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

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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.

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 ml⁻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.

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

**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.

**Abbreviation:** CAM, clarithromycin.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of the clinical *Legionella rubrilucens* isolate is AB537503.
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. 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 1⁻¹, 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* strain was flaA (10), pili (10), *mip* (28), *mompS* (16), *proA* (18), *neaA* (6) (Gaia *et al.*, 2005; Ratzow *et al.*, 2007). Because this was a new profile, it was sent to the EWG-L/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 (≤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.
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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