Mixed Lung Infection by *Legionella pneumophila* and *Legionella gormanii* Detected by Fluorescent in Situ Hybridization

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Abstract

A mixed infection by *Legionella pneumophila* and a non-pneumophila *Legionella* species was detected in a lung biopsy specimen obtained from a patient with atypical pneumonia by fluorescent in situ hybridization (FISH). This result was confirmed by polymerase chain reaction (PCR). Sequencing of PCR products confirmed mixed infection by *L. pneumophila* and *L. gormanii*. Culture for *Legionella* spp. was negative and serology showed a rise only in IgG anti-*Legionella pneumophila* titer. To our knowledge, this is the first report of a mixed infection by *L. pneumophila* and a non-pneumophila *Legionella* species detected by FISH. Because FISH is a rapid and culture independent method that detects specific microorganisms in biopsy specimens it is recommended, in particular, for the detection of fastidious bacteria.

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Introduction

Legionella spp. are ubiquitously distributed organisms [1] which cause a wide spectrum of disease, including self-limited influenza-like syndrome (Pontiac fever) and pneumonia [2]. Pneumonia is the predominant clinical finding in Legionnaires' disease. *Legionella pneumophila* has been recognized as a common etiologic agent for both community-acquired [3] and nosocomially-acquired pneumonia [4].

L. pneumophila can be separated into more than 15 serogroups. About 90% of *Legionella* infections are caused by *L. pneumophila* [5]. Clinically relevant non-pneumophila species are *Legionella micdadei, Legionella bozemanii, Legionella dumoffii, Legionella longbeachae, Legionella feeleii,* and *Legionella gormanii* [6]. Water distribution systems are the primary reservoir for dissemination of the organism. Both epidemics of pneumonia and nosocomial infections by contaminated aerosols have been described [7, 8]. The exact percentage of *Legionella* pneumonia of total pulmonary infections is still unclear.

The risk for *Legionella* pneumonia increases with age and diseases such as cancer, cardiac insufficiency, chronic lung diseases, diabetes, and alcoholism. Men are affected twice as often as women [9]. Immunosuppression and surgery, with transplant recipients at highest risk, have been consistently implicated as predisposing factors for nosocomial infections by both *L. pneumophila* and non-*pneumophila* species [10–14].

If antimicrobial treatment is delayed, mortality rates range between 50 and 80%, whereas appropriate and early therapy reduces this rate to 5 to 10%. Use of steroids or other immunosuppressive drugs and the presence of other diseases such as cancer, diabetes or renal disease are significantly associated with increased mortality from Legionnaires' disease [15].

Because Legionnaires' disease is not specific in its clinical and radiologic presentation, specialized laboratory tests are necessary to establish the diagnosis. The standard method for diagnosis of *Legionella* infection is isolation of the organism from bronchoscopy or lung biopsy specimens [16]. The organism grows slowly and requires 3 to 5 days to form macroscopically visible colonies. Serologic detection of antibodies is also of little use for rapid diagnosis because 2 to 12 weeks are often required to detect an antibody response. Detection of urinary antigen is a rapid and simple diagnostic test since it is often easier to obtain urine than adequate sputum or endotracheal aspirate in affected patients. The drawback of this type of test is that it can remain positive for months after the episode of pneumonia. The common culture-independent tests for rapid detection of *Legionella* infection are direct antibody staining (DFA) and

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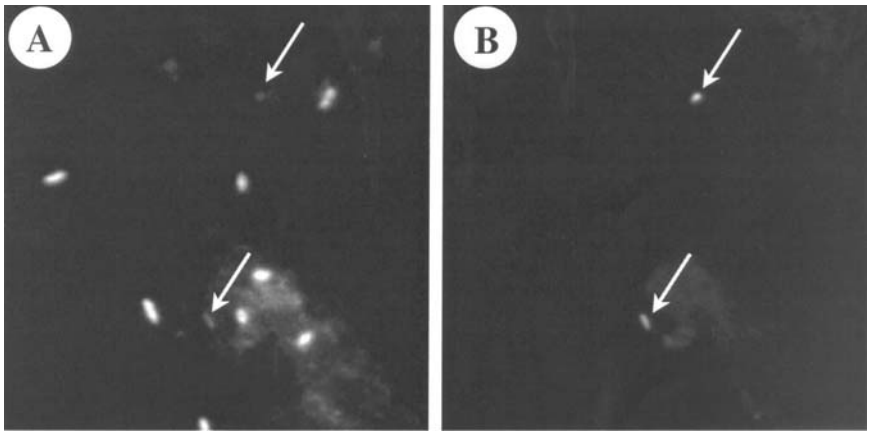

Figure 1. In situ hybridization of *L. pneumophila* in lung biopsy smears with LEG705 (B) and DAPI staining (A). Magnification × 1000.

polymerase chain reaction (PCR) [17]. The drawback of DFA is a cross-reaction with non-*Legionella* organisms [18]. Using these tests, it is not possible to tell if antibiotic treatment was successful because both living and dead organisms are detected.

Fluorescent in situ hybridization (FISH) of whole cells with rRNA-targeted oligonucleotide probes is a rapid method to identify microbial cells without cultivation [19, 20] and is a new alternative. Here we report a case of a mixed infection by *L. pneumophila* and a non-*pneumophila* species detected by FISH.

Patients and Methods

Case Report

A 75-year-old man with diabetes and former nicotine abuse underwent treatment for dermatosis. After ineffective therapy with corticosteroids for 3 months, treatment with methotrexate was started. Six weeks later he developed respiratory insufficiency. A chest radiograph showed a new infiltrate in the right upper lobe. The patient suffered from non-productive cough, fever (39 °C), and dyspnea. Subsequently, he developed bilateral basal infiltrates. Blood culture was sterile. Serology for *L. pneumophila* (serogroup 1–14) and non-*pneumophil*a species (including *L. gormanii*), *Mycoplasma*, and *Chlamydia* was negative (1:10 for *Legionella*, RIDA-Fluor Legionella IgG, r-biopharm, Darmstadt, Germany). Therapy with methotrexate was stopped, and antibiotic treatment with ceftazidime, gentamicin, and erythromycin was started. On day 6, fever subsided but the patient had to be intubated and a chest radiograph showed increasing interstitial infiltrates. Bronchoalveolar lavage was performed and *Candida tropicalis* (10^4/ ml) was cultivated. Serology gave no evidence of virus infection. Ceftazidime treatment was settled and an antimycotic treatment was started. On day 17, the anti-*L. pneumophila* serogroup 1 titer rose from 1:10 to 1:256. Antibiotic and antimycotic treatments were stopped and therapy with ciprofloxacin was started. On day 20, a lung biopsy specimen was obtained. The FISH technique and PCR detected *L. pneumophila* and a non-*pneumophila* species. Urinary antigen for *L. pneumophila* serogroup 1 was negative (Binax, Portland, United Kingdom). No microorganisms were grown from any of the samples. Pathology gave evidence of chronic consolidating pneumonia, possibly compatible with methotrexate treatment. In an endotracheal aspirate obtained on day 23, FISH was negative and PCR was positive only for *L. pneumophila* while DFA and culture were negative. A chest X-ray showed interstitial infiltrates and lung fibrosis. Rifampicin was added to the antibiotic treatment. On day 24, the patient died as a result of respiratory insufficiency.

Bacteria and Culture Conditions

The medium used in this study was buffered charcoal yeast extract medium supplemented with a-ketoglutarate (BCYE) [21]. Samples were inoculated both without prior incubation and after incubation at 56 °C for 10 min. A culture of the lung biopsy specimen was performed after grinding the sample with a sterile mortar and dilution in 1 ml 0.9% NaCl. Plates were incubated with 5% CO2 for 10 days and were checked daily by plate microscopy. Colonies with a morphology similar to that of Legionellaceae were subcultured on BCYE and on blood agar plates; those that grew on the former but not the latter medium were further characterized and tested by a DFA as previously described [22].

Antibody Detection

Serologic detection of antibodies was examined with an indirect fluorescent antibody test that covers three pools of polyvalent antigens (RIDA-Fluor Legionella IgG, r-biopharm, Darmstadt, Germany), performed according to the manufacturer's instructions. Pool I contains serogroup 1, 4, 6, 8, 10, 12, 14 of *L. pneumophila*, pool II serogroup 2, 3, 5, 7, 9, 11, 13 of *L. pneumophila,* pool III contains *L. bozemanii, L. dumoffii, L. gormanii, L. jordanis, L. longbeachae, L. micdadei*. A titer of > 1:16 was considered as negative and a titer of 1:64 – 1:256 as ambiguous. Diagnosis was based on a rise in antibody titer to >1:256 or a > 4-fold rise in subsequent sera samples.

Urinary Antigen

Detection of *L. pneumophila* serotype 1 antigen was performed with a specific commercial test (Binax, Portland, United Kingdom) following the manufacturer's protocol.

Direct Fluorescent Antibody (DFA) Staining

Aliquots of 20 µl of the sample were air-dried and heat-fixed on a microscopic slide. Then the sample was incubated for 10 min in 4% buffered formalin and washed for 10 min in distilled water. After air drying, the cells were incubated for 10 min in acetone and air-dried again. A portion of 20 µl of antibody against *L. pneumophila* serogroup 1 (Gull, Bad Homburg, Germany) was added to each well and incubated for 45 min. The slides were rinsed carefully with distilled water and washed twice with PBS buffer. Fluorescing cells were viewed with a microscope for epifluorescence microscopy (Leica, Heerbrugg, Switzerland), equipped with a standard filter set.

Fluorescent in Situ Hybridization (FISH)

The probes LEG705 and LEGPNE1 used for hybridization have been described previously [19, 23]. For whole-cell hybridization, bacteria were fixed with 4% paraformaldehyde phosphate-buffered saline solution at 4 °C for 1 h and washed once with phosphate-buffered saline (PBS). Cells were spotted on glass slides carrying

six wells (Paul Marienfeld KG, Bad Mergentheim, Germany) immediately before hybridization. Then the bacteria were dehydrated in an ascending ethanol series (50%, 80%, 100%). Fixed cells were hybridized by application of 10 µl of hybridization buffer (20% [vol/vol]) formamide, 0.9 M NaCl, 0.001% sodium dodecyl sulfate, 20 mM Tris-HCL (pH 8) containing 50 ng each of labeled probes LEG705 and LEGPNE1 on each well of the slide. As a positive control, *L. pneumophila* serogroup 1 (ATCC 33153) was used. The slides were incubated for 1.5 h in an isotonically equilibrated humid chamber at 46 °C. Then the hybridized cells were washed with washing buffer (20 mM Tris-HCL (pH 8), 0.01% sodium dodecyl sulfate, 180 mM NaCl) and incubated 15 min at 48 °C. Finally, the slides were carefully rinsed with PBS and air-dried. Then the cells were stained with 0.001% DAPI (Sigma Aldrich, Deisenhofen, Germany). Citifluor (CitifluorLtd., London, United Kingdom) was used as a mounting medium on hybridized slides. Fluorescing cells were visualized with a Leica (Heerbrugg, Switzerland) TCS NT scanning confocal microscope equipped with a standard filter set.

PCR Amplification and Sequence Analysis

The DNA was isolated with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. *Legionella*-specific primers and PCR conditions have been described previously [24]. For sequencing of PCR products of the lung biopsy, partial-length rDNA was amplified with primer pairs LEG 1036r and LNPN 614 v2, LEG 1036r and LPNE 614v2, respectively. One of each primer was biotinylated in each of two reciprocal reactions. Single-stranded DNA was obtained for direct sequencing by using the streptavidin-coated magnetic bead separation technique [25]. Single-stranded DNAs were sequenced by the *Taq* cycle DyeDeoxy terminator method, combined with an ABI PRISM 373A automatic sequencer (PE Applied Biosystems, Weiterstadt, Germany) and 320 bp were compared by BLAST.

Results

Cultures for *Legionella* spp. of the lung biopsy specimen (obtained on day 20) and endotracheal aspirate (obtained on day 23) were negative. A urine sample obtained on day 20 contained no detectable *L. pneumophila* serotype 1 antigen. DFA of endotracheal aspirate was also negative (Table 1).

Antibody Detection

On day 5, serology for *Legionella* was negative (titer < 1:16). On day 17, an anti-*L. pneumophila* serogroup 1 titer of 1:256 and an anti-*L. pneumophila* serogroup 12 titer of 1:64 was detected. Serum samples from days 5 and 17 were tested in parallel. No rise of antibody titer to pool III for non-*pneumophila* species (including *L. gormanii*) was observed.

Fluorescent in Situ Hybridization

Gram staining of lung biopsy smears revealed no microbial cells. Staining with DAPI showed many rod-like particles distributed among the sample. Nine cells hybridized with LEG705 and LEGPNE1 and could therefore be identified as *L. pneumophila*. Two additional cells only bound LEG 705 for non-*pneumophila* species. Figure 1 shows two rods hybridized with LEG705 and rods stained with DAPI. Hybridization of the endotracheal aspirate (obtained on day 23) revealed no fluorescent cells. Staining with DAPI was negative as well.

PCR

PCR amplification of the lung biopsy specimen detected *L. pneumophila* and a non-*pneumophila* species. Sequencing of PCR products resulted in identification of *L. pneumophila* (M59157, 100% similarity) and *L. gormanii* (Z32639, 100% similarity), respectively. Other similar species were *L. bozemanii* (Z49718 97.5% similarity), *L. cherii* (X73404, 96.25% similarity), *L. dumoffii* (X73405 93.7% similarity) and *L. micdadei* (M36032 77.5% similarity). PCR analysis of the endotracheal aspirate resulted in a signal only for *L. pneumophila*.

Table 1
Timetable of microbiological tests and results of the patient infected with *L. pneumophila* and *L. gormanii*.

Day	Material	Test	Result
5	Serum	*Legionella* serology	Serogroup < 1 1:16 (titer)
6	Bronchoalveolar lavage	Culture	*C. tropicalis*
6	Serum	*Legionella* serology	Serogroup 1 1:256 (titer)
17	Lung biopsy	FISH/PCR	*L. pneumophila*/ Non-*pneumophila* species
20	Urine	Urinary antigen	Negative
23	Endotracheal aspirate	FISH	Negative
		PCR	*L. pneumophila*
		DFA	Negative
		Culture	Negative

FISH: Fluorescent in situ hybridization; DFA: direct antibody staining

Discussion

Because Legionnaires' disease is a severe infection, especially in immunocompromised patients, rapid diagnosis and efficient antibiotic treatment are essential to decrease mortality.

Our case of atypical pneumonia showed risk factors for *L. pneumonia* such as male gender, old age, ex-smoker, diabetes, and immunosuppressive therapy. Initially, the chest X-ray showed a single unilateral infiltrate; bilateral infiltrates were observed later. Serial chest X-rays of 35 patients with confirmed Legionnaire's disease gave evidence that a unilateral, unilobar infiltrate was, in general, the initial radiographic finding. Progression to consolidation or to new areas of involvement was typical. Every lobe was involved, but lower lobe involvement was typical [26]. Although the clinical findings in our patient were not specific, they correlate with the findings in confirmed *L. pneumonia* cases. The first evidence for Legionnaires' disease was given by serology on day 17 after the onset of symptoms. In the lung biopsy obtained on day 20, many rods were visible after DAPI staining, but hybridizing cells were rare. We assume that rods de-

tected with DAPI staining but not with the probes were Legionellaceae which had lost ribosomal RNA by antibiotic treatment. Such a DAPI-positive/FISH-negative phenomenon has been demonstrated with penicillin G-treated streptococci *in vitro* [27]. The presence of *L. pneumophila* and a non-*pneumophila* species was confirmed by PCR. Sequencing of PCR products gave evidence for a mixed infection by *L. pneumophila* and *L. gormanii*. Endotracheal aspirate obtained on day 23 contained no visible rods after DAPI staining and no cells were hybridized. However, PCR was positive for *L. pneumophila*. This finding was due to released DNA of antibiotic-killed legionellae. Interestingly, a serum antibody response was only mounted against *L. pneumophila* indicating that the amount or antigenicity of *L. gormanii* was not sufficient.

The cause of death of this patient still remains unclear because, in addition to the infection by legionellae, pathology provided evidence for lung damage caused by methotrexate. We assume the damage to the lung had impaired penetration of the antibiotics into the infected tissue and that death was a result of combination of methotrexate injury and *Legionella* infection. Because of the immunosuppressive therapy with lung damage, prognosis of this patient was poor.

In conclusion, we demonstrated for the first time by the *Legionella*-specific FISH technique, PCR, and sequencing a double infection by two *Legionella* species which would be overlooked by bacterial culture and DFA. We could detect living Legionellaceae after antibiotic treatment for 15 days and conclude that FISH is useful to detect a *Legionella* infection and estimate the success of antibiotic treatment.

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