Occurrence of *Legionella* in groundwater: an ecological study

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Abstract The natural habitat of *Legionella* is the water environment. Little is known about their presence in groundwaters in spite of the fact that many millions around the globe regularly rely on groundwaters. This pilot study was aimed at evaluating the occurrence of *Legionella* in groundwater samples (water and biofilms) collected from various sites. Water and biofilm samples from selected groundwater sources were examined for *Legionella* using culture media (selective and non-selective) and a semi-nested PCR assay. Innovative approaches such as immunomagnetic separation (IMS) in combination with cultivation and flow cytometry were also evaluated. The findings available thus far show that (a) *Legionella* could be readily recovered from groundwater samples by cultivation even though their numbers showed considerable variations, (b) surprisingly, the PCR methodology was not yet as sensitive as cultivation and (c) flow cytometry was not directly applicable on natural samples because of debris and the high number of heterotrophic associated microflora from which some members were likely to cross-react with the monoclonal antibody used for separation procedures (IMS).

Keywords Microbial ecology; *Legionella*; groundwater; biofilm; wells; waterborne infections; human health

Introduction

Members of the genus *Legionella* are a cause of both community-acquired and nosocomial pneumonia (Legionnaires' disease, LD) acquired mainly by the inhalation of *Legionella*-containing aerosols. It is estimated that 25,000–100,000 cases of LD occur in the US annually. The bacterium can be recovered from waters that have a variety of physical, chemical and biological characteristics. Considering that legionellae are widespread in aquatic environments, the purpose of this study was to investigate if groundwaters may also act as natural reservoirs for these bacteria. Indeed, over 100 million residents in the US and many more in other parts of the world rely on groundwaters for their drinking water. In this study we proposed to detect and quantify *Legionella* in groundwater samples from representative regions of North America. Results presented here were obtained during the initial phase of a study aimed at first demonstrating (technical process validation) the presence of *Legionella* in selected groundwater wells. *Legionella* was recovered by cultivation (the gold standard) and a semi-PCR method was also used as comparative testing on water and biofilm samples. Attempts were also made to apply newer experimental approaches such as IMS using a genus-specific monoclonal antibody in combination with cultivation and flow cytometry. Additional work is now underway on samples from different locations in the US and Canada to determine if biotic or abiotic water quality parameters can act as potential indicators of *Legionella*.

Materials and Methods

"Routine approach"

Cultivation – the findings reported here are based on the analysis of water and biofilm samples from twelve wells located at two sites in the US. The process for the collection of biofilms was either by removing the end caps of each well and scraping the biofilm off

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from the well wall or from pipe sections of the feed lines of the wells. Water samples were taken at the same time as biofilm samples. Samples were placed in coolers with ice, sent to us by overnight courier and processed within a few hours of arrival at the laboratory. Waters were filtered through polycarbonate filters and the concentrates plated either directly or after acid and heat treatments on media with (CCVC, GVPA) or without antibiotics (BCYE). Any clumps of *Legionella* organisms and/or their aggregates (biofilms) were dispersed by vortexing at full speed (3×30 s) and by sonication before plating. All plates were incubated at 30ºC and 35ºC in humidified air (>50%) with 2.5–5% CO_2 for 18–20 d. Parts of the samples were incubated (1 week at 35ºC) as an enrichment method and processed as above. Altogether 120 plates per sample were inoculated. Suspected *Legionella*-like colonies were regarded as presumptive *Legionella* after the cysteine requirement was verified.

Semi-nested PCR – a semi-nested PCR assay (Miyamoto *et al.*, 1997) was used to directly detect *Legionella* in samples after concentration and DNA extraction. When necessary, separate PCR assays were also conducted after adding bovine serum albumin (BSA) to the PCR mix.

Identification of isolates – identification of some isolates was done by nucleotide sequencing of amplified fragments (using the first step of the semi-nested PCR assay, a 16S rRNA gene-targeted PCR) obtained from suspected colonies. Nucleotide sequences were compared with available sequences in the DDBJ, EMBL and GenBank databases with the BLAST program through the Internet (*http://www.ncbi.nlm.nih.gov*).

"Experimental approach"

The specificity of the monoclonal antibody (Mab) chosen as a candidate for *Legionella* detection by IMS was tested by flow cytometry against a strain of *Pseudomonas aeruginosa* as representative of strains frequently encountered in environmental samples and known to cross-react with *Legionella* when using direct fluorescent antibody typing. This Mab is known to recognise a *Legionella*-specific epitope of the Mip protein on all 34 *Legionella* species tested (Helbig *et al.*, 1995). The Mab 22/1 was received as cell culture supernatant and was purified using a protein-G column following manufacturer's instructions (Amersham Pharmacia). One fraction was FITC-labelled using a FluoReporter® FITC Protein Labeling Kit following manufacturer's instructions (Molecular Probes Inc.). An Epics XL™ (Coulter Corp.) cytometer equipped with a 15 mW, air-cooled, 488 nm wavelength argon laser light allowing a four-colour fluorescence analysis was used to measure forward angle light scatter (FSC) and fluorescence of the microbial cells. Set up of the flow cytometric assay was done using pure cultures of *L steigerwaltii* (strain SC-18-C9, ATCC 35302). Photomuliplier tube voltage and spectral compensation were initially set using cells single-stained with propidium iodide (PI) and FITC-labeled 22/1 antibody.

IMS was also tested in combination with cultivation to recover pure *Legionella* suspensions and to recover the bacteria from artificially contaminated field samples. Briefly, pure bacterial suspensions or filtered-artificially contaminated field samples were concentrated to 1mL by centrifugation. The Mab (15 µg) was added to the suspension and left at room temperature (30 min) with agitation. Dynabeads® (Dynal Inc) coated with a sheep anti-mouse antibody were added and the mixture agitated at room temperature for 30 min. Magnetic separation was performed using one washing step with the cells still attached to the magnetic support. Concentrated cells were then plated on BCYE in triplicates. Controls were run in parallel to determine the recovery rates.

Results

Samples (24) from 12 separate wells have been processed. Of these, 22 have tested positive for the presence of *Legionella* in the groundwater (10/12) and/or the biofilm (9/12). Concentrations of *Legionella* in water samples were 1×10^2–8.4×10^4 CFU/L. In biofilm samples, concentrations of 2–267 CFU/cm^2 were observed. Media that were more frequently associated with *Legionella* recovery were CCVC (37%, 23/61 positive plates) followed by BCYE (36%, 22/61 positive plates) and GVPA (26%, 16/61 positive plates). Both incubation temperatures were associated with *Legionella* recovery (35ºC – 9/12 positive samples; 30ºC – 10/12 positive samples). Some strains grew exclusively at 30ºC. The treatment that was more associated with *Legionella* recovery was acid plating then direct plating and heat treatment. Enrichment did not enhance *Legionella* detection by cultivation. Bluish-white and red auto fluorescent species were more frequently isolated than non-autofluorescent species. When carried out, partial 16S rRNA gene sequencing identified *L dumoffii* or *L gormanii* as the major species encountered. Red fluorescent isolates were more related to *L rubrilucens*. Some atypical strains or species have also been recovered. All isolates have been preserved for further study and characterization.

Of the 24 samples tested, 7 were PCR positive (3/12 water samples and 4/12 biofilm samples). All positive samples were obtained after enrichment. Six biofilms and two water samples were found to inhibit PCR possibly leading to false-negative results. Inhibitory substances detected in some enriched biofilms had been sufficiently diluted by the semi-nested PCR procedure to allow an efficient amplification. However, in other cases, the dilution between the two steps PCR was not sufficient to decrease the inhibitory effects of the extract.

Flow cytometry was used to evaluate the ability of the monoclonal antibody used to specifically detect *Legionella* spp. Specificity testing against *P aeruginosa* showed that the flow cytometric assay might be able to detect a very few bacteria belonging to the *Pseudomonas* genus. This is why IMS was being tested: (a) to partially eliminate background materials and (b) to evaluate the specificity of this approach to detect *Legionella* versus the heterotrophic associated microflora in the samples.

Results obtained so far from IMS cultivation showed that in the best case not more than a 36% recovery rate was to be expected (*L pneumophila*) in the range of 10^2–10^3 CFU/L. When testing other *Legionella* species (*L steigerwaltii* and *L rubrilucens*) recovery rates were about 30% for both species. However, the concentration ranges for optimal detection were 10^3–10^4 and 10^3 CFU/L respectively. When artificially contaminated samples were tested, similar recovery rates were obtained and a few non-*Legionella* colonies were also observed on inoculated plates suggesting that further refinement of this approach was needed.

Discussion

Our objective during this pilot study was to demonstrate the presence of *Legionella* in groundwaters. Two sites were selected with water temperatures (approximately 25ºC) that were believed to be compatible with *Legionella* survival and/or growth. Levels of contamination (measured by cultivation) in the samples analysed so far were relatively low in relation to concentrations of *Legionella* usually found in cooling tower waters or in tapwaters. However, and not surprisingly, the levels of contamination in the biofilm samples were usually much higher than those in the corresponding water samples. Our cultivation assay revealed information that should be interpreted with care because single samples were collected from the target sites. The concentrations observed may not have reflected the real levels of *Legionella* contamination within a particular well and local conditions might influence *Legionella* concentrations. Most of the positive samples were contaminated with members of the bluish-white auto fluorescent group of *Legionella*. Some samples showed a

double or triple contamination with red auto-fluorescent species and/or non-auto fluorescent species. This situation resembled that observed in a French study on the occurrence of *Legionella* in hot spring spas (Bornstein *et al.*, 1989). It must also be noted here that a definitive identification of the isolated bacteria was possible only on the basis of a combination of tests. Therefore, partial 16S rRNA gene sequencing might not be the ultimate tool to definitively assign a species name to an isolate (Fox *et al.*, 1992).

In view of the low levels of *Legionella* contamination seen in the samples tested thus far, it was not surprising to see only a few positive results by our PCR assay. It is also possible that the presence of inhibitors in the samples may have decreased the level of sensitivity of the PCR reaction. It is very likely that incorporation of Southern blotting of the PCR products would have increased the level of sensitivity of the assay and dramatically raised the number of positive results. In this study the Southern blotting procedure was not used as it was expected that a combination of 50 cycles of amplification (combining the two steps of the semi-nested PCR assay) would have been sufficient to achieve a high level of sensitivity.

Conclusions

IMS cultivation showed promise as a tool for the isolation of *Legionella* with recovery rates of 36–60% depending on the species. This approach, which could also reduce the number of culture plates needed for *Legionella* isolation, is under further investigation for the rapid recovery of *Legionella* from environmental samples. The use of Mab against a genus-specific and readily accessible epitope could, at least in theory, provide cleaner samples by reducing the levels of contamination with other organisms present in the samples although potential cross-reactions with unknown bacterial species in the samples may lead to unspecific detection. Such a clean-up step should also help in the removal of debris that could clog the flow cytometer injector and interfere with direct counting of labelled cells. The IMS procedure could be directed either at maximising the capture of target cells and accepting a high contamination level or at obtaining a smaller population of pure target cells. It was decided that, initially, the procedure should be directed towards maximising cell recovery. Thus, following cell–antibody interaction, the cell–antibody complexes were attracted towards the magnet and held at the side of the tube during washing.

We have demonstrated that *Legionella* can be recovered from biofilm and water samples collected from groundwater wells. While the role of surface water-related biofilms as a niche for *Legionella* is well recognized, our findings clearly showed that biofilms associated with groundwaters could also be sources of these bacteria in drinking waters.

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