Note

Growth in *Acanthamoeba* sp. and Antibiotic Susceptibility of *Legionella micdadei* Isolated from Hot Spring Water Samples

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As part of an epidemiological study on legionellosis, we attempted to isolate *Legionella* spp. from hot spring water samples, and were able to isolate *Legionella micdadei* from 3 (5.5%) of 55 samples. All of these isolates were able to grow within *Acanthamoeba* sp., suggesting that the isolates will be pathogens. We also confirmed that the K-2 strain from hot spring water grew in guinea pig monocytes. Sensitivity tests using 10 drugs showed that the isolates were most sensitive to imipenem, with the MIC₉₀ of 0.032 μ g/ml, were least sensitive to minocycline, with the MIC₉₀ of 4 μ g/ml, and were not sensitive to low amounts of other drugs.

Key words : *Legionella micdadei* / Growth in *Acanthamoeba* sp. / Antibiotic susceptibility / Hot spring water

A recent mass outbreak of legionellosis transmitted through hot spring water in Japan highlighted the importance of the hygienic management of bath water (Yabuuchi and Agata, 2004). Among the causative agents of legionellosis, a respiratory disease, *Legionella pneumophila* is well known, and extensive information is available on this bacterium (Edelstein and Meyer, 1980; Riffard et al., 1998; Albers et al., 2005; Amemura-Maekawa et al., 2008). On the other hand, a few cases of *Legionella micdadei* infection have been reported in the literature, but, to date, few studies have investigated the properties of this bacterium (Dowling et al., 1984; Koide et al., 1988; Goldberg et al., 1989; Bangsberg et al., 1991; Takiguchi et al., 1999). *L. micdadei*, a causative agent of legionellosis, was discovered by Tatlock (1944), and was referred to as the Tatlock agent. It was proposed as a new species, *Tatlockia micdadei*, by Garritty et al. (1980), but was later included in *L.*

micdadei (Hébert et al., 1980). In this study, we examined the pathogenicity and drug sensitivity of *L. micdadei* isolated from hot spring water.

Fifty-five test samples were collected into sterile polyethylene containers (500 ml) in nine prefectures in Japan between May and October 2008, stored in a refrigerator until being cultured, and tested as follows.

Two hundred-milliliter samples were concentrated to 1 ml by centrifugation at 6,000 rpm for 30 min. This concentrate was mixed with an equal volume of 0.2 M HCl-KCl solution (pH 2.2), and the mixture was incubated at room temperature for 15 min. A 0.1-ml aliquot of this mixture was spread over the surface of GVPC α agar medium (Merck Ltd. • Japan, Tokyo, Japan) with a Conradi stick, and cultured at 36 °C for 7 days. Subsequently, several colonies of each isolate suggestive of the genus *Legionella* were picked up, smeared onto a 2-compartment agar plate (Nikken Bio Medical Laboratory Inc., Kyoto, Japan) consisting of BCYE α and blood agar media, and subjected to pure culture and testing for cysteine requirement. In addition, strains that failed to grow in

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blood agar medium but grew in BCYE α agar medium alone were gram-stained, and long rod-shaped, gram-negative strains were presumed to belong to the genus *Legionella*. Next, strains were identified by latex agglutination (Kanto Chemical Co., Inc., Tokyo, Japan) and DNA-DNA hybridization (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan).

Legionella isolates were tested for intra-amoebic growth as follows, according to the amoeba-agar method developed by Miyamoto et al. (2003). The surface of BCYE α agar was sufficiently dried, and 3 ml of a suspension of the *Acanthamoeba* JAC/E1 strain that had been cultured in PYGC medium at 30 °C for 7 days was dropped onto and spread over the entire surface. The agar plate was incubated at 30 °C for 3 h to allow the amoebae to adhere firmly to it, and then the excess suspension was removed. Samples were applied simultaneously to this medium (referred to as amoeba-agar below) and BCYE α medium, and were cultured at 30 °C for 7 days. Samples that formed colonies in both culture media were regarded as being able to grow in amoebae. The clinical isolate *L. pneumophila* IID 5232 and attenuated strain *L. pneumophila* 25D (Horwitz, 1987) were used as positive and negative controls, respectively.

One milliliter of peripheral blood was collected from a guinea pig, and treated with heparin. This blood sample was infected with *L. micdadei* strain K-2 by the addition of 100 μ l of the bacterial suspension. After 24 h, blood smears were prepared, stained by the Gimenez method, and observed for *L. micdadei* within monocytes.

Drug sensitivity tests were performed using Etest (Aska Diagnostics Inc., Tokyo) according to the attached technical guide. The drugs tested were erythromycin (EM), clarithromycin (CAM), azithromycin (AZM), minocycline (MINO), levofloxacin (LVFX), ciprofloxacin (CPFX), piperacillin (PIPC), imipenem (IPM), gentamicin (GM), and rifampicin (RFP) (10 drugs). The bacterial suspension of each test strain (0.5 ml) was dripped on BCYE α agar medium (Merck Ltd. • Japan, Tokyo) (60 ml in a 150-mm dish (Corning Inc., USA)) and smeared over the surface using a Conradi stick, and an Etest strip was closely attached onto the medium. The plates were cultured at 30 °C for 5 days, and the growth inhibition zone

formed around the strip was observed. Minimum Inhibitory Concentration (MIC) was judged by macroscopically reading the gradation at which the end of the growth inhibition zone and the strip crossed.

L. micdadei was successfully isolated from 3 (5.5%) of the 55 samples of hot spring water (TABLE 1). Of these, samples F-1 and F-2 were collected from swimming pool and indoor hot spring water, respectively, from the T spa facility in Hiroshima Prefecture, and were simple alkaline spring water (pH 9.1). The numbers of bacteria detected in these samples were 6.0×10 and 5.0×10 CFU/100 ml, respectively. Sample K was collected from open-air hot spring water from the U spa facility in Kanagawa Prefecture, and was sodium-chloride spring water with a sodium ion concentration of 10,920 mg/L (pH 7.4), yielding 5.0×10 CFU/100 ml. Furthermore, *L. pneumophila* was isolated from 14 samples (25.5%). However, *L. micdadei* was not isolated from these samples.

Twenty-seven *L. micdadei* strains (12 F-1 and F-2 strains each and three K strains) isolated from hot spring water formed colonies on amoeba-agar medium, as in the positive control. These colonies did not differ at all from those formed on BCYE α agar medium used as a control, suggesting that these strains grew in *Acanthamoeba* sp. cells.

FIG. 1. Giménez staining of *L. micdadei* in guinea pig monocytes.

TABLE 1. Isolation of *L. micdadei* from hot spring water samples.

Samples	Prefecture	Facility	Sampling sites	pH	Quality	Viable numbers (CFU/100ml)
F-1	Hiroshima	T	Swimming pool	9.1	Simple alkaline	6.0×10
F-2			Indoor-spa			5.0×10
K	Kanagawa	U	Open-air bath	7.9	Sodium chloride	5.0×10

TABLE 2. Susceptibility of *L. micdadei* isolated from hot spring waters to antimicrobial agents.

Antimicrobial agents	MIC ($\mu\text{g/ml}$)														MIC ₅₀	MIC ₉₀
	<0.002	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8		
Erythromycin								4	9	11	3				0.5	1
Clarithromycin							1	10	11	4	1				0.25	0.5
Azithromycin					1	1	2	12	7	1	3				0.125	1
Minocycline										1	6	14	5	1	2	4
Levofloxacin									2	24	1				0.5	0.5
Ciprofloxacin										15	12				0.5	1
Piperacillin					19	2	3	1	2						0.016	0.125
Imipenem	4	1	8	3	7	2	2								0.008	0.032
Gentamicin								9	8		3	6	1		0.25	2
Rifampicin			1		2	13	10	1							0.032	0.064

n=27

Guinea pig monocytes in peripheral blood were infected with the K-2 strain of *L. micdadei*, and stained by the Giménez method. The stained image is shown in FIG. 1. Thus, it was confirmed that *L. micdadei* grew in the cytoplasm of monocytes, compressing the nuclei to the periphery. In addition, the growth of *L. micdadei* resulted in the destruction of some monocytes in the blood smear.

TABLE 2 shows the susceptibility of the 27 test strains to 10 drugs. The MIC values of EM, CAM, AZM, MINO, LVFX, CPFX, PIPC, IPM, GM, and RFP were in the ranges of 0.125-1, 0.064-1, 0.016-1, 0.5-8, 0.25-1, 0.5-1, 0.016-0.25, <0.002-0.064, 0.125-4 and 0.004-0.125 $\mu\text{g/ml}$, respectively. The MICs of 8 of the 10 drugs tested showed a unimodal peak, but those of IPM and GM exhibited bimodal peaks at 0.004, 0.016 $\mu\text{g/ml}$ and 0.125, 2 $\mu\text{g/ml}$, respectively. The MICs of all drugs for the test strains were 8 $\mu\text{g/ml}$ or less; thus, no strains showed a particularly low sensitivity.

In terms of MIC₉₀ values, the isolates were most sensitive to IPM at 0.032 $\mu\text{g/ml}$, followed by RFP (0.064 $\mu\text{g/ml}$), PIPC (0.125 $\mu\text{g/ml}$), CAM and LVFX (0.5 $\mu\text{g/ml}$), EM, AZM and CPFX (1 $\mu\text{g/ml}$), GM (2 $\mu\text{g/ml}$), and MINO (4 $\mu\text{g/ml}$). The sensitivities of the isolates to RFP, PIPC, CAM/ LVX, EM/AZM/CPFX, GM, and MINO were 2, 4, 16, 32, 64, and 128 times lower than that to IPM, to which the isolates were most sensitive (TABLE 2).

In this study, we isolated *L. micdadei* from hot spring water, and found that the bacterium inhabits hot spring water in Japan, although the rate of isolation was as low as 5.5% (3 in 55 samples). In a nationwide study in 2003 (Furuhata et al., 2004a), we isolated *L. micdadei* in only 2 (0.3%) of 710 samples, and the majority of the isolates belonged to *L. pneumophila*. In this study, two of the three samples, from which *L. micdadei* was isolated, were simple alkaline spring water with pH 9.1, and the remaining

one sample was sodium-chloride spring water with a high sodium-ion concentration of 10,920 mg/L. Sheehan et al. (2005) isolated *L. micdadei* from a eukaryotic algal mat community in a pH 2.7 geothermal stream in the Yellowstone National Park. Thus, it became clear that *L. micdadei* is distributed widely in aquatic environments that vary greatly in water quality.

To investigate the pathogenicity of *L. micdadei* from hot spring water, we examined its ability to grow intracellularly in *Acanthamoeba* sp. This method was developed by Miyamoto et al. (2003), and has been used to evaluate the pathogenicity of bacteria of the genus *Legionella* (Albers et al., 2005). The bacteria of the genus *Legionella* can grow intracellularly in protists that phagocytize bacteria, showing an ability to grow in human and animal macrophages. This property of the bacterium is reportedly important in considering the pathogenicity of the genus *Legionella*. Using a similar amoeba plate test on an agar containing *Acanthamoeba castellanii*, Albers et al. (2005) analyzed the gene expression in *L. pneumophila*. In this study, we confirmed that all *L. micdadei* isolates from the hot spring water samples were able to grow in *Acanthamoeba* sp., which strongly suggests that all isolates are pathogenic. Although the genes responsible for the pathogenicity of *L. micdadei* have not been identified, Morozova et al. (2004) reported that *L. micdadei* has genes analogous to the *icm/dot* genes of *L. pneumophila*. Although they examined the presence or absence of analogous genes by Southern hybridization, they did not clarify whether these genes were involved in intracellular growth.

Sensitivity tests against 10 drugs showed that these isolates were most sensitive to IPM and RFP, with an MIC₉₀ of 0.064 $\mu\text{g/ml}$, and were not sensitive to low amounts of other drugs. In a previous study (Furuhata et al., 2004b), drug sensitivity tests also

showed that *L. pneumophila* isolates from hot spring water were most sensitive to RFP, with an MIC₉₀ of 0.125 µg/ml. Edelstein and Meyer (1980) also tested the drug sensitivity of 33 clinical isolates of *L. pneumophila*, and reported that the MIC values of RFP were distributed between 0.025 and 0.125 µg/ml, indicating high-level sensitivity to RFP, similar to the results of this study.

Murakami et al. (2001) measured the MICs of various agents against clinical strain-derived *L. pneumophila* in Japan using the E test. The results did not show any drug-resistant bacteria. *L. pneumophila* was highly sensitive to macrolides and RFP, as reported in other countries

Thus, we considered that the *L. micdadei* isolates from hot spring water were pathogenic, and highly sensitive to IPM and RFP. Further studies are needed to identify the genes responsible for the pathogenicity of *L. micdadei* and to follow the changes in the drug sensitivity regarding the emergence of drug-resistant bacteria.

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