Multiplex detection and identification of pathogenic *Legionella* species and associated hosts using DNA array technology

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Scientia Terrae Research Institute successfully developed a DNA macro-array to simultaneously detect and identify a comprehensive set of *Legionella* species and its hosts. Each diagnosis can be achieved within 36 hours of sampling based on an objective technique utilizing an array of genus- and species specific DNA fragments. This strategy provides a substantial improvement in the diagnosis time from seven days typically experienced with conventional culture plating. Moreover, the '*Legionella* scan' could identify several different *Legionella* species in water samples like *L. anisa, L. bozemanii, L. gormanii, L. pneumophila* and *L. sainthelensi*. Culture-based analysis only recovered *L. anisa* and *L. pneumophila*. Further experiments should clarify whether this DNA represents viable bacteria or whether the classical plating is too selective. The diagnostic kit *now* contains specific detector probes for the detection and identification of 20 different *Legionella* species and 3 genera of Protozoa.

Introduction

Legionnaires' disease is a sporadic or endemic disease caused by legionellae, which are Gram-negative bacteria that are ubiquitous inhabitants of aquatic environments. Out of more than 50 *Legionella* species, *Legionella pneumophila* is reported as the most common cause of legionellosis. Nevertheless, other serogroups and other *Legionella* species are increasingly associated with human disease(Fields et al., 2002. CMR).

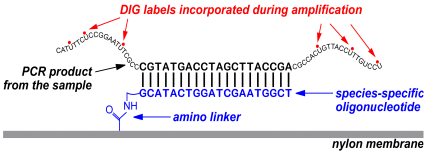
Accurate detection and identification of *Legionella* using conventional methods is complicated by their growth requirements, their ability to enter a viable non-culturable state, their association with protozoan hosts and the occurrence of *Legionella* within biofilms. These drawbacks can be circumvented by molecular detection methods. Currently, polymerase chain reaction (PCR)-based DNA array technology is one of the most suitable techniques to detect multiple organisms in a single assay, even if they differ in only a single to a few bases in the targeted sequence. The main objective of this study was to develop a DNA array (Lievens et al., 2003. FEMS Microbiol Lett) to specifically detect and identify 20 different *Legionella* species from water samples in a single assay.

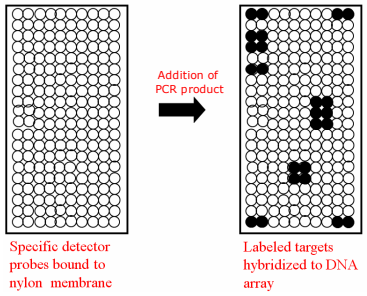
Material and methods

Development of a DNA array for *Legionella*, the '*Legionella* multiscan'

A collection containing about 120 *Legionella* isolates and more than 45 species of *Legionella* was obtained. Sequences of the 16S rDNA and the macrophage infectivity potentiator (mip) were aligned and multiple 19- to 25-mer taxon-specific oligonucleotides were selected, attempting to respect optimal and uniform hybridization kinetics. All oligonucleotides were synthesized with a 5'-C6-aminolinker and blotted onto nylon membranes.

For analysis of the water samples, 500ml water was filtrated. Genomic DNA was extracted and the target 16S rDNA and mip regions were amplified and simultaneously labeled with alkaline-labile digoxigenin using respectively *Legionella* specific 16S rDNA and mip primers (Ratcliff et al., 1998. JCM). Hybridization was conducted overnight at 54°C. Detection was performed using anti-digoxigenin alkaline phosphatase conjugate and CDP-Star substrate. Chemiluminescence was detected using a highly sensitive digital CCD video camera.





Classical plating

Water samples were analyzed on Buffered Charcoal Yeast Extract (BCYE, Oxoid) and on Glycine-Vancomycin-Polymyxin B-cyclohexamide (GVPC, Oxoid) agar after 7 days aerobic incubation at 37°C following the ISO norm 11731.

Future direction

Specific oligonucleotides have been developed for the detection of *Naegleria*, *Hartmanella* en *Acantamoeba* species. In the future, industrial water samples will be analyzed and evaluated on the presence of both *Legionella* sp. and these protozoa hosts.

Results and discussion

Design and development of the '*Legionella* multiscan'

All strains for which specific detector oligonucleotides were developed, are listed in Table 1. The mip oligonucleotides are very specific and able to discriminate the different species. Nevertheless, detection of the 16S rDNA is more sensitive and can detect up till 5 cfu, whereas the mip gene has a detection limit down to 10^3 cfu/ml in this research.

Table 1. Targets that are detected by the '*Legionella* multiscan'

Specificity	Target Gene	Specificity	Target Gene
Legionella sp.	16SrRNA gene	*L. longbeachae*	mip gene
L. anisa	mip gene	*L. maceachernii*	mip gene
L. birminghamensis	mip gene	*L. micdadei*	mip gene
L. bozemanii	mip gene	*L. oakridgensis*	mip gene
L. cincinnatiensis	mip gene	*L. pariensis*	mip gene
L. dumofii	mip gene	*L. pneumophila*	mip gene
L. erythra	mip gene	*L. sainthelensi*	mip gene
L. feeleii	mip gene	*L. tucsoniensis*	mip gene
L. gormanii	mip gene	*L. wadsworthii*	mip gene
L. hackeliae	mip gene	*Acantamoeba* sp.	18SrDNA
L. jordanis	mip gene	*Hartmanella* sp.	18SrDNA
L. lansingensis	mip gene	*Neigleria* sp.	18SrDNA

Analysis of drinking water samples

The '*Legionella* scan' revealed the presence of different *Legionella* species in drinking water samples. Beside *L. pneumophila* and the well-known *L. anisa*, also *L. bozemanii*, *L. gormanii* and *L. sainthelensi* were detected. Moreover, 30% of the random sampled water contained DNA that represents more than 10^3 cfu/ml *Legionella*. For another 38% of the samples, only genus specific oligos for *Legionella* lighted up. Consequently, either the concentration of *Legionella* is too low to be detected by the species specific oligonucleotides or there are *Legionella* species present, different form the 20 species on the membrane. Sequencing a clone library of these samples will clarify this observation.

Table 2. Results of DNA array analysis of 111 water samples

Legionella species	Number of samples	percentage
L. anisa	9	8
L. bozemanii	5	5
L. gormanii	2	2
L. longbeachae	2	2
L. pneumophila	14	13
L. sainthelensi	10	9
Only *Legionella* sp.	42	38
Legionella detected	76	68
No legionella detected	35	32
Totaal	111	100

Large differences were obtained between the classical plating and the macro-array (Table 3). Classical plating is more sensitive than the *Legionella* scan which explains the higher numbers of *L. pneumophila* with this technique. However, the DNA array detected *Legionella* spp. in many samples without culturing. Either this DNA is obtained from dead cells or the classical plating does not efficiently pick up other species than *L. pneumophila (*and *L. anisa*). The stability of DNA from dead *Legionella* bacteria is now being tested.

Table 3. Comparison between the results of the *Legionella* scan and the classical plating for 111 water samples

Target	DNA array	Classical plating
Legionella genus detected	76	38
Specific *Legionella* sp. without *L. pneumophila*	24	4
L. pneumophila	14	36
Negative	35	73

Conclusions

1.The DNA array analysis revealed the presence of several different *Legionella* species in water samples like *L. anisa, L. bozemanii*, *L. gormanii, L. pneumophila* and *L. sainthelensii*. Culture-based analysis only recovered *L. anisa* and *L. pneumophila*. Further experiments should clarify whether the DNA represents viable bacteria or whether the classical plating is too selective.

2.To our knowledge, the developed tool is the first to detect 20 different *Legionella* species in 1 analysis (of 36 hours).

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