X-ray film, we observed minimal infiltrative shadows on the left middle and lower lobes.

After admission, his febrile state did not change despite the administration of flomoxef at 2 g per day. On the 3rd day, he started to have a dry cough and fine crackles were audible in the left lower lobe. We found extended infiltrative shadows on the left middle and lower lobes in the chest X-ray (Fig. 1). The oxygen saturation by pulse oximetry (SpO_2) had declined to 93 % at this point.

During admission, it was reported that he had bathed in a hot spring twice, 11 days and 2 days before admission, and

Abbreviation: CAM, clarithromycin.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the clinical *Legionella rubrilucens* isolate is AB537503.

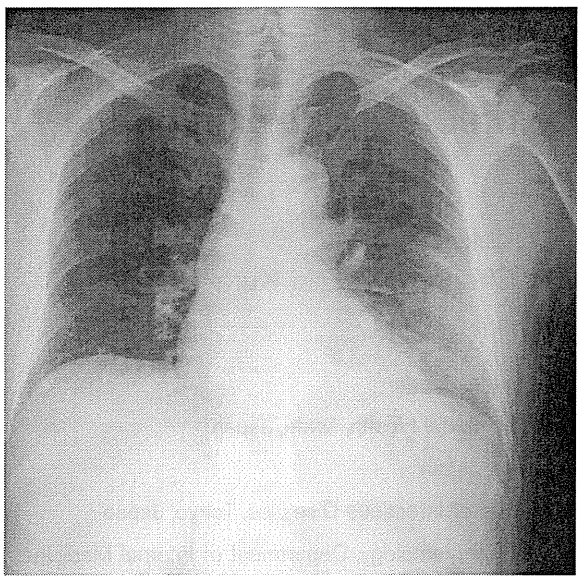

Fig. 1. Chest X-ray film taken on the 3rd day of admission showing ground glass shadows in the left middle and lower lung fields.

it was suspected that he might be infected with *Legionella* from the hot-spring water. A urine antigen test for *Legionella* (Biotest AG) was positive. Furthermore, we observed two *Legionella*-like colonies on GVPC plates (BCYE supplemented with glycine, vancomycin, polymyxin B and cycloheximide; Oxoid) from sputum obtained on the 4th day after admission. The *Legionella*-like colonies demonstrated red autofluorescence under 365 nm UV light and were identified as *Legionella* species (non-*L. pneumophila*) by a standard block-based PCR using LEG primers targeting the 16S rRNA gene (for the detection of members of the *Legionella* genus; Yamamoto *et al.*, 1993) and *Lmip* primers targeting the *mip* gene (for detection of *L. pneumophila*; Mahbubani *et al.*, 1990). The presence or absence of amplified products was determined following gel electrophoresis and the above colonies yielded a band of the expected size with the LEG primers but no band with the *Lmip* primers. Subsequently, the colonies were identified as *L. rubrilucens* using a DNA–DNA hybridization kit (Kyokuto Pharmaceutical Industrial) at the Iwate Prefecture Research Center for Environmental Health and by sequencing of the 5′-region of the 16S rRNA gene (100 % identity with the type strain WA-270A-C2; accession no. of the clinical isolate is AB537503; 488 bp in length) at the National Institute of Infectious Diseases in Japan. Unfortunately, no *L. pneumophila* strain was isolated from sputa. Real-time PCR of the remaining sputum targeting both the 5S rRNA gene for detection of the *Legionella* genus and the *L. pneumophila*-specific *mip* gene (CycleavePCR *Legionella* Detection kit; Takara Bio) was performed according to the supplier's instruction, using the SmartCycler System (Cepheid). For DNA extraction, the sputum was treated with 2 % Triton X-100 and 1 mg proteinase K ml^{-1} and purified using a MonoFas Column for the *Legionella* genome (GL Sciences). The detection limits for *L. pneumophila* were 1.2 c.f.u. per reaction when targeting the 5S rRNA gene, and 12 c.f.u. per reaction when targeting the *mip* gene, using the purified DNA of *L. pneumophila* 80-045 (Saito *et al.*, 1981). It was also confirmed that the detection limit for *L. rubrilucens* was 320 c.f.u. per reaction when targeting the 5S rRNA gene. About 200 µl sputum was used for DNA extraction. Each reaction sample (25 µl) contained 5 µl purified DNA. All reaction samples were negative except for one sample in which 5S rRNA DNA equivalent to 2 c.f.u. *L. pneumophila* was detected although *mip* DNA was negative. No inhibition was found throughout the test. The results suggested that DNA from *Legionella* species was present in the sputum sample at very low levels.

By environmental investigation conducted on the 9th day of admission, a sample from the bath water of the hot spring was collected. The chlorine concentration of the water was 0.1 mg l^{-1}, and *L. rubrilucens* and *L. pneumophila* were isolated from the sample at 60 c.f.u. per 100 ml and 40 c.f.u. per 100 ml, respectively. Bathwater was drained and changed every day and some bathtubs were equipped with hydrotherapy jet circulation. The *L. pneumophila* strain was identified by PCR and was determined as serogroup 15 by the Dresden panel of monoclonal antibodies (Helbig *et al.*, 1997). Monovalent antisera specific for *L. pneumophila* serogroups 1–15 (Denka Seiken) were non-reactive. Using the antisera, some strains of *L. pneumophila* assigned to serogroups 4, 10 or 15 by the Dresden panel have been grouped into non-reactive strains (unpublished data). The sequence type of the *L. pneumophila* was *flaA* (10), *pile* (10), *asd* (7), *mip* (28), *mompS* (16), *proA* (18), *neuA* (6) (Gaia *et al.*, 2005; Ratzow *et al.*, 2007). Because this was a new profile, it was sent to the EWGLI-SBT database (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php) and assigned the number ST768. The DNA restriction profile of *L. rubrilucens* from the patient's sputum and the hot-spring water revealed that the isolates were indistinguishable (Fig. 2a), while epidemiologically unrelated *L. rubrilucens* strains showed different profiles (Fig. 2b).

Subsequently, using a Biotest enzyme immunoassay, we examined the reactivity of *Legionella* soluble antigens (Okada *et al.*, 2002) from heat-killed McFarland 4 cell suspensions of the clinical and environmental *L. rubrilucens* strains and an environmental *L. pneumophila* strain. *L. pneumophila* serogroup 15 soluble antigen, but not *L. rubrilucens* soluble antigen, showed a positive enzyme immunoassay reaction, suggesting that the positivity of the urine *Legionella* antigen test might be due to the infection with the serogroup 15 *L. pneumophila* strain. Indirect fluorescence antibody titres against *L. rubrilucens* and *L. pneumophila* isolates of serum obtained 3 months after infection were examined, but they were very low ($\leqslant$1 : 16, interpreted as a negative response). The in-house formalin-killed *L. pneumophila* serogroup 1 Philadelphia-1 (type strain) was used as a control antigen. A titre of 1 : 256 positive antiserum was used as a control.

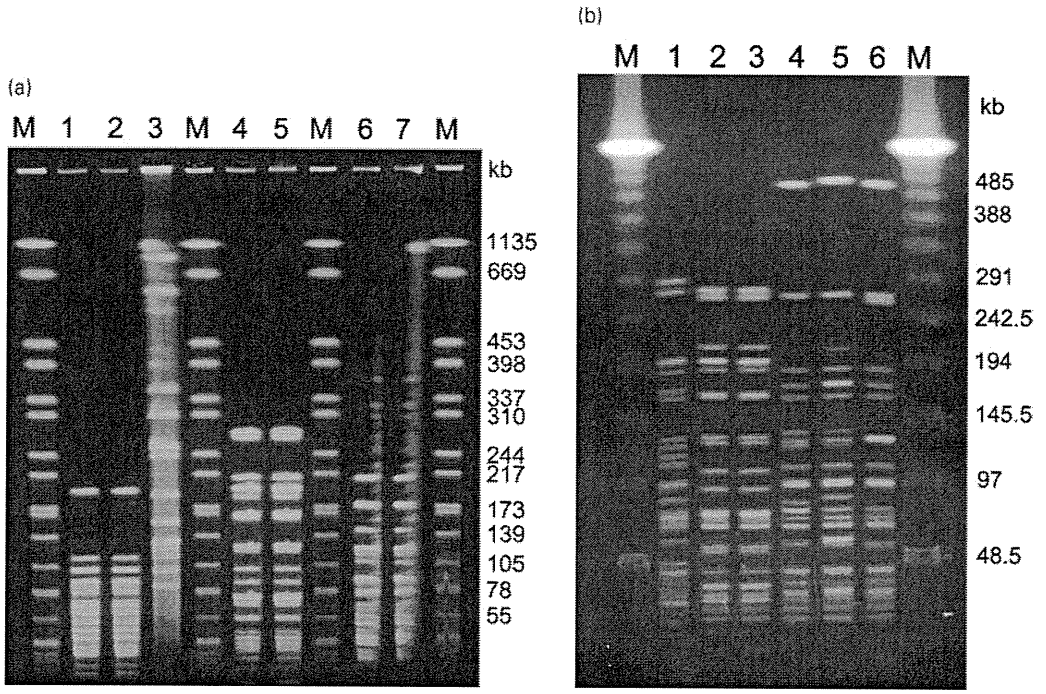

Fig. 2. (a) Restriction profiles of *L. rubrilucens* digested with *Sfi*I, *Xba*I and *Not*I using PFGE. The *L. rubrilucens* isolates from the patient's sputum and the hot-spring water were analysed. For the electrophoresis, a linearly ramped switching time from 5 to 50 s was applied for 19 h at 6 V cm^{-1} and 14 °C. Lanes: M, molecular marker (*Salmonella* Braenderup, *Xba*I digestion); 1, 4 and 6, *L. rubrilucens* from the patient's sputum; 2, 5 and 7, *L. rubrilucens* from the hot-spring water; 3, *L. pneumophila* serogroup 15. Lanes 1, 2 and 3, *Sfi*I digestion; lanes 4 and 5, *Xba*I digestion; lanes 6 and 7, *Not*I digestion. (b) Restriction profiles of *L. rubrilucens* isolates from the patient, the hot-spring water and unlinked environments digested with *Xba*I. A linearly ramped switching time from 0.3 to 30 s was applied for 16 h at 6 V cm^{-1} and 14 °C. Lanes: M, molecular marker (lambda ladder); 1, *L. rubrilucens* WA-270A-C2 (ATCC 35304); 2, *L. rubrilucens* from the patient; 3, *L. rubrilucens* from the hot-spring water; 4, *L. rubrilucens* NIIB 0335 (Thai NIH 8211) from cooling tower water; 5, *L. rubrilucens* NIIB 0337 (Thai NIH 8383) from cooling tower water; 6, *L. rubrilucens* NIIB 0345 (Thai NIH 8568) from cooling tower water.

From the 4th day of admission, we administered clarithromycin (CAM) at 400 mg per day and meropenem at 1 g per day. After 3 days of treatment, since the fever alleviated, we stopped meropenem but continued CAM. Finally, by the 10th day of administration, the patient's clinical symptoms had improved, and the laboratory data such as white blood cell count and C-reactive protein value, and the shadows in chest X-ray, returned to normal on the 13th day of administration.

Discussion

The causative agent of *Legionella* pneumonia is the genus *Legionella*, which is commonly found in hot springs, soil and water supply facilities. *Legionella* species accounted for 3–10 % of causative pathogens for all community-acquired pneumonia cases in a worldwide survey (Miyashita *et al.*, 2006).

Since a new infectious diseases control law in Japan was enacted in April 1999, legionellosis cases classified as Category-4-notifiable infectious diseases must be notified to the proper agencies as well as to the prefecture governor (Ministry of Health, Labour and Welfare in Japan, 1999). In the present case, since the urine *Legionella* antigen was positive, we reported this legionellosis case to the local health centre. In order to elucidate the source of the infection, *Legionella* was cultivated from the patient's sputum and the hot-spring water and DNA analysis of the *L. rubrilucens* strains by PFGE was performed. The clinical and environmental *L. rubrilucens* strains were indistinguishable. A final diagnosis is recommended to be made by criteria that include (a) isolation of *Legionella* from clinical specimens, (b) detection of *Legionella* DNA by PCR in clinical specimens, (c) a positive response for urine *Legionella* antigen and (d) an increase in serum anti-*Legionella* antibody titres. The final diagnosis was made by (a), (b) and (c) in our case. Urinary antigen detection is a rapid and easy test and can detect most cases of legionellosis caused by *L. pneumophila* serogroup 1. However, without isolation of clinical strains, the source of infection cannot be definitely confirmed and, as found in our study, the possibility of mixed infection remains unrecognized. Therefore, clinical specimens for *Legionella* isolation should always be cultured.

There has only been one report of *L. rubrilucens* infection as far as we know (Berger *et al.*, 2006). In that study, two patients had increased serum titres for the bacterium: one patient with seroconversion (high level of evidence, fourfold increase in antibody titre between acute and convalescent-phase serum samples or seroconversion from 0 to 1 : 100) and one patient with single high titre (low level of evidence, >1 : 400) for *L. rubrilucens* among 18 patients where infectious amoeba-associated agents were identified in a study of 157 intensive-care units patients with 210 episodes of pneumonia. However, to our knowledge, a culture-positive case of *L. rubrilucens* has never been reported.

In the present case, the patient was a healthy middle-aged man with no underlying disease. Since at first the patient had fever, shivering, weariness all over the body and muscle pain but no respiratory symptoms, we suspected that he was suffering from influenza. On the 3rd day of hospitalization, we heard a fine crackle sound in the left middle and lower lobes, and then we decided to reconsider the cause of his symptoms. After learning that the patient had been bathing in a hot spring, we examined the possibility of *Legionella* infection. This case emphasizes the importance of getting precise information about the patient's current history to detect unknown causes of pneumonia.

It is not easy to prove the pathological role of *L. rubrilucens* in the pneumonia in this case. The patient was thought to be apparently healthy; however, he had smoked for 34 years and had a habit of alcohol drinking. Both smoking and drinking alcohol are thought to be risk factors for infection. In this regard, it can be suggested that the patient was at risk of developing legionellosis when he was exposed to *Legionella* in the hot-spring water. *L. rubrilucens* was detected from the sputum obtained at least 6 days after bathing in the hot-spring water, suggesting that *L. rubrilucens* in the sputum was not merely exhibiting colonization and that this was a dual infection with *L. pneumophila* and *L. rubrilucens*. It is likely that the *L. pneumophila* was the primary infecting agent in the patient. Alternatively, *L. pneumophila* infection and temporal colonization by *L. rubrilucens* from spa bath water was possible.

In the early stages of *Legionella* pneumonia, some patients present various symptoms such as fever, cough, sputum, diarrhoea, impaired level of consciousness, etc., without respiratory symptoms (Yagyu *et al.*, 2003). In the present case, the patient complained only of fever and joint pain without respiratory symptoms. In two large outbreaks of legionellosis in Japan, the patients presented light or no respiratory symptoms at the first visit (Yagyu *et al.*, 2003; Sasaki *et al.*, 2008). In *Legionella* pneumonia, infiltrative shadows often appear 3 days after the first visit (Kirby *et al.*, 1979; Kroboth *et al.*, 1983). Taking into account these findings, it is important to note that in the early stages of *Legionella* pneumonia, some patients may have no respiratory symptoms.

Concerning the shadows in the chest X-rays of patients with *Legionella* pneumonia, interstitial shadows, infiltration and consolidation have been reported (Kirby *et al.*, 1979; Kroboth *et al.*, 1983). Pulmonary infiltrates are predominantly seen in the lower lobe, sometimes bilaterally, and there is rapid progression and pleural effusion (Kroboth *et al.*, 1983; Dietrich *et al.*, 1978; Tan *et al.*, 2000). High-resolution computed tomography also revealed that air-space consolidation, ground-glass opacity and pleural effusion were common features in patients with Legionnaires' disease and that the shadows show a peripheral and bilateral distribution in multiple segments (Yagyu *et al.*, 2003; Matsumoto *et al.*, 2008). In our case, we found ground-glass or light infiltrative shadows in the left middle and lower lobes.

Treatments for *Legionella* pneumonia have been described in the guidelines for the management of adults with community-acquired pneumonia (Miyashita *et al.*, 2006; Niederman *et al.*, 2001; Bartlett *et al.*, 1998; Committee for The Japanese Respiratory Society Guidelines for the Management of Respiratory Infections, 2006). Macrolides including erythromycin, CAM, etc., have been recommended for the treatment of *Legionella* pneumonia. In our case, the patient recovered completely with 13 days of CAM at 400 mg per day. Although *Legionella* pneumonia tends to progress rapidly to severe pneumonia, many patients can be cured if given appropriate early therapy.

In conclusion, in the present case, *L. rubrilucens* was isolated from the patient's sputum and from hot-spring water by culturing, and PFGE revealed that the isolates from these two sources were indistinguishable. To our knowledge, this is the first case in which human airway infection or colonization by environmental *L. rubrilucens* has been confirmed.

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Characterization of *Legionella pneumophila* isolates from patients in Japan according to serogroups, monoclonal antibody subgroups and sequence types

Junko Amemura-Maekawa,[1] Fumiaki Kura,[1] Jürgen H. Helbig,[2] Bin Chang,[1] Akiko Kaneko,[3] Yuko Watanabe,[4] Junko Isobe,[5] Masafumi Nukina,[6] Hiroshi Nakajima,[7] Kimiko Kawano,[8] Yuki Tada,[9] Haruo Watanabe[1] and the Working Group for *Legionella* in Japan†

Correspondence
Junko Amemura-Maekawa
jmaekawa@nih.go.jp

[1]Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan
[2]Institute of Medical Microbiology and Hygiene, TU Dresden, Dresden, Germany
[3]Yamagata Prefectural Institute of Public Health, Yamagata, Japan
[4]Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan
[5]Toyama Institute of Health, Toyama, Japan
[6]Public Health Research Institute of Kobe City, Hyogo, Japan
[7]Okayama Prefectural Institute for Environmental Science and Public Health, Okayama, Japan
[8]Miyazaki Prefectural Institute for Public Health and Environment, Miyazaki, Japan
[9]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan

We collected 86 unrelated clinical *Legionella pneumophila* strains that were isolated in Japan during the period 1980–2008. Most (80.2 %) belonged to serogroup 1, followed by serogroups 5, 3 and 2. Interestingly, the patients with *L. pneumophila* serogroup 1 had a significantly higher male-to-female ratio (12.4) than the patients with other *L. pneumophila* serogroups (2.0) (OR, 10.5; 95 % CI, 2.5–44.5). When the serogroup 1 strains were analysed by monoclonal antibody (mAb) typing, the most prevalent subgroup was Benidorm (34.9 % of all isolates). Moreover, 79.7 % of the serogroup 1 isolates were bound by mAb 3/1, which recognizes the virulence-associated epitope. When all 86 isolates were subjected to sequence-based typing (SBT) using seven loci, they could be divided into 53 sequence types (STs). The ST with the most isolates (seven) was ST1, to which most isolates from patients and environments around the world belong. However, six of the seven ST1 isolates were isolated before 1994. Other major STs were ST306 (n=6), ST120 (n=5) and ST138 (n=5). All ST306 and ST138 isolates, except for one isolate (ST306), were suspected or confirmed to be derived from bath water, which suggests that these strains prefer bath habitats. The sources of all ST1 and ST120 isolates remain unclear. By combining the SBT and mAb data, the 86 isolates could be divided into 59 types (discrimination index, 0.984). This confirms the usefulness of this combination in epidemiological studies.

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INTRODUCTION

Legionellosis is caused by *Legionella* species, which are environmental Gram-negative bacteria. To date, 52 species of *Legionella* have been described (Kuroki *et al.*, 2007). The species that is most commonly found in the environment and also causes the most disease is *Legionella pneumophila* (Yu *et al.*, 2002). To aid the epidemiological surveillance of legionellosis, *L. pneumophila* isolates can be divided into serogroups by antisera that recognize differences on the

†The members of the Working Group for *Legionella* in Japan are listed in Acknowledgements.

Abbreviations: EWGLI, European Working Group on *Legionella* Infections; SBT, sequence-based typing; ST, sequence type.

A supplementary table showing the sequence types, serogroups and monoclonal antibody subtypes of Japanese clinical isolates of *Legionella pneumophila* is available with the online version of this paper.

LPS molecules. At present, 15 serogroups have been identified (Brenner *et al.*, 1988). Serogroup 1 is responsible for the majority of human infections (Yu *et al.*, 2002) and its subgroups can be delineated by six monoclonal antibodies (mAbs) that recognize specific epitopes (Helbig *et al.*, 1997). *L. pneumophila* isolates can also be characterized by sequence-based typing (SBT) using the six loci (*flaA*, *pilE*, *asd*, *mip*, *mompS* and *proA*) proposed by the European Working Group on *Legionella* Infections (EWGLI; http://www.ewgli.org/) (Gaia *et al.*, 2005). Recently, to enhance the delineation of *L. pneumophila* strains, a seventh allele, *neuA*, has been added in SBT (Ratzow *et al.*, 2007).

In Japan, National Epidemiological Surveillance of Infectious Diseases data indicate that hot springs and public baths but not cooling towers are the major sources of *Legionella* infections (Infectious Disease Surveillance Center, 2000). Indeed, there have been four large outbreaks in public bath facilities (Kuroki *et al.*, 2009).

In our previous study (Amemura-Maekawa *et al.*, 2005), we analysed 27 epidemiologically unrelated *L. pneumophila* serogroup 1 isolates (ten from cooling towers, ten from public spas and/or hot spring baths, and seven from patients with public bath-related infections) from Japan by SBT using the six alleles proposed in 2005 by the EWGLI. The 27 isolates could be divided into 14 sequence types (STs). Notably, the 10 isolates from the cooling towers all showed the same allele types, namely *flaA* (1), *pilE* (4), *asd* (3), *mip* (1), *mompS* (1) and *proA*(1), whereas the public bath-derived isolates were more diverse.

Here, to further characterize clinical *L. pneumophila* isolates from Japan and to confirm the usefulness of the mAb- and SBT-based classification methods, we analysed 86 isolates by both typing methods.

METHODS

***L. pneumophila* strains.** We analysed 86 clinical isolates of *L. pneumophila* that were isolated in Japan during the period 1980–2008. Of these, 42 clinical *Legionella* isolates were from the *Legionella* Reference Center, which collects *Legionella* isolates obtained in six representative prefectural/municipal public health institutes of each district in Japan. The remaining 44 isolates were from the collection of the National Institute of Infectious Diseases, Department of Bacteriology I (NIIB). Of the 86 isolates, 84 were from unrelated cases. The remaining two isolates were obtained from the same patient but belonged to different serogroups (NIIB 2136 and NIIB 2137; Supplementary Table S1 in JMM Online). Nosocomial cases were defined as those in patients who had been hospitalized, and travel-associated cases were defined as those in patients who had spent at least one night away from home before onset of the symptoms. The incubation period was set to 2–10 days, but this depended on the discretion of the physician who notified a patient with legionellosis. Because previous studies (Infectious Disease Surveillance Center, 2000; Kuroki *et al.*, 2009) have indicated that the major sources of *Legionella* infections are public baths without taking into consideration whether or not the infected individuals had engaged in previous travelling or not, the relationship between travelling and legionellosis has not been under active consideration. Outbreaks were defined as two or more cases for which there was strong epidemiological evidence of a common source of infection, with or without microbiological evidence. The 86 isolates included one representative isolate from each of the 12 *Legionella* outbreaks; all of the outbreaks were community-acquired, of which four were major outbreaks (Kuroki *et al.*, 2009).

Serogrouping and mAb subgrouping. Serogrouping of the isolates was performed by slide agglutination tests using a monovalent serum for *L. pneumophila* serogroups 1–15 (Denka Seiken). Serogroup 1 and 5 isolates were then subtyped serologically by using mAbs as described previously (Helbig *et al.*, 1997).

DNA manipulation, sequencing and sequence typing. Genomic DNA was extracted by using a High Pure PCR Template Preparation kit (Roche Diagnostics) and amplified by using the GeneAmp PCR System 9700 (Applied Biosystems) and previously described reaction mixtures and conditions (Amemura-Maekawa *et al.*, 2005; Gaia *et al.*, 2005). Both strands of the amplicons were sequenced by a model 3100, 3130 or 3130xl ABI Sequencer (Applied Biosystems). The nucleotide sequences obtained were confirmed and the allele numbers were determined using the online Sequence Quality Tool of the EWGLI website (Underwood *et al.*, 2006). Putative novel variants were submitted to the site 'Sequence Quality Tool' for verification and assignment of new allelic numbers. New combinations of allelic numbers were also submitted to the curators via the EWGLI website for assignment of new ST numbers. In this study, the isolates that failed amplification of *neuA* (indicated allele number as '0') were not given ST numbers but were allocated arbitrary numbers that start with J (J1, J2, etc.). A minimum spanning tree was generated by BioNumerics software (version 5.1; Applied Maths) using as parameters the categorical coefficient of similarity and the priority rule of the highest number of single-locus variants.

RESULTS AND DISCUSSION

Patient age, gender and sources of isolates

The mean and median age of the 85 legionellosis patients (72 males, 10 females and 3 unknown cases) was 59.2 and 60 years, respectively (range 0–81; excluding the three patients whose ages were unknown). It has been shown previously that many more males than females contract legionellosis (Infectious Disease Surveillance Center, 2008; Neil & Berkelman, 2008; Ng *et al.*, 2008), although the reasons for this are unclear. There were seven nosocomial cases (8.2 %), eight travel-associated cases (9.4 %), 49 community-acquired cases (57.6 %) and 21 cases for which the source could not be identified (24.7 %). The source of infection for 35 of the 85 patients was suspected to be public bathing facilities (n=30, 35.3 %), many of which apparently had circulation systems and 25 of which used hot-spring waters, domestic baths (n=2), a shower (n=1), a humidifier (n=1) and a cooling tower (n=1). The sources of the infection for 16 of these 35 cases were indeed confirmed to be public baths (n=15) and a humidifier (n=1) by comparing the PFGE DNA patterns (Amemura-Maekawa *et al.*, 2005) of the clinical isolates to those of environmental isolates from the suspected origin of the infection at prefectural/municipal public health institutes. The sources of infection of the remaining 50 cases (61.2 %) are unknown. In addition, 12 isolates (14.1 %) were

derived from 12 individual outbreaks, of which 9, 1, 1 and 1 outbreaks occurred at public bath facilities (hot-spring waters were used at eight of the nine facilities and tap water was used at the remaining one), a nursing home (Maesaki *et al.*, 1992), the cooling tower of a waste-processing plant (Isozumi *et al.*, 2005) and the spa of a cruise ship (Kura *et al.*, 2006) (Supplementary Table S1), respectively.

Serogroups

The majority of the isolates (n=69, 80.2 %) belonged to serogroup 1, including 11 of 12 outbreak-derived isolates. Two, five, one, seven (including the remaining one outbreak-derived isolate), one and one isolate(s) belonged to serogoups 2, 3, 4, 5, 6 and 9, respectively (Supplementary Table S1). The majority of the male patients (87.5 %) were infected with serogroup 1 strains whereas only 40 % of the female patients were infected with serogroup 1 strains. Thus, the serogroup 1-infected patients were significantly more likely to be male (male : female ratio=12.4) than the patients infected with isolates belonging to other serogroups (male : female ratio=2.0; OR, 10.5; 95 % CI, 2.5–44.5). The reasons for this difference remain to be elucidated.

mAb subgroups

Of the 69 serogroup 1 isolates, 79.7 % (n=55) had the virulence-associated epitope that is recognized by mAb 3/1 and is not present on any other serogroups (Helbig *et al.*, 2002). Thus, of the 86 *Legionella* isolates, 64.0 % were mAb 3/1-positive, which is similar to the frequency of 66.8 % reported by the pan-European study. The 69 serogroup 1 isolates belonged to the Benidorm (44.9 %), Allentown/France (17.4 %), OLDA (14.5 %), Philadelphia (10.1 %), Knoxville (7.2 %), Oxford (4.4 %) and Bellingham (1.5 %) mAb subgroups. The distribution of mAb subgroups in Japanese isolates differs from that in pan-European isolates, which most frequently have the Philadelphia subgroup (28.5 %) and then the Benidorm subgroup (20.0 %) (Helbig *et al.*, 2002). Eleven of the 12 outbreak-derived isolates were mAb 3/1-positive (the single serogroup 5 outbreak-derived isolate lacked this marker).

STs

The 86 clinical isolates could be divided into 53 STs (discrimination index, 0.979) (Hunter & Gaston, 1988), though amplification of the *neuA* target failed for eight isolates of serogroups 2, 4 and 5 (Table 1 and Supplementary Table S1). The minimum spanning tree illustrates the distribution of the STs (Fig. 1). The tree has seven clonal complexes. The ST with the largest number of isolates was ST1 (seven isolates). ST1 is the most prevalent ST in the world (Borchardt *et al.*, 2008; Cazalet *et al.*, 2008; Harrison *et al.*, 2009; Kozak *et al.*, 2009; Reimer *et al.*, 2010), although a Canadian study has reported that the prevalence of ST1 clinical isolates has decreased dramatically during the past 12 years (Tijet *et al.*, 2010). Indeed, in our study, six of the seven known clinical ST1 strains were isolated before 1994, and it is now unusual to isolate clinical ST1 strains; thus, it is striking that the majority of environmental isolates from cooling tower water still belong to ST1 (Amemura-Maekawa *et al.*, 2005; Cazalet *et al.*, 2008). The next major STs were ST306 (six isolates), ST120 (five isolates) and ST138 (five isolates). Notably, all of the ST138 and ST306 isolates but one (ST306) were suspected or confirmed to be derived from public bath water, which suggests that these strains prefer bath habitats. Both ST138 and ST306 are unique to Japan according to data submitted to the EWGLI SBT database as of 12 January 2010. In contrast, the sources of all but one of the Japanese isolates belonging to ST1 and ST120 remain unclear (one isolate was suspected to come from shower water). Four isolates were ST23 strains, two of which (Supplementary Table S1, NIIB 292 and NIIB 374) were derived from two large public bath facility-associated outbreaks that affected hundreds of people (Nakamura *et al.*, 2003; Okada *et al.*, 2005). In Europe, clinical isolates often belong to ST23 (Borchardt *et al.*, 2008; Cazalet *et al.*, 2008). These major STs (ST1, ST23, ST120 and ST306), except for ST138, belonged to clonal complexes, and these STs were distinct from each other, with the exception of ST23 and ST120 (Fig. 1). ST430 and J2 had three isolates each, and the other seven STs had two isolates each (Table 1). The remaining 39 STs each had one isolate. Thirty-four of the 53 STs were unique to Japan as of 12 January 2010, including two SBT profiles that could not be assigned to STs because they failed *neuA* amplification (Table 1). With regard to the 12 outbreak-derived strains, two belonged to each of ST23, ST138 and ST139, and the remaining six belonged to different STs (ST2, ST36, ST89, ST142, ST434 and J4). Notably, for ST142, there was not only an outbreak-derived isolate but also another unrelated non-outbreak-derived isolate. Similarly, ST2, ST36 and ST89 isolates have also been found in patients elsewhere in Japan (unpublished results) and/or abroad, as Philadelphia 1 was typed to ST36 (Ratzow *et al.*, 2007).

The discrimination index (0.979) in our investigation was higher than that described in previous reports which were based on isolates from England and Wales (0.901; Harrison *et al.*, 2009), USA (0.946; Kozak *et al.*, 2009) and Canada (0.964; Reimer *et al.*, 2010). This may reflect the different infection sources in Japan: the main infection source was public bath water (Supplementary Table S1), whereas those of outbreaks in Europe were hot- or cold-water systems and cooling towers (Ricketts *et al.*, 2007). (There have been no reports for the dataset of the infection sources of sporadic cases in Europe, as far as we know.) The water of Japanese public baths is often obtained from hot springs. The characteristics of hot spring water, namely chemical features such as pH and temperature, are highly variable, whereas the water from hot- or cold-water systems and cooling towers tends to

Table 1. Sequence types of 86 Japanese clinical isolates of *Legionella pneumophila*

ST, Sequence type: seven-allele profile; J2 and J5, arbitrary numbers allocated to each unique six-allele profile without *neuA*; Others, STs each presented by only a single isolate.

ST	No. of isolates	%	mAb (no. of isolates)
Serogroup 1	69	80.2	
1	7	8.1	OLDA (6), Oxford (1)
306*	6	7.0	Benidorm
138*	5	5.8	Benidorm (4), Allentown/France (1)
120	5	5.8	Benidorm
23	4	4.7	Allentown/France (2), Philadelphia (1), Oxford (1)
122*	2	2.3	Benidorm
42	2	2.3	Benidorm
118*	2	2.3	Philadelphia
123*	2	2.3	Benidorm
139*	2	2.3	Allentown/France (1), Benidorm (1)
142*	2	2.3	Allentown/France
Others†	30	34.9	(Not shown)
Other serogroups (serogroup)	17	19.8	
430* (3)	3	3.5	
J2 (5)	3	3.5	Dallas (2), Cambridge (1)
J5 (5)	2	2.3	Dallas
Others‡ (2, 3, 4, 5, 6, 9)	9	10.5	(Not shown)
Total	86	100.0	

*Described only in Japan as of 12 January 2010.
†Nineteen of 30 STs only in Japan as of 12 January 2010.
‡Seven of nine STs were described only in Japan as of 12 January 2010.

have rather similar characteristics due to similar water treatment procedures in addition to the environmental selective pressure. Thus, reflecting the wider array of *Legionella*-suitable environmental niches in Japan, the Japanese clinical isolates may be more genetically variable than Western isolates. This idea is supported by previous reports from our laboratory, which showed that Japanese isolates derived from public baths differ genetically from Japanese isolates derived from cooling towers (Amemura-Maekawa *et al.*, 2005, 2008).

Combining sequence typing and mAb subgrouping

Some STs were composed of isolates belonging to the different mAb subgroups (and vice versa). Thus, six of the seven ST1 isolates were OLDA subgroup (n=6) and the remaining ST1 isolate was an Oxford subgroup, while two of the four ST23 isolates were Allentown/France subgroup and the other two were Philadelphia and Oxford subgroups. All six ST306, all five ST120 and four of the five ST138 isolates were Benidorm subgroup. Moreover, in six of the seven STs, each of which consisted of two isolates, both isolates belonged to the same subgroup. By combining the SBT and subgrouping data, we could divide the 86 isolates into 59 types (discrimination index, 0.984) (Table 1 and Supplementary Table S1).

Importance of *Legionella* isolation

Based on the Infectious Diseases Control Law, legionellosis in Japan has been classified as a category IV notifiable infectious disease and has been monitored by the National Epidemiological Surveillance of Infectious Diseases (NESID) since 1999. Consequently, in Japan, physicians must notify the authorities about legionellosis cases. In 2008, 884 legionellosis cases were reported, which represents a remarkable fivefold increase in the previous 5 years (Infectious Disease Surveillance Center, 2008). However, this increase is thought to be due to the widespread use of a highly accurate urine antigen assay, which is easier to perform than the laborious and time-consuming process of isolating *Legionella* from the patients. From January 2003 to September 2008, 2460 legionellosis cases were reported, of which only 97 cases were diagnosed by the isolation of *Legionella* (Infectious Disease Surveillance Center, 2008). We collected 29 isolates during the same period. Therefore, our study may not entirely faithfully delineate the strains responsible for legionellosis in Japan. Health workers should isolate the bacterium from the patient and identify the infection source by genotyping the organism and comparing this genotype to those of environmental isolates from the surroundings of the patient.

The legionellosis incidence in Japan did not vary in a seasonal fashion (Infectious Disease Surveillance Center,

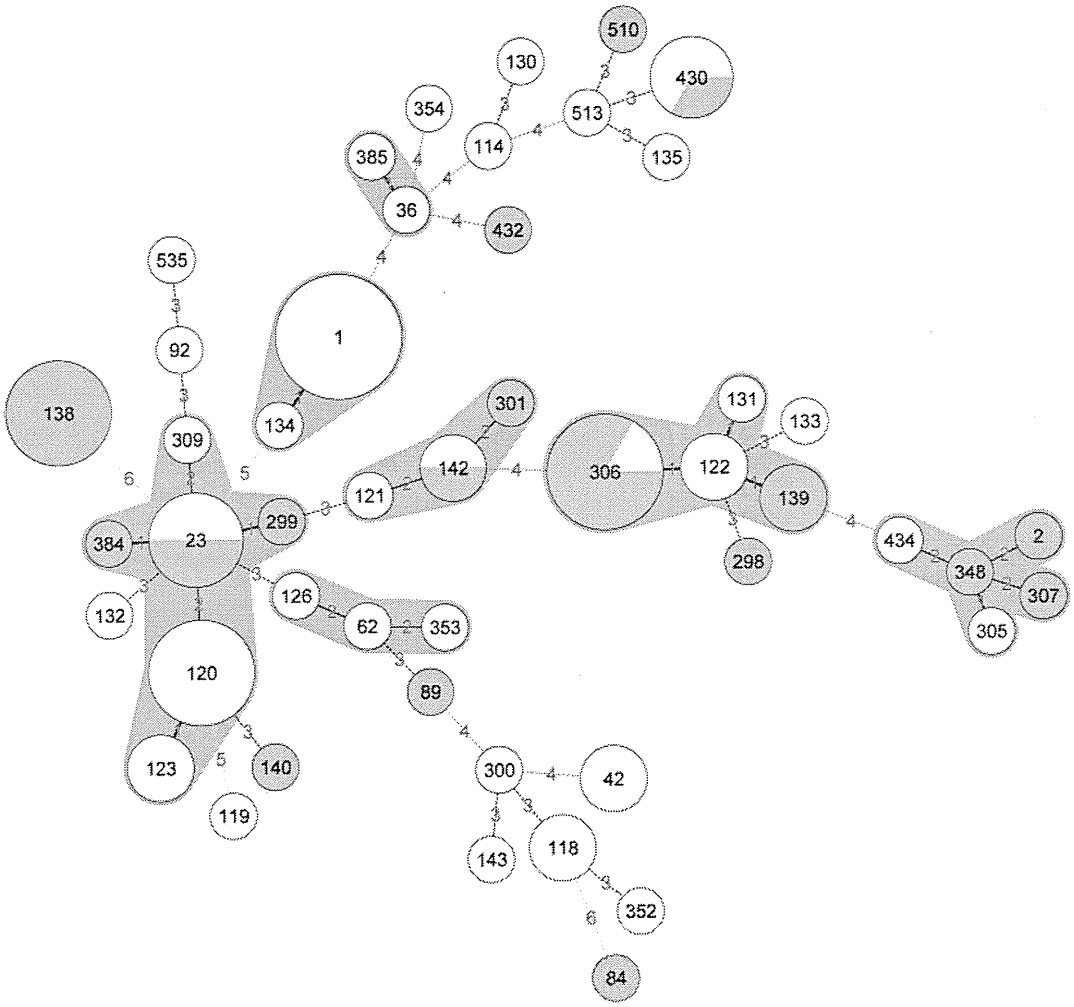

Fig. 1. Minimum spanning tree showing how the *L. pneumophila* clinical isolates, with seven determined alleles, are distributed in terms of their STs. The ST number is shown in the circle and the size of the circle indicates the number of isolates. Short thick lines connect single-locus variants, thin lines connect double-locus variants, black broken lines connect triple-locus variants and grey broken lines connect more than three different STs. The grey parts of the circles (pie charts) indicate the clinical isolates associated with bath water. The clonal complexes that were generated with single-locus variants and double-locus variants are indicated by the shaded backgrounds.

2003) until 2005, which is when the number of legionellosis cases started peaking in July (Infectious Disease Surveillance Center, 2008). This seasonality relates to humidity: rainfall increases microbial contamination in source waters or water distribution systems (Fisman *et al.*, 2005; Ng *et al.*, 2008). However, the epidemiological profile of sporadic legionellosis remains poorly understood. It has recently been proposed that legionellosis can be acquired from puddles on asphalt roads on rainy days (Sakamoto *et al.*, 2009a) and from the air-conditioning systems of motor cars (Sakamoto *et al.*, 2009b). It is likely that there are many as yet unrecognized infection sources of *Legionella*. It is possible that groups of isolates with particular genotypes inhabit distinct infection sources including unrecognized sources.

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解　説　基　礎

レジオネラ感染の分子機構と診断法の進歩

前川　純子　　倉　文明

要　旨　レジオネラ属菌は本来アメーバや繊毛虫の細胞内で増殖する環境細菌であるが，経気道感染し，“accidental pathogen”としてヒトのマクロファージ内で増殖することができる。遺伝子解析技術の進展に伴い，代表菌種である*Legionella pneumophila*について病原性の解明が進んだ。*L. pneumophila*は，本来の宿主である原生生物から水平伝播により獲得したと考えられる100種類以上のエフェクタータンパク質を宿主細胞内に分泌して，自己の増殖に有利になるように宿主の細胞伝達系を操作するという複雑な機構を有する。また，菌株間の遺伝的多様性が極めて高く，エフェクタータンパク質のレパートリーが株間で大きく異なっていることが分ってきた。臨床分離株の遺伝子型別の結果からもその多様性が明らかになり，遺伝子型別法は感染源の解明にも有用だと考えられる。

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はじめに

レジオネラ症の起因菌であるレジオネラ属菌は本来，河川，湖沼，土壌などに生息し，環境中では自由生活性アメーバや繊毛虫などの細菌捕食性単細胞生物の細胞内に寄生し，増殖する。本菌を含むエアロゾルをヒトが吸い込み，経気道感染を起こすことがある。その場合，アメーバと似た食作用をもつ肺中のマクロファージのなかで増殖し，肺炎が引き起こされる(レジオネラ肺炎)。感冒様の症状のポンティアック熱という病態で留まる場合もある。ヒトからヒトへの感染はない。

レジオネラ属菌の代表菌種である*Legionella pneumophila*は，1976年7月に米国フィラデルフィアのホテルで開催された在郷軍人会(the American Legion)の州総会で起きた重症肺炎の集団感染の起因菌として発見された[1]。近年，水冷式空調設備，給湯設備等の人工水系が発達してきたが，それらの適切な衛生管理が行われなかった場合，本菌の増殖を招き，レジオネラ症の発生へとつながる。したがって都市生活の人工水系に起因するとされるレジオネラ症が認識されているのは先進国のみであり，発展途上国での実態は殆ど不明である。米国での集団感染もホテル空調の冷却塔水が感染源であった。その後，現在までに50種以上のレジオネラ属菌が同定されているが[2]，本菌種の半数以上は環境中からのみ分離されており，ヒトへの病原性が報告されているのはおよそ4割である。レジオネラ症の起因菌のおよそ9割が*L. pneumophila*で，さらにその8割以上が15ある血清群のうちの血清群1によるものである[3]。

Ⅰ．病原性

レジオネラ属菌の病原性については*L. pneumophila*での解析が進んでいる[4]。病原性の本質はマクロファージ内での殺菌抵抗性にある。*L. pneumophila*がマクロファー

Molecular mechanism of *Legionella* infection and improved diagnosis of legionellosis
国立感染症研究所細菌第1部
Junko Amemura-Maekawa and Fumiaki Kura
Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

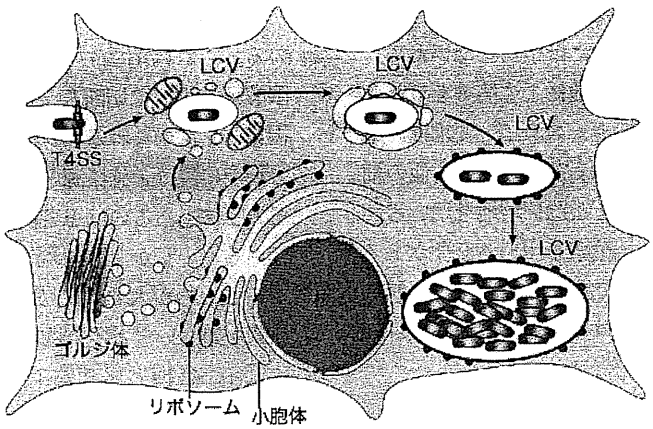

図1 *L. pneumophila* のマクロファージあるいはアメーバ内での増殖の様子
T4SS：type four secretion system, LCV：*Legionella*-containing vacuole
(Isberg RR, *et al*[5]. *Nature Reviews* 7:2009 より引用, 改変)

ジに取り込まれて形成される食胞(*Legionella*-containing vacuole, LCV)は，小胞体から分泌される小胞と融合し，小胞体マーカータンパク質を獲得し，初期においては酸性化やリソゾームとの融合が阻止され，殺菌されない。LCV の周囲にはミトコンドリアが接近している像も観察される。さらに *L. pneumophila* が増殖している LCV には粗面小胞体が集積し，LCV 膜にはリボソームが点在しており，栄養を供給していると考えられる。LCV は *L. pneumophila* が自ら作り出したその増殖にふさわしい環境であり，宿主細胞内が菌でいっぱいになるまで増殖が進むと LCV 膜および宿主細胞膜が破裂し，菌体が飛び出し，感染を広げてゆく(**図1**)[5)6)]。

L. pneumophila は感染に際して，自身の作るⅣ型分泌装置(T4SS)を用いて，エフェクターと呼ばれる複数のタンパク質を宿主細胞に分泌する。T4SS は *dot/icm* 遺伝子群にコードされる 20 数種のタンパク質からなる複雑な構造体で，菌体の細胞膜を貫通している。*dot/icm* 遺伝子群は，そこに突然変異が生じると，LCV の周りに細胞内小器官が集まってこなくなり(defect in organelle trafficking, *dot*)，宿主細胞内で増殖(intracellular multiplication, *icm*)できなくなることから，レジオネラの病原性に寄与する遺伝子群として見出された[7)8)]。*dot/icm* 変異体が病原性を失っているのは，その変異によって T4SS が不完全になり，宿主細胞に働きかけるエフェクタータンパク質が宿主細胞質に分泌されなくなるためである。エフェクタータンパク質の多くは，宿主膜輸送系にかかわる因子を様々な段階で制御するものである。似た機能をもつエフェクタータンパク質がゲノム上に複数コードされているため，突然変異体を用いた解析は進まなかったが，ゲノム解析技術の進展により，現在では 100 種類以上のエフェクタータンパク質が存在し，そのレパートリーは株により異なることが分ってきた[9)10)]。エフェクタータンパク質は真核生物特有のモチーフを有しているものが多く，本来の宿主であるアメーバや繊毛虫のゲノムから水平伝播により遺伝子を獲得したと考えられている[11)]。そのために宿主細胞の情報伝達系に働きかけることができ，*L. pneumophila* の増殖に都合のよい環境を作り上げる。

Ⅱ．臨　　床

レジオネラ肺炎は消化器症状を伴う率が高く，中枢神経症状を起こすこともある点などに特徴があるが，他の細菌性肺炎との区別は困難である。したがって，発症の 2～10 日前にレジオネラ属菌に汚染された水で経気道感染を起こす機会がなかったか，また，通常の細菌性肺炎治療薬が功を奏さないことなどがレジオネラ肺炎診断の契機となる。患者は 50 歳以上の高齢者が多く，小児は極めて少ない。また男性の患者が 8 割強を占め，その理由は不明だが，危険因子である喫煙，飲酒歴と関連があるという指摘もある。その他，糖尿病などの基礎疾患が危険因子となる。適切な治療を行わないと急速に悪化することがあるため，成人市中肺炎診療ガイドラインにおいては，エピソードにかかわらず，入院治療患者へのレジオネラ尿中抗原検査が奨励されている[12)]。

レジオネラ属菌はグラム陰性桿菌だが，細胞内寄生細菌であるため，塗抹標本ではグラム染色されにくく，ヒメネ