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Immunogold and Fluorescein Immunolabelling of *Legionella pneumophila* within an Aquatic Biofilm Visualized by Using Episcopic Differential Interference Contrast Microscopy

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Biofilms containing diverse microflora were developed in tap water on glass and polybutylene surfaces. *Legionella pneumophila* within the biofilms was labelled with monoclonal antibodies and visualized with immunogold or fluorescein isothiocyanate conjugates. Development of a differential interference contrast technique in an episcopic mode enabled simultaneous visualization of the total biofilm flora and gold-labelled legionellae. The legionellae occurred in microcolonies within the biofilm in the absence of amoebae, suggesting that the bacterial consortium was supplying sufficient nutrients to enable legionellae to grow extracellularly within the biofilm.

Colonization of water systems by *Legionella pneumophila* has been implicated as a cause of nosocomial Legionnaires' disease (3), and the organism is known to be widely distributed within drinking water systems (4, 14) and cooling towers (1, 10). Legionellae may proliferate in hot water systems, and polybutylene pipe, which is commonly used in such systems, appears to encourage their growth (20). Detection of low numbers of legionellae in aquatic environments is achieved classically by concentration of water samples by filtration and culture on selective media (2, 5). Adherent microbial consortia or biofilms occur within the water system and contain numbers of legionellae that are higher than those in the water phase (8); therefore, examination of this sessile phase might not necessitate a concentration step to detect legionellae in such environments.

Concern that culture may not detect all viable legionellae has led to increasing use of more direct methods to confirm culture results (7). Immunofluorescence detection offers a more rapid method for identifying legionellae in water samples in addition to supplying serotyping of the recovered strains (19). Immunological methods for detection of legionellae in water samples with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies were found to be more rapid and sensitive than conventional culture methods (16).

We previously evaluated the growth of *L. pneumophila* in biofilm and planktonic phases by culture (13). Biofilm was removed aseptically from the surface under investigation, and the microorganisms were dispersed into water and then enumerated by culture. The use of a direct method for the evaluation of the legionellae within the biofilm would allow detection of legionellae in situ, rapidly confirm culture results, and enable the investigation of biofilm structure and legionella ecology.

Immunogold labelling of the legionellae in the biofilm in situ may have advantages over using fluorescein-labelled antibodies, since gold is inherently more stable; samples can be viewed for longer periods and reviewed after storage. Moreover, the technique should allow both unlabelled biofilm microorganisms and immunogold-labelled legionellae to be viewed simultaneously by light microscopy rather than by visualizing the FITC-labelled legionellae alone by UV fluorescence microscopy. In this report, we describe the feasibility and suitability of these direct immunostaining procedures for detecting legionellae growing in biofilms on plumbing material surfaces.

Generation of biofilms. Biofilms were generated on the surfaces of previously sterilized coupons of glass or polybutylene pipe sections (1 cm^2) by suspending them in a two-stage chemostat model of a water distribution system as previously described (13). The inoculum for the complex microbial consortium was derived from an outbreak of Legionnaires' disease and contained an indigenous population of pathogenic *L. pneumophila* serogroup 1 (Pontiac), along with the associated bacteria and protozoa. The growth medium for the model was filter-sterilized, river-derived tap water of moderate hardness that had no additional supplements. The vessels were constructed only of titanium and glass to prevent leaching of iron, manganese, chromium, etc., from conventional stainless steel into the water phase. The dilution rate in the vessel used to generate biofilms was 0.2 h^{-1} (mean generation time, 3.5 h), the temperature was controlled at 40 ± 0.1°C, the agitation rate was 245 ± 15 rpm, and the dissolved oxygen tension was maintained at 20% ± 2% of air saturation by using microprocessor controllers (Brighton Systems, Hove, United Kingdom). Coupons of materials with attached biofilms were removed aseptically from the chemostat after 14 days for the immunolabelling procedures.

Biofilms were also generated on similar surfaces within a chemostat at 50°C. Culture studies showed that these biofilms contained very few legionellae, and therefore, they were used as negative controls for the immunolabelling

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procedures. Legionellae and other bacteria were isolated by culture on buffered charcoal-yeast extract (BCYE) agar (11) and low-nutrient R2A agar (12), after dispersion of biofilm into 1 ml of sterile tap water.

Immunogold labelling. Biofilms on the tiles were immediately fixed in 2% (vol/vol) formaldehyde in 0.01 M phosphate-buffered saline at pH 7.4 (PBS) for 1 h and then dehydrated in acetone for 15 min. A monoclonal antibody specific for the lipopolysaccharide of *L. pneumophila* (Sethi/LP 45; Cogent Ltd., Edinburgh, United Kingdom) (19) was diluted 1/40 in PBS for use. A 100-μl aliquot was applied to the 1-cm^2 biofilm surface at 4°C for at least 8 h. Unbound monoclonal antibody was removed by three washes in 20 ml of PBS with gentle stirring. A 100-μl volume of 1/40 goat anti-mouse immunoglobulin G conjugated with 5-nm gold particles (Biocell Research Laboratories, Cardiff, United Kingdom) was then applied to the treated biofilm and incubated at 4°C for at least 8 h. Excess gold conjugate was removed from the biofilm by three washings in 20 ml of PBS with gentle stirring. The gold-labelled cells were visualized after treatment with a silver enhancing kit (Biocell) until sufficient resolution was achieved, as judged by microscopy (approximately 3 min). The biofilm was washed under tap water to stop further reaction.

L. pneumophila was isolated from the chemostat by culture on BCYE agar for a working positive control for the immunolabelling procedure. A 100-μl aliquot of a suspension in PBS was placed onto a glass slide and air dried. A strain of *Pseudomonas paucimobilis* was isolated from the planktonic and sessile phases of the complex microbial consortium and used as a negative control, prepared in the same manner. The legionellae and *Pseudomonas* strains were identified by using a Biolog gram-negative identification system (Biolog Inc.) with an additional data base for the identification of legionellae (9). Further negative controls included biofilms generated under the same conditions, known to contain *L. pneumophila*, and subjected to the same staining protocol but without either the monoclonal antibody or the gold conjugate.

Fluorescein labelling. The immunogold staining procedure was repeated, except that FITC-conjugated goat anti-mouse immunoglobulin G (Sigma Chemical Co., Poole, United Kingdom) was used to visualize the immunolabelled cells. The FITC conjugate was diluted 1/40 in PBS for use.

Microscopy. The microscope used was a Nikon Labophot (Nikon, Telford, United Kingdom) equipped with both transmitted visible light and episcopic UV illumination. The brightness of the episcopic UV light from a mercury lamp was controlled by a combination of 1/2 and 1/4 neutral density filters. For visualization of FITC labelling, the EFD filter block contained an excitation filter of 450 to 490 nm bandwidth, a dichromic mirror at 510 nm, and a barrier filter of 520 nm. The differential interference contrast (DIC) analyzer was removed for observation of FITC labelling. For DIC microscopy, the filter block contained a polarizer and a 1/4 wave plate. The conventional immunogold staining block also contained a DIC analyzer. The microscope had an adjustable analyzer fitted into the episcopic fluorescence attachment main body between the eyepiece and the DIC or immunogold staining block. Comparison of these configurations indicated that the DIC block could also be used to view the gold labelling. Removable DIC prisms were also present within the objectives, which were long-working-distance, noncontact lenses usually used for metallurgical purposes. The objective lenses used were M Plan Apo 150/0.95 210/0, M Plan 100/0.80 ELWD 210/0, and M Plan 40/0.5 ELWD 210/0 (Nikon).

Biofilms and control pure cultures were examined with both the immunogold staining block (without the analyzer) and conventional DIC microscopy (with the analyzer fitted before the eyepieces). The immunogold staining block and the DIC block were readily interchangeable on a carriage within the episcopic illumination main body.

Microscopy of unstained biofilms. Before immunolabelling, the biofilms developed on the glass surface were viewed under transmitted light and by episcopic DIC microscopy to determine whether the staining procedures result in large losses of biofilm flora. Microscopical evaluation of unstained and unfixed biofilms showed that the biofilm consisted of a low background of microorganisms embedded in extracellular material with tall stacks of microorganisms and matrix material rising from the surface. The fine-focus adjustment of the microscope was fitted with an incremental scale that enabled the focusing distances to be determined between the surface and the top of the biofilm mosaic structure. The basal biofilm layer was approximately 5 μm in depth, and the taller stacks exceeded 100 μm in height. Within both zones of the biofilm there were areas that contained tight clusters of morphologically similar microorganisms, suggesting that the organisms within the biofilm were growing as microcolonies. Although the first chemostat vessel, which operated at 30°C, contained mixed populations of protozoa that could be seen grazing the biofilm under the microscope, there were no detectable protozoa on the biofilms developing at 40 or 50°C.

Use of DIC microscopy to view biofilm. The combination of episcopic light Normarski DIC and non-coverslip-corrected, extra-long-working-distance lenses allowed visualization of the biofilm on opaque polybutylene surfaces at up to ×1,500 magnification at the eyepiece. Even if the plastic had been translucent, these biofilms could not have been viewed under transmitted incident light with conventional biological lenses because the curved surfaces of the pipe prevented complete sample visualization (the sides of the pipe touched the lens before focusing could be achieved). The use of episcopic DIC was most successful when the biofilm was viewed directly without oil or a coverslip; these interfaces reflected episcopic light from their surfaces, interfering with the light returning from the specimen. A particular attribute of DIC microscopy was that the method allowed rapid, clear visualization of microbial cells within the biofilm without the need of any staining procedure.

Immunogold labelling. A pure culture of *L. pneumophila* from the chemostat used as the positive control was clearly labelled with the immunogold staining protocol, and there was minimal background nonspecific staining (Fig. 1a). The negative controls of biofilms containing legionellae incubated in the absence of either the monoclonal antibody or the gold conjugate were all unlabelled by the gold conjugate (Fig. 1e). Similarly, a pure culture of *P. paucimobilis* was also unlabelled (Fig. 1b). Observation of biofilm treated with immunogold indicated that the legionellae in the biofilm were also successfully labelled with gold particles (Fig. 1c). Microscopic observation of the immunolabelled biofilm showed that although the fixing procedure resulted in the dehydration and compression of the biofilm, there was no appreciable loss of biofilm or apparent loss of bacterial cells. The legionellae appeared as short rods on the biofilms developing in tap water; the labelled pure cultures contained both short rods and much longer, pleomorphic rods. The legionellae were found dispersed individually over the surface of the biofilm but were more commonly observed in distinct groups

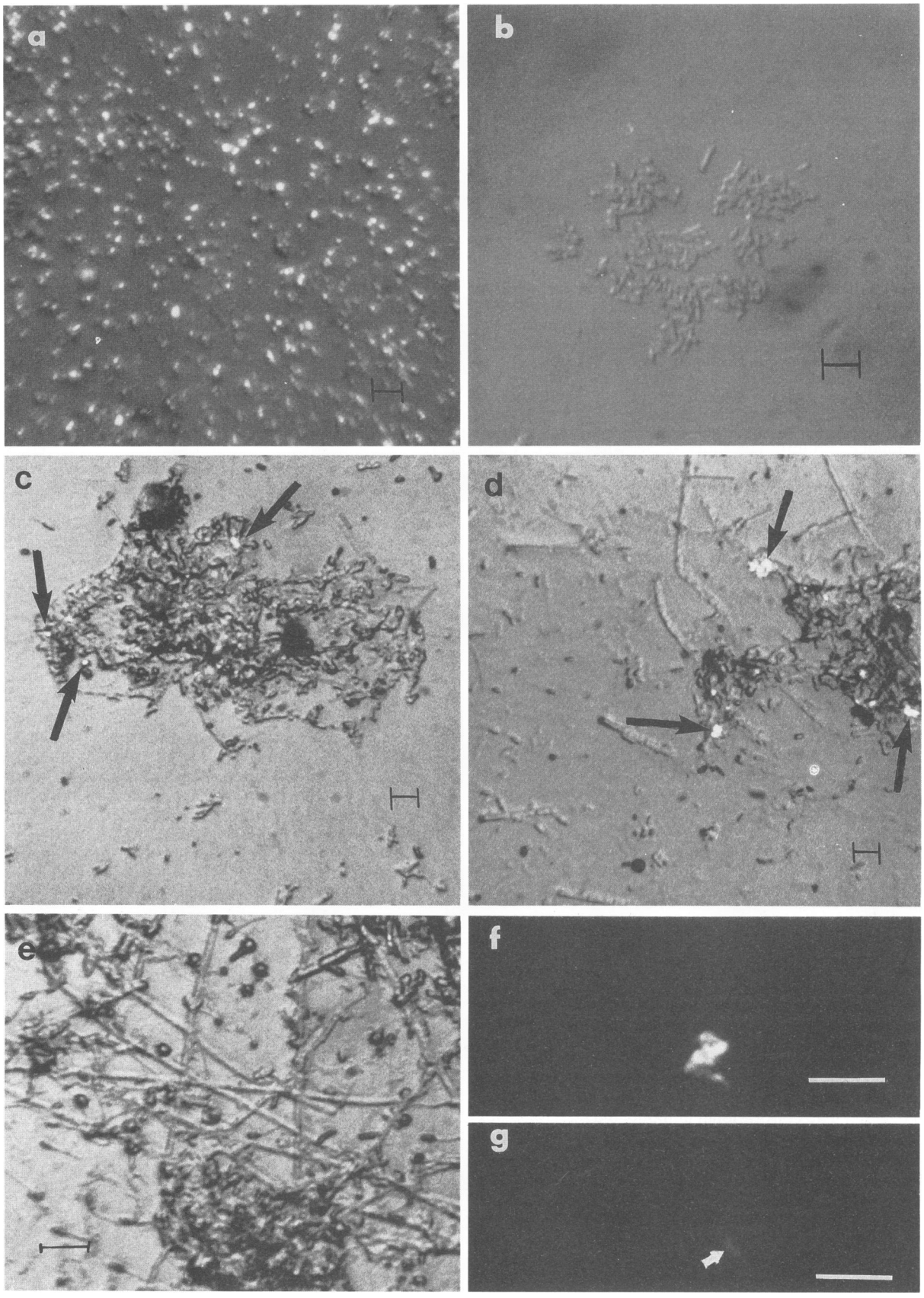
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FIG. 1. The positive control for the immunogold staining procedure was *L. pneumophila* (a), and the negative control was *P. paucimobilis* (b). The *L. pneumophila* was detected within the biofilm as single cells (c) and within microcolonies (d), denoted by the arrows. Biofilm incubation in the absence of gold conjugate showed little nonspecific binding (e). The existence of microcolonies of legionellae was confirmed by FITC labelling (f), although the surrounding biofilm cannot be observed with this method. The calcium carbonate deposits on the biofilm were faintly observable (g; arrow), but their lack of intensity and morphological distinction from the FITC-labelled legionellae was apparent. Bars, 10 μm.

and microcolonies (Fig. 1d). There was little evidence of nonspecific binding of the monoclonal antibodies or the gold particles, despite theoretical concern over the sequestering properties of the biofilm matrix. The appearances of the immunogold-labelled bacteria were similar when they were viewed with either the DIC block or the immunogold staining block. The DIC block afforded the advantage of adjustment of the analyzer, which improved contrast between the gold-labelled legionellae and the unlabelled biofilm bacteria. The absence of legionellae in biofilms generated at 50°C was confirmed by the absence of gold-labelled cells and provided an additional negative control for this study.

The use of gold labelling to detect legionellae in the environmental model samples with DIC microscopy allowed the simultaneous observation of total biofilm flora and the labelled legionellae, so that estimation of the proportion of legionellae in the flora population was practicable. The gold-labelled legionellae were quickly and easily recognized in the biofilms generated in the moderately hard river water. Some calcium carbonate was deposited onto the surface of the biofilm at 40°C and had a goldlike diffuse appearance under episcopic DIC light. However, these deposits were clearly morphologically different from those of the bacterial cells, and the two could be easily distinguished. Culture of biofilm flora on BCYE or low-nutrient R2A agar showed that legionellae composed a low proportion (less than 2%) of the total biofilm flora; however, the immunogold-labelled *L. pneumophila* cells were readily detectable within the biofilms in comparable numbers.

The immunogold-labelled biofilms that were on glass surfaces were also examined under transmitted incident light. The immunogold-labelled cells had a dense black appearance, which made them more difficult to differentiate from the biofilm flora with this method than with DIC microscopy.

FITC-labelled biofilm. The legionellae in the biofilm were successfully labelled apple green by the alternative FITC method, and there was minimal nonspecific binding (Fig. 1f). The biofilm contained some organisms that autofluoresced red, probably due to chlorophyll-containing cyanobacteria or algae; calcium carbonate deposits were diffusely and nonspecifically labelled a light apple green. As with the gold-labelled legionella-containing biofilm, nonlegionella fluorescent microstructures within the biofilm were easily recognized by their differences in color and morphology and could be discounted (Fig. 1g). The positive control pure culture of legionellae was labelled strongly, and the bacterial cells appeared apple green on a black background. The negative controls did not stain.

Viewing of biofilm directly by immunological methods allowed the rapid and accurate evaluation of the microbiological quality of the aquatic environment of this experimental plumbing system. Sample preparation can be achieved within 24 h and may provide useful information at least 48 h before culture results are available. The biofilm often contains numbers of both microbial flora and pathogens that are considerably higher than those in the water surrounding the surfaces (6), and direct viewing of the biofilm reduced the time required to sample the environment by avoiding a concentration step and time for incubation of agar plates. For rapid processing and visualization of individual legionellae on surfaces, it was much easier to locate the FITC-immunolabelled cells than the gold-labelled cells in biofilm. This was because the fluorescent stain contrasted more with the black background than did the gold-labelled particles when viewed by episcopic DIC microscopy. In the visualization of biofilm flora on the surface of metals with DIC microscopy, it may be impossible to distinguish between the immunogold-labelled cells and components of the metal surface. FITC labelling of organisms in the biofilm developed on the surface of metals or, in particular, dirty samples may be more appropriate than the immunogold labelling. However, the advantage of immunogold labelling of bacterial cells within the biofilm consortium is that the immunolabelled *L. pneumophila* and the biofilm flora can be viewed simultaneously, which may lead to increased information about the aquatic habitat.

The present study shows that the commercially available monoclonal antibody is suitable for detecting *L. pneumophila* serogroup 1 Pontiac in an environmental model system. This suggests that the lipopolysaccharide epitope that is expressed by the bacteria grown on BCYE agar, before the monoclonal antibody is generated, is conserved in the aquatic biofilm described herein. This monoclonal antibody and other monoclonal and/or polyclonal antibodies could now be evaluated against biofilms generated under differing environmental conditions.

Legionellae survive poorly and appear incapable of growth alone in sterilized tap water (20). There is considerable evidence that legionellae grow intracellularly within free-living amoebae (17), and it is now accepted that amoebae and other protozoa provide an important means of increasing the numbers of *L. pneumophila* within the aquatic environment. The presence of small cells of *L. pneumophila* in tight microcolonies among the biofilm flora suggests that the organisms are actively growing within the consortium and that growth of the legionellae could be sustained in the absence of host organisms. Although the biofilm generated within the chemostat model system at lower temperatures contains a diverse range of protozoa, at 40°C protozoa were not detectable by either culture on bacterial lawns or light or electron microscopy. At the higher temperatures, therefore, the legionellae may continue to grow extracellularly as part of the complex microbial consortia, receiving essential nutrients from several genera of bacteria (15, 18). In this regard, *L. pneumophila* has been described under some laboratory conditions to be a microaerophile (9). Extracellular growth within microcolonies of aerobic respiring aquatic species may thus provide a suitable low-oxygen environment for enhanced growth.

It is likely that both the biofilm microflora and the protozoan hosts play important roles in the extracellular and intracellular amplification of numbers of *L. pneumophila* within the aquatic environment. Further work with lower temperatures and increased times of incubation of coupons

in the chemostat model may provide more information on the interactions that occur between the biofilm flora, grazing protozoa, and *L. pneumophila*.

The method described herein could be useful for the routine detection and visualization of *L. pneumophila* if coupons of appropriate plumbing materials were inserted into cooling towers or biofilm sampling devices were fitted into pipe systems. A modification of the methods described here, with appropriate monoclonal or polyclonal antibodies, may be useful for the detection of other aquatic microorganisms, including *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Escherichia coli*, whose persistence in biofilms may pose health problems (8).

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