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Isolation of *Legionella pneumophila* from Pluvial Floods by Amoebal Coculture

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ABSTRACT

Viable *Legionella pneumophila* bacteria were isolated by amoebal coculture from pluvial floods after intense rainfall and from water collected at sewage treatment plants. Several isolated *L. pneumophila* strains belonged to sequence types that have been previously identified in patients.

TEXT

The specific *Legionella* strains that are found most often in patients are rarely isolated from (patient-related) environmental samples (4, 5, 10), and the sources of the infecting bacteria often remain unknown (3). A possible explanation for this discrepancy may be that exposure to pathogenic *Legionella* strains has occurred through yet-unknown sources. Alternatively, pathogenic *Legionella* bacteria are missed because of the failure of methods used for the detection of *Legionella* in the environment. The standard culture method for detection of *Legionella* is plating of samples on buffered charcoal yeast extract (BCYE) plates. However, plates are easily overgrown by other bacteria present in the samples. Furthermore, it is known that *Legionella* bacteria can enter a viable-but-not-culturable (VBNC) state (22, 23). Amoebal coculture has been applied as a method to isolate *Legionella* bacteria that could not be recovered by plating on BCYE plates (1, 6, 15, 20). In this study, an amoebal coculture procedure (15, 20) was applied in order to identify yet-unknown sources for *Legionella*. For the coculture procedure, *Acanthamoeba castellanii* cells (ATCC 30234; American Type Culture Collection [ATCC], Rockville, MD) were used. After cocultivation of the sample with amoebae, the amoebal suspension was plated on BCYE plates (Oxoid Ltd., Hampshire, United Kingdom), which were inspected for *Legionella* colonies after incubation at 32°C for 7 days.

The ability of several *Legionella* strains to be cocultured with *A. castellanii* was investigated. Eleven *Legionella pneumophila* strains that tested positive with the Dresden monoclonal antibody (MAb) 3/1 (MAb 3/1 positive) (16), 10 *L. pneumophila* strains that tested negative (MAb 3/1 negative), eight *Legionella anisa* strains that were collected from clinical and environmental sources, and an *L. pneumophila* Philadelphia strain that was retrieved from the ATCC (ATCC 33152) were tested by amoebal coculture. The MAb 3/1-positive strains, which are considered to be pathogenic for humans (11, 16), replicated as well as MAb 3/1-negative strains, which are considered nonpathogenic. The enrichment of all tested *Legionella* strains was at least 5 to 6 $\log_{10}$ units after 6 days of cocultivation. The *L. pneumophila* Philadelphia strain retrieved from the ATCC could not be cocultured on *A. castellanii* cells. The eight *L. anisa* strains replicated well. This is in contrast with the findings of Neumeister et al. (17), who showed that *L. anisa* does not replicate in the presence of *A. castellanii* cells. It is possible that a difference in sensitivity between strains of the same *Legionella* species for cocultivation with *A. castellanii* exists. As a consequence, some *Legionella* strains will be missed by amoebal coculture (9, 17), a limitation of the method.

Thirteen samples were taken from water of pluvial flooding incidents in residential areas at locations where more than 100 m^2 of the residential area was flooded or at locations where buildings were flooded. The flood water originated from surface runoff or from flooded sewers. Twenty-four water samples from five sewage treatment plants (STPs) were taken, both from influent and from the aeration ponds. Surface water samples were taken from six locations on a small river where combined sewer overflows enter that river. In addition, four fountains that were fed by surface water were sampled. Since most samples contained soil or sludge, these were not concentrated by filtration. For each sample, 100 µl was investigated in duplicate with the amoebal coculture procedure (15, 20).

Legionella strains that were isolated from the environmental samples were identified by sequence analysis of the *mip* gene with the following degenerated primers: Mip-FP1 (5′-GAASARCAAATGAAAGAYGTTC-3′) and Mip-RP8 (5′-CCAGGRATAACTTGYGAWAC-3′). Sequences were analyzed with BioNumerics software, version 6.6 (Applied Maths, Kortrijk, Belgium), and compared to *Legionella* sequences present in the NCBI database. *L. pneumophila* strains were further typed by serotyping (3) and by sequence-based typing (SBT) (8, 19).

Legionella was detected in 50% of pluvial flood water samples from surface runoff (three out of six) (Table 1). One sample contained a mixture of an *L. pneumophila* serogroup 1 (SG1) strain and an *L. pneumophila* SG8 strain, one sample contained a mixture of *Legionella fallonii* and *Legionella wadsworthii*, and one sample contained only *L. pneumophila* SG8. For two *L. pneumophila* strains, a sequence type (ST) could be assigned by SBT, namely, ST46 and ST1079. ST46 has been found in 20 out of 471 patients from whom *Legionella* has been isolated and typed in The Netherlands in the period from 2002 to 2011. *Legionella* was detected in 1 out of 7 pluvial flood samples (14%) originating from flooding of the sewerage and typed as *L. wadsworthii*.

Table 1

Table 1 Isolation and typing of *Legionella* from pluvial floods[a]

Sample no.	Sampling date (mo-day-yr)	Origin of flooding	*mip* gene sequence analysis result[b]	Serotyping result	ST
1	6-7-11	Runoff	*L. pneumophila* (12)	SG1	46
			L. pneumophila (8)	SG8	
2	6-28-11	Runoff	–		
3	6-28-11	Runoff	*L. falloni* (1); *L. wadsworthii* (2)		
4	8-23-11	Runoff	*L. pneumophila* (3)	SG8	1079
5	8-23-11	Runoff	–		
6	8-23-11	Runoff	–		
7	6-28-11	Sewer	–		
8	6-28-11	Sewer	–		
9	6-28-11	Sewer	*L. wadsworthii* (1)		
10	6-28-11	Sewer	–		
11	6-28-11	Sewer	–		
12	8-23-11	Sewer	–		
13	8-23-11	Sewer	–		

[a] –, negative result; SG, serogroup; ST, sequence type.

[b] The numbers of *Legionella* isolates that were identified by *mip* gene sequencing are indicated in parentheses.

Legionella was found in 38% of the STP samples (9 out of 24) (Table 2). One plant contained *L. pneumophila* on all three sampling days in the aeration pond and once in the influent pond. Of the five *L. pneumophila* strains that were isolated from STPs, two strains could be typed by SBT. Both types, ST93 and ST625, have been found in 3 out of 471 patients in The Netherlands in the period from 2002 to 2011. For comparison with amoebal coculture, 16 STP samples were also tested directly on BCYE agar plates with antibiotics (Oxoid). However, *Legionella* could not be isolated due to overgrowth of the plates with other bacteria. The presence of *L. pneumophila* in water samples from the aeration ponds of STPs is of importance for workers at the plant, since aerosols are generated and can be dispersed into the environment (2, 14, 18). Only one surface water sample contained *Legionella* (*Legionella gormanii*). No *L. pneumophila* was detected in surface water samples.

Table 2

Table 2 Isolation and typing of *Legionella* from sewage treatment plants[a]

STP no.	I/A	Typing results on each sampling date (mo-day-yr)[b]		
		3-23-11	**5-23-11**	**6-8-11**
1	I	*Legionella* sp.	ND	ND
	A	–	ND	ND
2	I	–	–	–
	A	–	–	*Legionella lytica* (1); *L. fallonii* (2)
3	I	–	*L. pneumophila* (6) (SG3, ST93)	–
	A	*L. pneumophila* (2) (SG4, SG9); *L. lytica* (1)	*L. pneumophila* (6) (SG3)	*L. pneumophila* (3)
4	I	*L. lytica* (1)	–	–
	A	–	–	–
5	I	ND	–	*L. pneumophila* (2) (SG2, ST625)
	A	ND	*Legionella sainthelensi* (1)	–

[a] I, influent; A, aeration pond; ND, not done; –, negative result; SG, serogroup; ST, sequence type.

[b] The numbers of *Legionella* isolates that were identified by *mip* gene sequencing are indicated in parentheses.

This is the first publication showing that potentially pathogenic *Legionella* strains can be detected in a very unsuspicious environmental source, such as water from pluvial floods after intense rainfall. The implication for public health is not clear. An association between increased rainfall and patients with Legionnaires' disease exists (7, 12, 13). Also, viable *L. pneumophila* bacteria have been found in rainwater on the road (21). Air sampling is required to investigate whether *Legionella* bacteria in pluvial floods are aerosolized and if a risk of exposure during and shortly after periods of heavy rainfall exists. Soil and air samples can be analyzed to determine the origin of pathogenic *Legionella* bacteria.

Through the selective enrichment of intracellular bacteria in the amoebal coculture step, there is a higher probability of isolating *Legionella* bacteria from samples with a high background of microbial flora. This method can contribute to the tracing of yet-unidentified sources of *Legionella* exposure and disease.

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