

Detection of *Legionella pneumophila* in environmental water samples using a fluorescein conjugated monoclonal antibody

T. MAKIN AND C. A. HART

*Department of Medical Microbiology, University of Liverpool, P.O. Box 147,
Liverpool L69 3BX*

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SUMMARY

Sixty-three environmental water samples from various sources were examined for the presence of *Legionella pneumophila* with a commercially available direct fluorescent monoclonal antibody (GS), an indirect fluorescent antibody test (IFAT) and culture. GS detected *L. pneumophila* in 94% and 100% of environmental water samples which were culture and IFAT positive for *L. pneumophila*, respectively. IFAT detected 69% of *L. pneumophila* culture positive samples. Cultures of *L. pneumophila* serogroups 1 to 12, 14 and non-*L. pneumophila* bacteria which may be found in water, and bacteria containing non-specific binding proteins, were stained by GS and IFAT. GS identified all serogroups of *L. pneumophila* and did not cross react with any non-*L. pneumophila* bacteria. *L. pneumophila* in environmental samples was easy to detect against a clear dark background when stained with GS.

INTRODUCTION

There is an urgent need for reliable, sensitive and rapid tests for the detection of *Legionella pneumophila* in environmental water samples (1). Detection of viable bacteria by culture is the method most commonly employed but this can take up to 2 weeks, especially in the presence of sub-inhibitory concentrations of biocides or other bacteria that produce inhibitory compounds (2). An alternative method is to detect the antigens of *L. pneumophila*. The direct fluorescent monoclonal antibody test (GS) recognizes a species-specific *L. pneumophila* antigen and has proved to be highly sensitive and specific in detecting *L. pneumophila* in clinical samples (3–5). We have adapted this test for detection of *L. pneumophila* in environmental water samples and compared its specificity and sensitivity with that of culture and of the indirect fluorescent antibody test (IFAT).

MATERIALS AND METHODS

Bacterial strains

Legionellae used in this study were supplied by the Legionella Reference Unit, Division of Microbiology Reagents and Quality Control (DMRQC), Central Public Health Laboratory, London, with the exception of two *L. pneumophila* serogroups,

which were isolated from a patient (serogroup 12) and the environment (serogroup 6) at the Royal Liverpool Hospital. Non-legionella bacteria were stock cultures from clinical and environmental sources held in the Department of Medical Microbiology, University of Liverpool. All bacterial strains used are listed in Tables 1 and 2.

Legionella antisera

GS, manufactured by Genetic Systems Corporation as Legionella Immuno-fluorescent Antibody Test Kit was obtained from Syva UK Ltd (Maidenhead, Berks). Polyvalent antisera to *L. pneumophila* serogroups 1–6 used in the IFAT, and monovalent antisera to *L. pneumophila* serogroups 7–12 were provided by DMRQC and in each case used at the recommended dilution of 1/100. ATAB (UK) Ltd (Winnersh, Berks) supplied the fluorescein antibody conjugate used with the IFAT.

Environmental water samples were collected from sites which had a history of colonization with legionellae. These were hot water taps, showers, cooling towers, calorifiers, and ice-making machines. Samples were centrifuged and all of the supernatant removed. Deposits were resuspended in 3 ml of filter sterilized deionized water. A portion of each deposit was heated to 50 °C for 30 min. Heat-treated and untreated deposits were inoculated in 0.2 ml volumes across the surface of buffered charcoal yeast extract agar (6) (BCYE) with and without selective supplements (SR111 and SR118 Oxoid Ltd, Basingstoke). Plates were incubated at 37 °C in a humid atmosphere for 10 days.

Untreated deposits were also inoculated onto two columbia agar plates containing 10% horse blood, and incubated at 20 °C for 4 days and at 37 °C for 2 days.

Presumptive legionella colonies were initially identified by their failure to grow on BCYE media lacking cysteine and subsequently with polyvalent and monovalent *L. pneumophila* antisera.

All non-*L. pneumophila* bacteria isolated on blood agar or BCYE media from samples that were positive by GS or IFAT were subsequently tested with both immunofluorescent reagents.

GS and IFAT methods

Duplicate smears were prepared with 50 µl of untreated deposit from each sample. These were air dried, heat fixed and covered with 10% formol saline for 10 min. Subsequent washing was in deionized water for GS and phosphate buffered saline (pH 7.2) for IFAT. The GS staining procedure took 30 min and was performed according to the manufacturer's instructions. IFAT staining procedures have been fully described elsewhere (7). All reagents, buffers and fixatives used in the staining procedures were prepared with deionized water which had been passed through a 0.22 µ Millipore filter to remove any possible legionella contaminants. Bacteria for staining by GS and IFAT were suspended in 1% formol saline to the turbidity of a McFarland No. 1 standard, before applying to a slide. Smears were air dried, heat fixed and stained as before.

All slides were examined with a Zeiss epifluorescence microscope, fitted with a BP 450-490 excitation filter, an FT 510 dichroic filter, an LP 520 barrier filter and

Table 1. Reactions of legionellae examined with GS and IFAT

| Organism | GS | IFAT |
|------------------------------|----------------|----------------|
| <i>L. pneumophila</i> | | |
| Serogroup | | |
| 1 (3) | + ³ | + ³ |
| 2 | + | + |
| 3 | + | + |
| 4 | + | + |
| 5 | + | + |
| 6 (2) | + ² | + ² |
| 7 | + | + |
| 8 | + | + |
| 9 | + | — |
| 10 | + | — |
| 11 | + | — |
| 12 (2) | + ² | + ² |
| 14 | + | + |
| <i>Legionella</i> spp. | | |
| <i>L. bozemanii</i> | — | + |
| <i>L. dumofii</i> | — | — |
| <i>L. feelii</i> | — | — |
| <i>L. micdadei</i> (Heba) | — | + |
| <i>L. micdadei</i> (Tatlock) | — | — |

Single strain tested except where indicated by parentheses; +ⁿ, number of fluorescence positive strains; +/—, presence or absence of fluorescent bacteria.

an HBO 50 mercury vapour lamp. Smears were only scored as positive when fluorescent rod-shaped bacteria were clearly seen (Figure 1).

The relationship between immunofluorescence and culture in detecting L. pneumophila in water

A fresh isolate of *L. pneumophila* serogroup 6 was suspended in 10 ml of filter sterilized deionized water to approximately 10⁴ organisms/ml, and left at room temperature. Twenty-five microlitre aliquots were removed and inoculated over the surface of two BCYE plates without selective supplements, and onto duplicate slides for IFAT and GS staining. This procedure was repeated after 11, 22 and 30 days. BCYE plates were read after 10 days incubation and colony counts compared with the number of fluorescent bacilli in each well of the stained slides.

Statistical Analysis

Sensitivity, specificity and positive and negative predictive values were determined according to Galen & Gambino (8). Analysis of difference was by the Chi-squared test with Yates' correction for small numbers, where appropriate.

RESULTS

The GS detected *L. pneumophila* serogroups 1–12 and 14, whereas the IFAT did not detect serogroups 9, 10 and 11 (Table 1). In contrast the GS did not detect any of the non-pneumophila legionella whereas IFAT detected *L. bozemanii* and

Table 2. Reactions of non-legionella bacteria examined with GS and IFAT

| Organism | GS | IFAT | Organism | GS | IFAT |
|------------------------------------|----|----------------|----------------------------------|----|----------------|
| <i>Acinetobacter calcoaceticus</i> | — | — | <i>Salmonella typhimurium</i> | — | + |
| <i>Aeromonas hydrophilia</i> | — | — | <i>Shigella boydii</i> | — | — |
| <i>Aeromonas</i> sp. (3) | — | — | <i>Shigella flexneri</i> | — | — |
| <i>Campylobacter jejuni</i> | — | — | <i>Shigella sonnei</i> | — | — |
| <i>Escherichia coli</i> | — | — | <i>Staphylococcus 'albus'</i> | — | — |
| <i>Hafnia alvei</i> | — | — | <i>Staphylococcus aureus</i> (3) | — | + ³ |
| <i>Klebsiella aerogenes</i> | — | — | <i>Streptococcus faecalis</i> | — | — |
| <i>Proteus mirabilis</i> | — | — | <i>Streptococcus, group G</i> | — | + |
| <i>Plesiomonas</i> sp. | — | — | <i>Streptococcus milleri</i> | — | + |
| <i>Pneumococcus</i> (2) | — | — | <i>Vibrio cholerae</i> | — | — |
| <i>Pseudomonas aeruginosa</i> | — | — | <i>Vibrio parahaemolyticus</i> | — | — |
| <i>Pseudomonas cepacia</i> (2) | — | + ¹ | <i>Bacillus mycoides</i> | — | — |
| <i>Pseudomonas diminuta</i> | — | + | <i>Bacillus subtilis</i> | — | — |
| <i>Pseudomonas fluorescens</i> | — | — | <i>Alcaligenes faecalis</i> | — | — |
| <i>Pseudomonas mendocina</i> | — | — | <i>Flavobacterium devorans</i> | — | — |
| <i>Serratia marcescens</i> | — | — | <i>Proteus vulgaris</i> | — | — |
| <i>Salmonella</i> sp. (2) | — | — | <i>Lactobacillus</i> sp. | — | — |

Single strain tested except where indicated by parentheses; +ⁿ, number of fluorescence positive strains; +/—, presence or absence of fluorescent bacteria.

Table 3. Results of environmental samples examined by culture, GS and IFAT, for *L. pneumophila*

| Reference method | Positive samples detected by reference method | No. of reference method positive samples detected by | | |
|------------------|---|--|----------|---------|
| | | Culture | GS | IFAT |
| Culture | 36 | — | 34 (94) | 25 (69) |
| GS | 47 | 34 (72) | — | 35 (74) |
| IFAT | 35 | 25 (81) | 35 (100) | — |
| IFAT or culture | 46 | — | 44 (96) | — |

Figures in parentheses indicate sensitivity calculated against reference method.

L. micdadei (Heba). The GS was highly specific for *L. pneumophila* since it did not detect any of the 35 other bacterial species or strains examined, whereas the IFAT did produce fluorescent bacteria when some pseudomonads, *Staphylococcus aureus*, *Streptococcus milleri* and group G streptococci were used (Table 2).

GS was easier to use than IFAT in detecting *L. pneumophila* in water. This was in part due to the darker background obtained with GS (Fig. 1). *L. pneumophila* stained with IFAT appeared slightly brighter than those stained with GS but were less obvious against a brighter background. *L. pneumophila* stained with GS was easily detected against a plain dark background and could often be observed with a lower power objective ($\times 40$).

Of the 63 environmental samples tested, 49 (78%) contained *L. pneumophila* as identified by immunofluorescence, culture, or both. Thirty-six samples were positive by culture, 47 by GS and 35 by IFAT (Table 3). Thirty-four were positive

Table 4. Detection of *L. pneumophila* in distilled water by fluorescence microscopy and plate culture

| Time of sampling | No. of <i>L. pneumophila</i> per well | | No. of <i>L. pneumophila</i> colonies per plate Culture |
|----------------------|---|-----|---|
| | IFAT | GS | |
| Day 1 | 182 | 174 | 163 |
| Day 11 | 132 | 159 | 104 |
| Day 22 | 155 | 139 | 38 |
| Day 30 | 115 | 103 | 56 |
| Percentage reduction | 37 | 41 | 66 |

by both culture and GS, and 25 by culture and IFAT. The sensitivity, specificity, positive and negative predictive values for GS using culture as the standard were 94, 52, 72 and 87% respectively, whereas those for IFAT compared to culture were 69, 74, 71 and 61% respectively. Thirteen (21%) of the 63 samples were positive by GS but culture negative. In each case fluorescent bacteria of the correct size and shape for legionella were seen. Ten of these culture-negative samples were also positive by IFAT. One of the GS positive but IFAT and culture negative samples contained non-cultivable bacteria that fluoresced with antiserum to *L. pneumophila* serogroup 10. Non-pneumophila legionella that were cultured from GS positive samples did not fluoresce with GS. GS and IFAT failed to identify *L. pneumophila* in 2 and 11 samples respectively from which *L. pneumophila* had been cultured.

Fluorescence microscopy and culture

In order to determine the relationship between viable counts and number of bacteria detected by the immunofluorescence methods, *L. pneumophila* (serogroup 6) was suspended at 10^4 c.f.u./ml as described in the materials and methods section. The numbers of bacteria seen by GS, IFAT, and culture, were very similar at day 1 (Table 4). The number of bacteria detected by each of the methods declined over a 30 day period. By GS 59% of the original inoculum was detectable after 30 days, by IFAT 63% and by culture 34%. The reduction in viable count was significantly greater ($P < 0.001$) than for the detection of fluorescent whole bacteria by IFAT or GS.

DISCUSSION

L. pneumophila is commonly isolated from environmental water samples (9, 10). Although precise figures have not been defined, high numbers of *L. pneumophila* are considered to be suitable cause for implementing procedures to eradicate the organism from a water system (1). Culturing *L. pneumophila* from water samples can be a lengthy exercise and growth can be inhibited by biocides or other bacteria (2). Thus there is a need for a rapid method to detect *L. pneumophila* in environmental samples. A monoclonal antibody developed by Gosting *et al.* (3) against a *L. pneumophila* species-specific antigen is highly sensitive at detecting *L. pneumophila* in clinical samples.

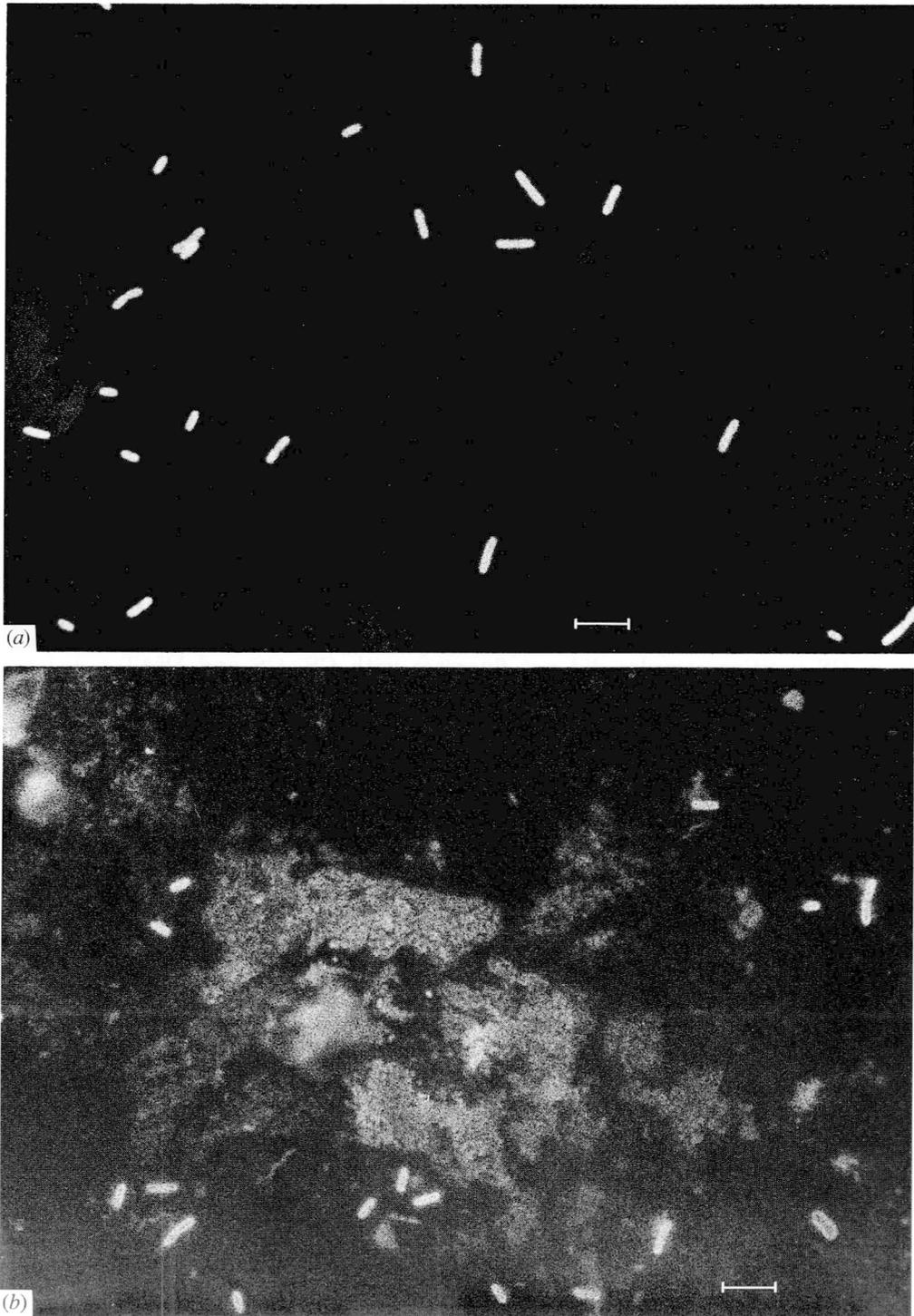


Fig. 1. *L. pneumophila* in environmental water stained by (a) GS and (b) IFAT. Scale marker represents 5 μ .

We have confirmed and extended observations on the specificity of this monoclonal antibody by demonstrating that in a direct immunofluorescence assay it could detect each of the available 13 *L. pneumophila* sero-groups. We have compared this GS with an indirect immunofluorescent method and with culture in detecting *L. pneumophila* from environmental water samples. GS detected *L. pneumophila* in 34 of the 36 water samples from which it was grown, whereas IFAT was positive in only 25 of these. The two culture-positive but GS-negative samples contained low numbers of bacteria (2 and 4 c.f.u./ml respectively); however, it must be noted that only a quarter of the sample volume used for culture was examined by GS or IFAT. According to some guidelines (1, 11) such small numbers of bacteria might not be considered significant.

Although the sensitivity of GS compared with culture was high (94%) its specificity was less good (52%). This was because GS detected fluorescent bacteria in 13 environmental samples from which *L. pneumophila* was not grown. It is also noteworthy that IFAT and monovalent antisera confirmed the presence of *L. pneumophila* in 11 of those 13 culture-negative samples. The performance of GS against legionella and non-legionella bacteria (Tables 1 and 2) suggest a high specificity, notably in excluding non-*L. pneumophila* bacteria which may share the same environment.

We have demonstrated that GS is as effective as IFAT or culture in demonstrating *L. pneumophila* serogroup 6 in artificially contaminated water (Table 4). In addition it was observed that the viable counts of *L. pneumophila* dropped during storage over a 30-day period more rapidly than the ability of GS or IFAT to detect whole bacteria. It is not known whether antigenicity and viability would decline at the same rates in environmental water samples. Direct counts of *L. pneumophila* in environmental samples stained by GS or IFAT may therefore not represent the number of viable bacteria present, but do indicate that conditions had been suitable for multiplication of the organism.

In certain situations where there may be cause for concern over the presence of *L. pneumophila* in a water system it has been recommended that the water be tested for the bacterium (1, 11). The delays inherent in culturing for *L. pneumophila* from environmental samples are unavoidable but frequently undesirable. The use of antigen detection systems may circumvent this problem. A rapid ELISA system has been devised (12) but this may have problems of even lower specificity since it would detect free as well as bacterium-associated antigen. In addition the antibody used in this test system is directed only against the Pontiac subgroup of *L. pneumophila* serogroup 1. This would preclude its use in areas where other serogroups of *L. pneumophila* have been associated with respiratory illness (13–17). Although GS would not detect the non-pneumophila legionella occasionally associated with Pontiac Fever (18), it is more rapid and more sensitive than IFAT, and as sensitive and certainly more rapid than culture in detecting *L. pneumophila* in environmental water samples. A combined attack using both culture and GS would provide a more complete picture of the role of *L. pneumophila* in water systems.

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