Epidemiol. Infect. (2001), **126**, 147–152. Printed in the United Kingdom

SHORT PAPER

Presence of *Legionellaceae* in warm water supplies and typing of strains by polymerase chain reaction

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(*Accepted 18 October 2000*)

SUMMARY

Outbreaks of Legionnaire's disease present a public health challenge especially because fatal outcomes still remain frequent. The aim of this study was to describe the abundance and epidemiology of *Legionellaceae* in the human-made environment. Water was sampled from hot-water taps in private and public buildings across the area of Göttingen, Germany, including distant suburbs. Following isolation, we used polymerase chain reaction in order to generate strain specific banding profiles of legionella isolates. In total, 70 buildings were examined. Of these 18 (26%) had the bacterium in at least one water sample. *Legionella pneumophila* serogroups 1, 4, 5 and 6 could be identified in the water samples. Most of the buildings were colonized solely by one distinct strain, as proven by PCR. In three cases equal patterns were found in separate buildings. There were two buildings in this study where isolates with different serogroups were found at the same time.

Legionella pneumophila were first recognized as the aetiological agent of Legionnaire's disease in 1977 following an epidemic of acute pneumonia at an American Legion convention in Philadelphia [1, 2]. Since that time many different serogroups and related species of this bacterium have been detected [3, 4]. Known as especially relevant for transmission are contaminated warm water supplies, cooling towers, evaporative condensers, whirlpool spas and respiratory therapy equipment [5]. Surveys of lakes, ponds, streams, and soils have indicated that this bacterium is also a common inhabitant of natural waters [6]. Because of the widespread distribution several methods have been used to discriminate between different strains of *Legionella pneumophila*. Most frequently used is typing with monoclonal antibodies [7], but polymerase chain reaction methods [8], and analysis of restriction fragment length polymorphisms [9] are also common.

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Recent information about the frequency as well as the distribution of different or equal legionella strains in the water systems of a restricted area in Germany are scarce and may differ from other parts of the country or the world. Thus, the aim of this study was to describe the abundance and epidemiology of *Legionellaceae* in water systems of different buildings in a German town in Lower Saxony. Special interest was directed to the presence of distinct strains in hospitals and old people's homes in contrast to other buildings. This was done to evaluate the question whether nosocomial infections can be securely differentiated from community-acquired types using a newly developed PCR analysis to further differentiate between strains on the serogroup level.

Water samples. Water was sampled from private and public buildings between February and September 1999 in Göttingen and area, Germany. In private buildings water was taken in the bath room from the hot-water taps. Water samples from public buildings were obtained by turning on the hot-water tap (mostly from showers heads) and taking the first water and then taking a second portion when the water reached the highest temperature. In total 129 samples from 70 buildings were collected.

Water supply. All sampled buildings were supplied by the water plant of Göttingen (Stadtwerke Göttingen AG). The distribution system could be divided into three main pressure zones according to different elevations of the city and several small higher or more distant zones. Water was supplied to the lowest zone by three facilities that combine water from a transport pipe from the Harz Mountains (about 80%) and their local well [10]. Out of this zone water was pumped up to the other zones.

Bacteriological assay. Water samples (1 l volume) were filtered through 0·45 μm pore-size cellulose-mixed ester filters with a diameter of 50 mm (Schleicher and Schuell, Dassel, Germany) using a vacuum pump according to Pabst et al. [11] and the German federal health agency [12]. Then 10 ml of a KCl/HCl buffer (0·2 M, adjusted to pH 2·2) was poured onto the filter and removed again after 5 min. The filters were placed on a MWY agar plate (following Wadowski and Yee, modified by Edelstein, supplied by Oxoid, Wesel, Germany) and incubated at 37 °C in a humidified atmosphere (plastic bag) for 7 days and examined daily. Additionally 1 ml of water was added to 1 ml of a KCl/HCl buffer and after 5 min 0·5 ml of the solution was used to inoculate the surface of the MWY agar. This was done in duplicate.

Colonies that morphologically matched legionella colonies were subcultured onto blood and MWY agar. Representative colonies (1–2) of those that failed to grow on blood agar were examined by direct fluorescent antibody technique using *L. pneumophila* serogroups 1–6 rabbit globulin and a combined *L. bozemanii*, *L. dumofii*, *L. gormanii*, *L. jordanis*, *L. longb.* 1+2, *L. micdadei* rabbit globulin supplied by Viramed, Planegg/Steinkirchen, Germany and *L. pneumophila* serogroups 1–14 monoclonal antibodies (mouse) supplied by Pro-Lab Diagnostics (Mast Diagnostica), Reinfeld, Germany. Isolates were stored at –70 °C (Microbank, Mast Diagnostica).

Polymerase chain reaction. To identify different strains of legionella we used different primers to amplify DNA fragments in crude bacterial lysates to generate banding profiles. The used method was based on a development of Wiese et al. (unpublished results). The stored isolates were cultured on MWY agar plates at 37 °C for 3 days. Next, colonies of each isolate were picked from the plates and suspended in 200 μl of 5% Chelex 100 (Biorad, Münden, Germany), vortexed for 15 sec and incubated in a heating block for 30 min at 99 °C. After centrifugation at 14 500 ***g*** for 5 min TE buffer (20-fold concentration) was added to the supernatent. These crude lysates were stored at –20 °C and used in PCR reactions after adjusting them to a DNA concentration of 10 μg/ml with TE buffer (10 mM Tris–HCl, pH 8·0; 1 mM EDTA, pH 8·0). DNA concentrations in the lysates were determined by UV spectroscopy (wavelength 260 nm). PCR reactions were carried out in a final volume of 25 μl containing 5 μl sample DNA, 2·5 μl primer (0·01 nM/μl) and 17·5 μl H_2O. This mixture was added to 'Ready To Go Analysis Beads' (Pharmacia Biotech Europe, Freiburg) each containing Ampli-Taq DNA polymerase, 0.4 mM deoxynucleotide triphosphates, 2·5 μg BSA and buffer (3 mM $MgCl_2$, 30 mM KCl, 10 mM Tris [pH 8·3]).

Primers were ERIC2 (5′-AAGTAAGTGACTGG-GGTGAGCG-3′, [8]) and a combination of Lpm-1 (5′-GGTGACTGCGGCTGTTATGG-3′) and Lpm-2 (5′-GGCCAATAGGTCCGCCAACG-3′) [13]. ERIC2 is an enterobacterial repetitive intergenic consensus motif. Lpm-1 and Lpm-2 are part of the macrophage infectivity potentiator (mip) gene of legionella. The primers were synthesized and cleaned by HPLC by Biometra (Göttingen, Germany).

Thermal cycling was carried out in a Crocodile III thermal cycler (Appligene Oncor, Heidelberg, Germany). After an initial denaturation at 94 °C for 5 min, 45 cycles of 60 s at 94 °C, 110 s ramp to 36 °C, 60 s at 36 °C and 120 s at 72 °C. This was followed by 1 cycle of 72 °C for 10 min.

The following reference strains were used as control: *L. pneumophila* ATCC 33152 (serogroup 1), ATCC 33156 (serogroup 4), ATCC 33216 (serogroup 5) and ATCC 33215 (serogroup 6) for banding patterns.

Gel electrophoresis. Gels were stained by adding ethidium bromide to the agarose gel and banding patterns were visualized under ultraviolet light. A 100 bp ladder (Pharmacia Biotech, Freiburg, Germany) was utilized as a size marker.

Band patterns were compared visually. Isolates of a serogroup were considered to have the same PCR type when the patterns obtained with both primers were indistinguishable. Very weak bands (not apparent on the photographs and/or not detected reproducibly) were not taken into account. In doubtful cases, the amplifications were repeated, and the patterns were compared after comigration on the same agarose gel.

Table 1. *Detected* Legionellaceae *in different types of buildings*

Type of building	Number of tested buildings	Buildings with at least one sample containing *Legionellaceae*	Range of colony forming units (c.f.u.)/l	Found serogroups (all isolates *L. pneumophila*)
Sports halls and swimming baths	24	5 (21%)	22–5000	1, 4, 5, 6
University buildings	19	5 (26%)	3–8000	1, 4, 6
Hospitals and old people's homes	8	5 (63%)	4000–78000	1, 4, 6
Halls of residence	4	2 (50%)	5–68	1, 5
Hotels	4	0 (0%)	—	—
Other buildings	11	1 (9%)	2000	1
Total	70	18 (26%)	3–78000	1, 4, 5, 6

Legionella contamination: Out of 70 tested buildings 18 (26%) contained *Legionellaceae* in at least one sample. Referring to the types of the buildings the results are depicted in Table 1. It is remarkable that hospitals and old people's homes used by a very sensitive population group had the highest rate of contamination among all tested buildings. Though it has to be taken into account that the tested number of these buildings was limited and the results thus may not be representative.

The distribution of the maximum found colony-forming units (c.f.u.) of legionella for all buildings was as follows. Legionella not detectable in 52 buildings; 1–100 c.f.u./l in 7 buildings; 101–1000 c.f.u./l in 1 building; 1001–10000 c.f.u./l in 6 buildings and 10001–100000 in 4 buildings. The overall trend was that the larger the buildings plumbing had been, the more samples were positive for legionella and the more bacteria could be found. The highest detected concentration of bacteria was 78000 c.f.u./l. Temperatures of contaminated second water samples were in many cases below 45 °C (Table 2). Also in Lower Saxony, Germany, Habicht and Müller [14] found that 70% of the 103 hospitals and 18% of the 62 hotels investigated were positive for legionella. These findings are similar to our results testing only one supply area more than 10 years later. Boschek et al. [15] were able to culture *Legionellaceae* in warm water from 11 out of 12 sampled hospitals in a German town. In Canada a frequency for legionella contaminated hospitals of 32% was found [16]. It has to be taken into account that methods used vary between these studies. A high constancy (regular detection, only a few minor genetical changes) of legionella colonization was found by Lück et al. [17] in a hospital connected with a ring pipe warm water system over 7 years. It is important to know that hospitals and old people's homes used by a risk group for infections frequently had a high rate of contamination including this study.

Detected serogroups. Only *Legionella pneumophila* serogroups 1, 4, 5 and 6 could be identified in the water samples. No other *Legionella* species was found. The distribution of the isolates among the serogroups is given in Table 2. There were two buildings in this study where isolates with different serogroups were found at the same time (hospital/old people's home E and F). Four isolates of serogroup 1 (G6, G10, G12, G13) were reanalysed at the University of Dresden (Dr P. C. Lück) for their classification into serogroups. This study was more concentrated on the epidemiology of different contaminated buildings than on detecting a minor colonization with distinct strains in the same building. Because only 1–3 colonies per sample were analysed with direct fluorescent antibody technique it is possible that a (minor) co-colonization with different serogroups may have been overlooked.

Referring to the frequency of serogroups of *L. pneumophila* the results of this study were similar to results of other studies [14, 15, 18]. In contrast to this some authors found non-pneumophila strains to be present also in the general environment [6, 19]. This may be due to the existence of different legionella strains in various habitats or due to the use of different selective media. In a comparative test Ta et al. [20] were only able to detect non-pneumophila species in water samples with culture on BCYE medium. Non-pneumophila species grew poorly on all selective media used. In other studies our laboratory has cultured legionella from water also using BCYEα combined with BMPA supplement parallel to the method described above. We have never isolated a non-pneumophila species from our water samples, but non-pneumophila reference strains can be grown on

Table 2. *Source and PCR pattern of legionella isolates used in this study*

Designation	Source	Date of sampling	Serogroup	PCR pattern
G1	Hospital/old people's home F, 2nd water	15 Mar. 1999	1	1A
G2	Hall of residence C, 2nd water	17 Feb. 1999	1	1B
G3	Sports hall/swimming bath I, 1st water	9 Jul. 1999	1	1C
G4	Sports hall/swimming bath I, 2nd water, 36 °C	9 Jul. 1999	1	1C
G5	Sports hall/swimming bath X, 1st water	23 Jul. 1999	1	1D
G6	University building H, 2nd water, 51 °C	3 Sep. 1999	1	1E
G7	Private/company building J, 1st water	9 Sep. 1999	1	1A
G8	Hospital/old people's home H, 1st water	9 Sep. 1999	1	1F
G9	Hospital/old people's home H, 2nd water, 42 °C	9 Sep. 1999	1	1F
G10	Hospital/old people's home E, 2nd water, 48 °C	15 Sep. 1999	1	1A
G11	Hospital/old people's home E, 2nd water, 36 °C	15 Sep. 1999	1	1A
G12	Hospital/old people's home E, 1st water	15 Sep. 1999	1	1A
G13	Hospital/old people's home E, 2nd water, 40 °C	15 Sep. 1999	1	1A
G14	Sports hall/swimming bath V, 1st water	23 Jul. 1999	4	4A
G15	Hospital/old people's home G, 1st water	9 Sep. 1999	4	4B
G16	Hospital/old people's home G, 2nd water, 46 °C	9 Sep. 1999	4	4B
G17	Hospital/old people's home G, 2nd water, 40 °C	9 Sep. 1999	4	4B
G18	University building M, 2nd water, 49 °C	10 Sep. 1999	4	4C
G19	Hospital/old people's home A, 1st water	11 Sep. 1999	4	(4B) cf. to text
G20	Hospital/old people's home E, 1st water	15 Sep. 1999	4	4D
G21	Hospital/old people's home E, 1st water	15 Sep. 1999	4	4E
G22	Hall of residence A, 2nd water	15 Mar. 1999	5	5A
G23	Sports hall/swimming bath A, 2nd water, 36 °C	25 Jun. 1999	5	5B
G24	Hospital/old people's home F, 2nd water, two isolates cultured: G24a and G24b	15 Mar. 1999	6	6A
G25	Sports hall/swimming bath O, 1st water	15 Jul. 1999	6	6B
G26	Sports hall/swimming bath O, 2nd water, 31 °C	15 Jul. 1999	6	6B
G27	University building B, 2nd water, 51 °C	31 Aug. 1999	6	6C
G28	University building E, 1st water	31 Aug. 1999	6	6C
G29	University building E, 2nd water, 46 °C	31 Aug. 1999	6	6C
G30	University building I, 2nd water, 47 °C	3 Sep. 1999	6	6D
X1	Hospital/old people's home E, cold water installation	6 Aug. 1997	1	1A
X2	Hospital/old people's home E, cold water installation	7 Oct. 1996	1	1A
X3	Hospital/old people's home E, cold water installation	6 Aug. 1997	5	5C
X4	Hospital/old people's home E, cold water installation	25 Aug. 1997	5	5D
R1	Reference strain ATCC 33152		1	1R
R2	Reference strain ATCC 33156		4	4R
R3	Reference strain ATCC 33216		5	5R
R4	Reference strain ATCC 33215		6	6R

our media. At present no official external quality assurance programme for legionella exists in Germany.

Results concerning the number of colony forming units found in Göttingen were similar to other studies [14, 15].

PCR patterns: In total 31 isolates have been stored and typed with PCR based techniques (Table 2). None of our isolates produced a pattern identical to the reference strains. In the following the reference strains were not mentioned as separate banding types any more.

Isolates of serogroup 1 sampled in the area of Göttingen showed six different banding patterns. Six isolates (G1, G7, G10–G13) of this serogroup had an identical profile. These were cultured from water sampled in three different buildings, located in two different supply zones. Two additional samples (X1, X2) from one of these buildings that were found about 2 years prior to this study had an identical pattern. Banding profiles of serogroup 4 isolates can be grouped in five patterns. There was one building that was colonized with two strains of serogroup 4 differing in PCR analysis (G20, G21). These samples were found in hospital/old people's home E in water sampled on the same day in different parts of the building. Four isolates (G15–G17, G19) of this serogroup produced an identical profile. They were cultured from two hospitals/old people's homes with a distance of more than 1000 metres and located in separate supply zones. Because there is a very fine additional banding in the isolate (G19) of one building that could be reproduced in a second PCR test it is possible that these buildings also have different strains.

The two isolates (G22, G23) of serogroup 5 had different banding patterns. Two additional samples (X3, X4) from a hospital in Göttingen that were found about 2 years prior to this study also had different banding characteristics.

Isolates of serogroup 6 gave four different banding patterns. Three isolates (G27, G28, G29) that showed identical patterns were found in two separate university buildings with a distance of about 500 metres and were supplied by the same pressure zone. A different pattern of serogroup 6 was found in an other building of the same complex (G30). It can be summarized that only in three cases identical patterns were found in separate buildings in Göttingen. In two cases the same banding pattern was produced by samples from two different buildings and in one case isolates cultured from water samples from three buildings had identical patterns. Two isolates with equal bandings came from samples of the same building in four cases. In two cases different strains were found in the same buildings at the same time.

Except for one (G30, discrimination by ERIC2) isolate both used primer Eric2 and the combination of Lpm-1 and Lpm-2 were able to differentiate between the mentioned strains.

Repeating the analysis of some isolates and reference strains over several years with different lots of reagents no changes in the resulting gel patterns were observed. However, there are reports that changes in equipment and reagents may result in a loss of reproducibility in DNA fingerprinting methods [21, 22]. To ensure reproducibility of this PCR testing all reagents and primers were used from identical lots and equipment was not changed during the study. Testing a set of 10 serogroup 1 reference strains primer pair Lpm-1/Lpm-2 were able to distinguish between 9 strains and primer ERIC2 was able to distinguish between 8 strains (data not shown). Bansal and McDonell [23] used PCR-based DNA fingerprinting technique with a combination of two random primers (double RAPD) to study its discriminatory ability with 67 well-defined legionella reference strains (representing 39 species) and 120 outbreak-related environmental and clinical isolates. For reference strains they obtained a unique strain-specific array of fragments for each species, serovar, and subtype. The band definition was adequate for confident visual comparison of the fingerprints located on the same or on different agarose gels. They concluded that this method was low-cost but sufficiently powerful and reliable to type individual strains. Other authors that made a methodological comparison of DNA fingerprinting and other epidemiological typing methods mostly found comparable discriminatory ability between the different methods [24–27].

The isolates from 18 buildings found in this study could be classified into four serogroups of *L. pneumophila*. The variety of serogroups could further be divided in many more strains using PCR method. Only in three cases equal patterns were found in separate buildings. In one case the presence of equal strains could be shown for a hospital, an old people's home and a private building. This is an important finding with regard to the question whether an infection is nosocomial or community-acquired.

No association of serogroups or identical strains and water supply zones was found. So the exact evolution and origin of these populations remains unclear.

The main conclusion of our study is that there exists a great diversity of legionella strains below the serogroup level detectable by PCR analysis.

The high frequency of legionella contamination in hospitals and old people's homes (revealed not only by this study) shows that regular controls and protection measurements can be an important part of prevention against Legionnaire's disease and Pontiac fever.

ACKNOWLEDGEMENTS

The authors thank J. Ebert, B. Gerhart, Dr S. Kevekordes and M. Leister for their technical support and Dr S. Wagner for reviewing the manuscript.

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