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Development of PCR-based detection assays for *Legionella pneumophila* in water

by

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Submitted in partial fulfilment of the requirements for the degree of
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In the department of Microbiology and Plant Pathology
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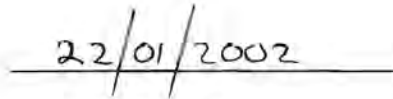
DECLARATION

I declare that the dissertation, which I hereby submit for the degree Magister Scientiae Agriculturae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

COENIE GOOSEN



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CHAPTER 1

Introduction

The bacterium *Legionella pneumophila* is a common cause of nosocomial and community-acquired pneumonia, particularly among individuals with impaired immunity. The organism was first isolated in 1977 after an outbreak in 1976 in Philadelphia, U.S.A. (McDade *et al*, 1977). *L.pneumophila* is found in natural aquatic habitats such as rivers and lakes (Fliermans *et al,* 1981) as well as industrial and domestic water systems. These include potable water systems, cooling tower systems, thermal waters for rehabilitation (spas) and dental water units (Barlett *et al*, 1983; Colbourne *et al*, 1988; Dondero *et al*, 1980; Atlas, 1999). Currently, detection of the pathogen largely relies on culturing on a semi-selective solid media such as buffered charcoal yeast extract (BCYE). Isolation may be preceded by heat or acid treatment to limit the growth of other non-legionellae. Several problems are encountered with this method, including the presence of viable but non-cultureable cells (Hussong *et al*, 1987), loss of viability of bacteria after collection, difficulties in isolation from biocontaminated samples and the time required for culture and confirmation, which can span several days. To avoid these problems, several hybridization methods employing specific DNA probes were developed (Edelstein, 1986; Grimont *et al*, 1985). These techniques, however, displayed poor sensitivity, and for this reason, have been considered inadequate for direct detection of legionellae in water sources.

The polymerase chain reaction (PCR) provides a possible alternative towards specific and rapid detection of *L.pneumophila* in environmental samples. Amplification within the macrophage infectivity potentiator (*mip*) gene (Engelberg *et al*, 1989) enabled specific detection and identification of *L.pneumophila*, and the application thereof have been shown in a previously available commercial system. Complexity of industrial and environmental water samples can however inhibit target amplification, leading to reduced

sensitivity and false-negative results (Koide *et al*, 1993, Maiwald *et al* 1994, Ng *et al.* 1997). Modification to the basic PCR approach (nested PCR) increases the sensitivity of the assay by dilution of inhibitory substances coupled to a increased number of cycles, but could produce false-positive results from minute amounts of carry-over contamination (Oshiro *et al*, 1994). A modification to the standard PCR approach, namely pit-stop PCR, has enabled sensitive and specific amplification of *Vibrio cholerae* from seeded environmental water samples (Theron *et al*, 2000). This approach enhances the detection limit of the standard 30-cycle amplification approach by using a two step approach consisting of the same total number of amplification cycles.

Although modifications to standard PCR approaches could improve the sensitivity of detection in inhibitory water sources, highly concentrated samples may still produce false-negative results. It is therefore necessary to improve the quality of target template by using a purification approach. Standard multi-step chemical-based purification assays could prove laborious in a diagnostic setup, and could lead to valuable target template loss. Alternative methods of target isolation, e.g. immunomagnetic separation (IMS) and solid-phase sequence capture have proven useful for detection of various pathogenic bacteria and viruses from PCR inhibitory substances (Hultman *et al*, 1989; Rimstad *et al*, 1990; Luk *et al*, 1991; Cudjoe *et al*, 1994; Olsvik *et al*, 1994; Mangiapan *at al*, 1996; Pyle *et al*, 1999). The former is based on isolation of intact *L.pneumophila* cells, enabling DNA release in the absence of inhibitory substances, were the latter enables target DNA specific capture from lysed cells in samples using probe-labelled magnetic beads

Considering the widespread application of standard detection assays and the shortcomings they present, the study primarily focused on further development and adaptation of the abovementioned technologies (PCR, pit-stop PCR, IMS and solid-phase sequence capture) to enable sensitive and specific detection of *L.pneumophila* in industrial and environmental waters.

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CHAPTER 2

Literature overview

2.1 Legionellae: an overview

Bacteria in the family *Legionellaceae* are representatives of a group of organisms which maintained their anonymity until 1977, primarily because of their fastidious nature and unique ecology. Initially, a single member of the group, *Legionella pneumophila*, was identified as the causative agent for Legionnaires' disease (McDade *et al*, 1977). This disease primary affects the respiratory system and may, in a percentage of susceptible individuals, proceed to be fatal.

Water is the major reservoir for legionellae, and inhalation of legionellae in aerosolised droplets is the primary means of transmission for Legionellosis. This fact has contributed to the importance of this pathogen with regards to industrial environments with nearby human activity. It has been suggested that protozoa, especially certain Amoebal species (*Hartmanella vermiformis*, *Acanthamoeba* spp., *Naegleria* spp. and *Tetrahymena pyriformis*) play a supportive role in the survival and multiplication of *Legionella* and may act as natural hosts and amplifiers for the organism (Rowbotham, 1980; Fields *et al*, 1984).

2.2 Taxonomic status

Members of the genus *Legionella* are characterised as motile, aerobic Gram negative bacilli, approximately 0.3-0.9 µm in width by 1-20 µm in length (Brenner, 1987). Energy and carbon are derived from amino acid metabolism, and not from carbohydrate fermentation or oxidation. Culturing in a laboratory

can only be achieved by means of specialised growth media such as buffered charcoal yeast extract agar (BCYE).

The genus *Legionella* was initially defined in 1979 by Brenner and co-workers (Brenner *et al*, 1979). Since then, the genus has been re-defined several times to take into account the addition of several species as well as subspecies. The initial description of the group was based on two methods: Oligonucleotide cataloguing of 16S rRNA and DNA hybridisation studies. Using the former technique, Brenner *et al* (1979) demonstrated that the legionellae are not closely related to other groups of organisms, but that they are closely related to each other at the ribosomal level. This study further showed that legionellae are distantly related to the purple sulphur bacteria and their non-photosynthetic relatives. DNA hybridisation studies also demonstrated uniqueness of the species. Brenner (1987) demonstrated that, based on rDNA hybridisation studies, the legionellae were most closely related (approximately 60%) to members of the *Enterobacteriaceae* and to *Pseudomonas*. To date, it would appear from the literature that the taxonomic status of the genus *Legionella* are agreed on, where 39 species and 61 serologically distinct groups have been identified within this genus (Brenner *et al*, 1984).

2.3 Legionellosis and Legionnaires' disease

Legionellosis is a collective term to describe two distinct disease patterns caused by species of the genus *Legionella*: Legionnaires' disease, the severe pneumonic form, and Pontiac fever, with flu-like symptoms, similar to influenza virus infection. These two forms were named after the first documented outbreaks in Philadelphia (McDade *et al*, 1977) and Michigan (Kaufmann *et al*, 1981) respectively, in the United States during the late 1970's. Advances in development of growth media during the early 1980's enabled isolation and further characterisation of this group of organisms.

Proliferation of legionellae and resultant human disease is promoted by human activities with environmental impact. Such activities include industrialization (with associated cooling systems) as well as indoor climate manipulations brought about by climate control and humidification. The disease occurs when a sufficient number of virulent legionellae are aerosolised followed by inhalation by a susceptible host. Multiplication has also been found to occur within alveolar macrophages in humans and forms an essential part in the pathogenicity of this group (Fields, 1996; Horwitz, 1993). There seems to be no definite correlation between disease occurrence and the number of legionellae present in any given water sample (Hoage and Breiman, 1991). Generally, monitoring for legionellae is only carried out to identify the source of an outbreak of Legionellosis.

Currently, 18 or more species of the genus *Legionella* have been implicated in human disease (Fields, 1997). The U.S. Centers for disease control (CDC, Atlanta, USA) estimates that more than 85 % of all *Legionella* pneumonias are caused by a single species of the genus, namely *Legionella pneumophila*, within which serogroup 1 is most prevalent (Fields, 1997).

2.4 Invasion in mononuclear phagocytes

Although *L.pneumophila* is a facultative intracellular pathogen, it does not grow in tissue culture media; any growth thus far observed in cultured cells is due to intracellular multiplication. As in the case of protozoan species, *L.pneumophila* infects alveolar macrophages, wherein the organism survives and replicates within a specialised phagosome. (*Legionella*-specialised phagosome, LSP). Work carried out by Horwitz and Silverstein (1980) regarding the cell biology and genetics of the host-pathogen interactions created the basis for understanding the entry and survival of the organism in human monocytes (Figure 1.1). With regards to the aims of the study, the precise mechanisms and pathways associated with infection will not be discussed in detail.

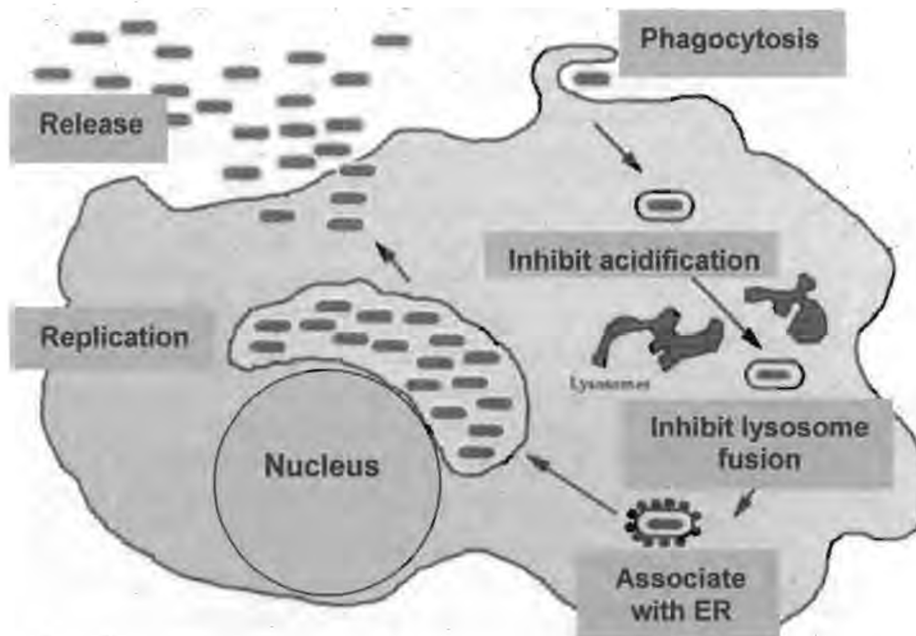

Figure 1.1. Infection cycle of wild-type *L.pneumophila* in mononuclear phagocytes. The bacterium enters the cell via coiling phagocytosis, where it sequentially interacts with smooth vesicles, mitochondria and ribosomes. The organism multiplies in the ribosome-lined phagosome until the monocyte is filled with bacteria and disintegrates. (www.legionella.com)

2.5 Ecology of the Legionellae

The major reservoir for legionellae is water, including environmental, industrial as well as potable waters. Although it has been shown throughout literature that legionella could be detected from almost any type of water sample (Fliermans *et al*, 1979), the organisms are most frequently found in close association with algae and protozoan species. Controversy still exist regarding the precise mode of survival of this fastidious organism in complex environments; currently, evidence exists supporting intracellular survival in amoebal species (Fields, 1996), the protozoan *Hartmanella vermiformis*

(Fields *et al*, 1993) as well as replication in ciliated protozoa such as *Tetrahymena thermophila* (Tyndall and Domingue, 1982).

Previous studies revealed that between 40 and 80% of environmental waters tested positive depending on the detection procedure used (culture based or PCR respectively) (Fields, 1997). Because of their ubiquitous nature, controversy exists around the purpose of testing various water sources for their presence. Generally, monitoring is carried out to identify the source of an outbreak of Legionellosis or to evaluate biocide performance. Regular microbiological analysis (as a preventative measure) of high-risk areas such as organ transplant units and hospitals are thus of utmost importance (reviewed in Atlas, 1999).

As mentioned in section 2.3, man-made environments also play a substantial role in the survival and proliferation of *L.pneumophila*. Outbreaks of Legionellosis have been traced to a wide variety of sources including cooling towers, whirlpools and spas, fountains, ice machines, vegetable misters, shower heads and dental water sources linked to potable water supplies. Outbreaks have also occurred in a range of man-made environments such as cruising ships, hotels and offices (Atlas, 1999). To date, these sources have been recognised and targeted as major contributors of sporadic outbreaks of the disease.

2.6 Detection strategies for *L.pneumophila*

2.6.1 Culture-based detection

A large number of different diagnostic techniques are available today for *Legionella* detection. Initially, detection procedures relied on inoculation of susceptible guinea pig hosts (McDade *et al*, 1977), which proved to be quite selective but very expensive and time consuming. Culture based isolation was first performed on a nutrient rich bacteriological medium. It consisted of Mueller-Hinton agar supplemented with haemoglobin and IsovitaleX which supplied the essential amino acid L- cysteine. After several improvement

steps Feeley-Gorman agar was developed. This medium provided increased recovery from tissues compared to Mueller-Hinton agar (Feely *et al*, 1978). Activated charcoal later replaced the starch in the medium for detoxification, and the amino acid source was changed to yeast extract, resulting in the presently used buffered charcoal yeast extract agar [BCYE] (Edelstein, 1981). Because of the fastidious nature of this bacterium, and long incubation periods needed on artificial culture media, problems are usually experienced when working with highly contaminated samples. Sample preparation methods eliminate the bulk of heterotrophic organisms that might interfere with detection. For this reason, pre-treatment of samples with acid buffer or heat are recommended to limit the heterotrophic count, thus enabling culturing and identification of *L.pneumophila*. Pre-treatment, however, have an effect on the survival and culture ability of the organism on media, and could therefore produce false-negative results in the case of low bacterial loads (Bartie, 2001). Presumptive identification after culturing is commonly achieved by confirmation of cystein dependence of individual colonies, which should be followed by a confirmational technique.

Direct immunofluorescence is most often used for confirmation of *Legionella* species from environmental samples and is specific for *L pneumophila SG 1-6* and *L micdadei*. Antigen from the sample is heat- or acetone- fixed on a microscope slide and covered with fluorescein-isothiocyanate (FITC) labelled globulin. Antigens in the sample bind to the labelled globulin and the resulting antigen-antibody complexes are visible under UV light.

2.6.2 Polymerase chain reaction-based detection

Culture based detection of legionellae (semi-selective culturing followed by confirmation) remains the method of choice, primarily because these methods do not require specialized apparatus and large numbers of samples could be screened in a standard microbiology laboratory setup. Although these techniques are widely applied, definite results can only be obtained after several days. By this time, spreading of the bacterium could have occurred

which could result in ongoing outbreak. Pre-treatment of samples, as mentioned above to eliminate other heterotrophic flora could also inhibit certain legionellae, thus resulting in lower plate count numbers and a false picture of the situation. The development of nucleic acid based techniques based on DNA probe hybridization (Grimont *et al*, 1985) provided some improvement as far as detection time is concerned. However, the sensitivity of these methods is not sufficient for direct detection of legionellae in many environmental samples.

Amplification of specific gene sequences using the polymerase chain reaction (PCR) enhances the sensitivity of detection, and provides a rapid and economical alternative towards routine *Legionella* detection. Amplification of a target sequence followed by hybridization with a specific probe has been shown to permit detection of a single bacterial cell (Mahbubani *et al*, 1990), and has been applied for the detection of *L. pneumophila* (Bej *et al*, 1991). Although the detection of amplified products by hybridization is sensitive, the process is tedious with regards to hybridization time and interpretation of results, thus making it difficult to apply in a diagnostic setup with a high sample throughput. Modification to the standard amplification approach, namely nested, hemi-nested and pitstop hemi-nested PCR, provides a sensitive means for direct visualization of the amplified product using ethidium-bromide-stained agarose gel (Catalan *et al*, 1997; Miyamoto *et al*, 1997; Theron *et al*, 2000). Using hemi-nested PCR, one of the primers of the first reaction is used together with a new internal primer to generate an internal amplicon, thus increasing both sensitivity (additional reaction cycles) and specificity (internal sequence match) of the assay. However, an increased number of amplification cycles (such as during nested and hemi-nested PCR) has been shown to promote the occurrence of false-positive results due to DNA template contamination (Oshiro *et al*, 1994; own observations).

In previous applications of PCR technology for the detection of *L.pneumophila*, a commercial system, the EnviroAmp *Legionella* kit, was developed by the Perkin Elmer Corporation (Foster city, California, U.S.A.).

Although this system displayed it's use as a diagnostic tool in various studies (Ng *et al*, 1997; Palmer *et al*, 1993; Palmer *et al*, 1995), the kit has been permanently withdrawn from the commercial market early in 1998. Using this system, DNA from the genus *Legionella* as well as *L. pneumophila* could be detected in environmental water samples by means of a multiplex PCR targeting both 5S ribosomal RNA (detection of the genus Legionella) as well as a chromosomal gene, the macrophage infectivity potentiator or *mip* (detection of *L. pneumophila*). The *mip* gene is conserved and specific to the genus Legionella, but *mip* like genes have been detected in other members of this genus (Cianciotto *et al*, 1990). An internal positive control (IPC) was also included in the reaction. The IPC is a synthetic DNA sequence that is co-amplified with the degenerate biotinylated primers used for the *mip*-gene and was included at a fixed copy number in the PCR kit. The subsequent amplified product could be detected by means of a reverse dot-blot procedure using probes immobilized on a nylon strip membrane. Hybridized biotinylated PCR product is detected calorimetrically using the enzyme conjugate, horseradish peroxidase- strepavidin (HRP-SA) followed by the HRP substrate, tetramethylbenzidine (TMB). Immobilized positive and negative control probes are also included, where the positive control probe display complimentarity with a sequence within the IPC, and the negative control probe a one base mismatch with another defined sequence also in the IPC.

PCR based detection strategies normally display a high degree of sensitivity as well as specificity (although the latter may depend on primer design as well as the target area). Sensitivity of a PCR-based detection strategy can be influenced by numerous variables including the efficacy of cell lysis, DNA availability, competition from non-target DNA and inhibition of the reaction by inhibitors present in the sample. Sensitivity could be significantly increased by filtering larger volumes of sample. This procedure could however also increase the concentration of inhibitors. It is therefore necessary to isolate the target organisms or DNA from these samples to obtain sensitive detection.

2.6.3 Immunomagnetic based detection

In order to increase the sensitivity of PCR-based assays from complex water samples, purification of target DNA should be performed. Although multiple purification steps can produce target DNA suitable for amplification, they could be laborious in a diagnostic setup, and could also lead to loss of valuable template DNA. Developments in magnetic-based separation assays such as immunomagnetic separation (IMS) and solid-phase sequence capture opened doors in the field of PCR-based detection strategies by separating target–organisms or DNA from complex, PCR inhibitory samples, enabling cultivation and concentration of target DNA respectively.

Several magnetic-based systems are commercially available today, and their application range from the isolation of target organisms for microbiological purposes to enrichment of proteins and nucleic acids for specific biochemical and molecular applications. Most of these particles are para-magnetic of nature, i.e. they retain magnetic properties in a magnetic field, but lose them as soon as the field disappears. This ability is necessary to ensure an even distribution of beads as soon as the magnetic field is removed. The shape and size of these particles are also important, where identical dimensions allow for optimization of sedimentation and inter-molecular binding kinetics.

The most commonly available magnetic-separation systems consists of uniform magnetic spheres (Fe_2O_3 and Fe_2O_2) coated by a polymer surface to prevent iron leakage. Cells bound to these beads remain viable/cultureable and unchanged, and could be used for further studies based on culturing as well as molecular-based techniques. Commercially available systems, manufactured by the Norish company DYNAL, apply two different sizes of beads (2.8 and 4.5μm in diameter) to a vast range of target isolation applications. Beads could be coated with either a primary antibody (IgM class) a secondary antibody directed to a specific primary. In the former approach, the secondary coated beads are pre-coated with a primary antibody directed against an epitope on the target organism. These coated beads are thus directly added to the sample followed by isolation. During the indirect

approach, the primary antibodies are added to the sample, allowing binding of the antibodies to the free cells. By using a secondary antibody on the bead surface, the possibility of stearical hindrance between primary IgG class antibodies could be minimized. These antibody-cell complexes can thus be isolated from the sample by allowing binding interaction between the primary antibody and the bead-bound secondary antibody.

The use of IMS over standard culturing for enrichment purposes, especially when looking at a fastidious slow grower such as *L.pneumophila*, is quite obvious. These organisms are easily out-competed, even on semi-selective media by faster heterotrophic growth, thus delaying confirmation assays. Using IMS, selective enrichment of the target bacteria could be achieved, where the captured organisms remains viable and unaltered for further applications. This method is however severely dependent on the availability of target-specific antibodies; for this reason, the use thereof is limited to only a small number of bacterial (*E.coli* O157, *Salmonella*, *Listeria*) and protozoan pathogens (Dynabeads anti-*Cryptosporidium* -and *Giardia*).

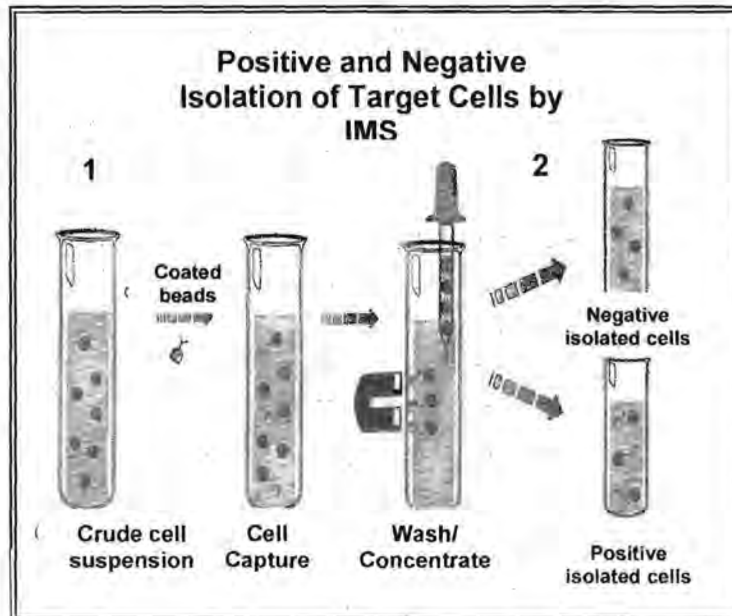

Figure 1.2. Positive (**1**) and negative (**2**) isolation assays used during immunomagnetic separation of target cells. The above approach depicts a primary antibody coated on the bead surface. Taken from DYNAL Bioscience Product Catalogue (DYNAL, Oslo, Norway).

2.6.4 Solid-phase sequence capture of *L.pneumophila*

Magnetic based isolation of target nucleic acids (solid phase sequence capture) have found a wide range of applications in the molecular biology field (Hultman *et al*, 1989; Rimstad *et al*, 1990, Olsvik *et al*, 1994). This approach allows the isolation and subsequent handling of target molecules in a highly specific manner which enables molecular based detection, such as those based on PCR. Commercially available systems, including that from the company DYNAL (Oslo, Norway) rely on strepavidin coated paramagnetic beads (2.8μm in diameter) and target or probe labeled with biotin. Strepavidin is a protein that consist of four identical subunits, each containing a high affinity binding site for biotin ($K_D = 10^{-15}M$).

Two different approaches could be followed for sequence capture, i.e. the direct and indirect method (Figure 1.3). The former relies, as in the case of IMS, on ligand pre-coated beads to capture target sequences, where the latter focus on the capturing of target hybridized ligand (biotinylated polynucleotide probe in the case of sequence capture) using uncoated Strepavidin beads.

Three major advantages of using sequence capture as a diagnostic tool are: 1) Using labelled oligonucleotides which can be specifically designed towards target capture, where the use of mono- or polyclonal antibodies could produce non- specific cross reactivity; 2). Amplification of target DNA directly from large quantities of non-target DNA which could produce a masking effect leading to reduced amplification sensitivity (Mangiapan *et al*, 1996) and 3) to eliminate PCR inhibitory substances from the reaction which could lead to reduced amplification sensitivity as well as false-negative results. Using this system, effective capturing of target DNA from *Mycobacterium tuberculosis* in clinical specimens containing large numbers of human cells and subsequent interfering DNA was achieved (Mangiapan *et al*, 1996).

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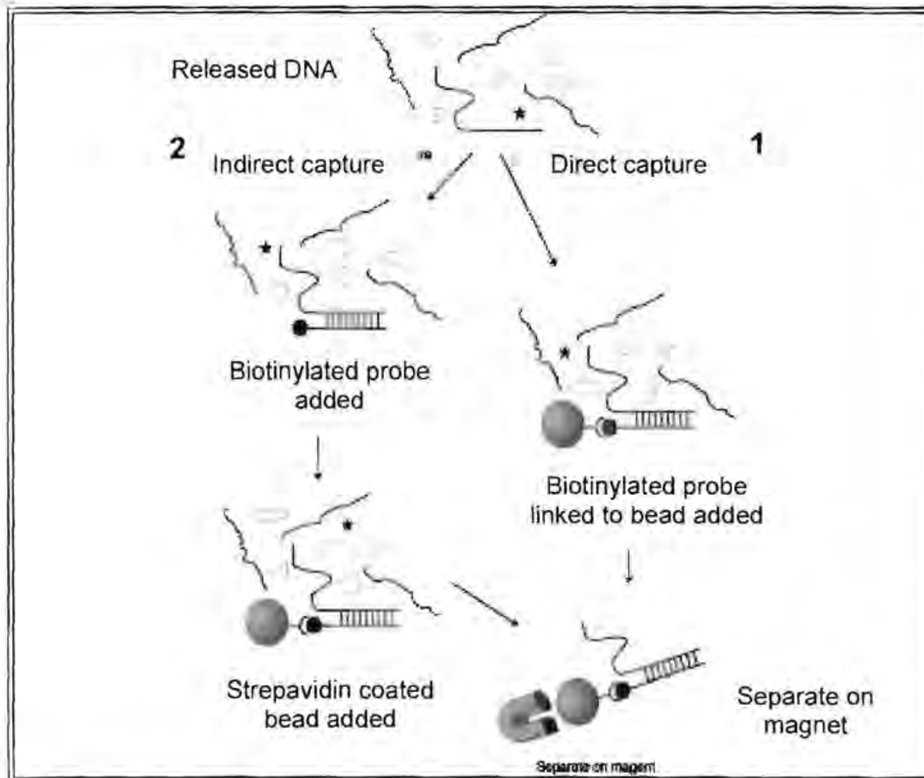

Figure 1.3. Solid-phase capture of target DNA using either the direct (1) or indirect (2) sequence capture approach. (Taken from DYNAL Bioscience Product Catalogue (DYNAL, Oslo, Norway).

Although most of the applications of this technology focussed on the capturing of labelled amplified products for diagnostic (Holmberg *et al*, 1992; Muir *et al*, 1993; Rimstad *et al*, 1990) and DNA sequencing applications (Brytting *et al*, 1992; Holmberg *et al*, 1992; Seesod *et al*, 1993), few concentrated on capturing bacterial or viral nucleic acids; further developments in this field of study could provide useful alternatives in purification of target nucleic acids for downstream applications.

CHAPTER 3

Detection of *L. pneumophila* in Industrial and Environmental Waters Using a Two-Step Polymerase Chain Reaction Approach

3.1 Introduction

Legionella pneumophila, the causative agent for Legionnaires' disease, was first isolated in 1977 after a documented outbreak in 1976 in Philadelphia (McDade *et al*, 1977). Currently, detection of the pathogen largely rely on culturing using semi-selective solid media such as buffered charcoal yeast extract (BCYE), which may be preceded by heat or acid treatment to limit other non-*Legionellae* growth. Although this approach has been proven to be effective for routine detection, it requires several days of incubation before presumptive results could be obtained. With the introduction of a commercially available multiplex polymerase chain reaction detection system in the early 1990's (EnviroAmp Legionella Kit, Perkin-Elmer Corp, U.S.A.), rapid and specific detection of *L.pneumophila* was made possible. In this approach the *L.pneumophila* specific macrophage infectivity potentiator (*mip*) gene as well as the genus specific 5SrDNA was targeted, followed by a reverse dot blot assay enabling direct visualization. In the light of the recent withdrawal of this system this study focused on the development a sensitive and specific PCR based detection assay for *L.pneumophila* in industrial and environmental waters.

Inhibition of the PCR reaction from industrial and environmental waters has previously been shown (Koide *et al*, 1993, Maiwald *et al* 1994, Ng *et al.* 1997). In such cases, PCR negative but culture positive results might be obtained which could be detected after standard culturing. To overcome such results, hemi-nested PCR might provide increased sensitivity of the assay by inhibitor dilution, as well as specificity by secondary internal amplification. This chapter will

therefore discuss the application of PCR as detection method in inhibitory waters, the use of a hemi-nested approach to increase specificity and sensitivity, and the development and application of a revised PCR method (modification of the PitStop PCR method, as described by Theron *et al*, 2000) to overcome some of the limitations of both the aforementioned approaches.

3.2 Materials and methods

3.2.1 Bacterial strains applied in the study

L.pneumophila serogroup 1 (ATCC 33152) was used as test organism. The strain was cultured on buffered charcoal yeast extract agar (without antibiotics) at 37°C for 4-7 days. To evaluate PCR specificity, 27 non-*Legionella* as well as *L.micdadei* (ATCC 33218) and *L.dumoffi* (ATCC 33279) were used (Table 3.2). Cultures were grown and maintained on nutrient agar plates (Oxoid) or on BCYE plates (without antibiotics) at the appropriate temperatures.

3.2.2 PCR primers

Detection of *L.pneumophila* was achieved by using primers (positions 948 to 965 and 1092 to 1115) complimentary to sequences within the *mip* gene (Engleberg *et al*, 1989); Forward: PT69: 5'-GCATTGGTGCCGATTTGG -3'; Reverse (degenerative primers): PT70: 5'-GCTTTGCCATCAAATCTTTCTGAA–3' and PT181:5'-GTTTTGCCATCAAATC TTTTTGAA-3'. Primers were manufactured by MWG (Boehringer Mannheim, Germany), received in lyophilized form, dissolved in sterile distilled water and aliquatted in working dilutions of 50pmol/μl.

3.2.3 DNA extraction

Two DNA extraction methods were compared. For the one method DNA extraction was performed as described in the EnviroAmp kit protocol: *L. pneumophila* was cultured on buffered charcoal yeast extract agar (BCYE without antibiotics) for 5 days at 37°C. A single colony was suspended in 500 μl of ultra high quality (UHQ) water, vortexed and centrifuged for 3 minutes at 12 000 rpm. Five hundred μl of lysis reagent (guanidinium thiocyanate based) was added, followed by votexing and boiling at 99°C for 20 minutes. The heat-treated sample was then pelleted by centrifugation at 12 000 rpm for 30 seconds. DNA precipitation was performed by adding 100% isopropanol to supernatant in equal amounts, followed by the addition of 10 μl of a kit supplied carrier reagent (4.0 mg/ml of RNA homopolymer poly(A)). The reaction mixture was mixed well and left for 10 minutes at room temperature for DNA precipitation to occur. The precipitate was centrifuged out at 12 000 rpm for 10 minutes, followed by a second precipitation step using 500 μl of a 75% isopropanol solution added to the pellet. After vortexing and centrifugation (12 000 rpm, 10 minutes) the pellet was resuspended in 160 μl sterile water in a water bath (70°C, 3 minutes). The suspension could be stored at -20°C for up to 14 days.

To determine the effect of an alternative DNA release method on the sensitivity of PCR detection, the efficiency of the freeze-thaw lysis method was also compared to that of the EnviroAmp DNA purification method. Serial dilutions of a pure culture of *L.pneumophila* was set up, ranging from 10^{-1} to 10^{-7}. One hundred microlitres of dilutions 10^{-3} to 10^{-6} (duplicate) was plated on BCYE (non-selective) agar and incubated at 37°C for 5 to 10 days to check viability. The same volume (100μl) of each of the dilutions was subjected to either the EnviroAmp extraction procedure or five freeze-thaw lysis cycles using 1 minute liquid nitrogen or 5 minutes -70°C freezing followed by a 3 minute thaw in a 37°C water bath. Twenty microlitres of each lysate was used in subsequent amplification reactions.

3.2.4 PCR optimization

For PCR optimization, a modified Taguchi approach was followed (Cobb and Clarkson, 1994). Table 3.1 displays the combinations used for the 8 positive reactions:

Table 3.1. Reaction conditions used during PCR optimization. $MgCl_2$, dNTP's and primer concentrations were altered in each reaction

Reaction nr.	$MgCl_2$ (mM)	dNTP's (uM) each	Primer (pmol)
1	3.75	200	100
2	3.75	200	50
3	2.50	150	50
4	2.50	150	100
5	1.50	100	50
6	1.50	100	20
7	1.50	100	100
8	3.75	200	20
Negative (no DNA)	2.50	150	50

Twenty microlitres of cell lysate was used in the optimized 50µl reaction mixture that included 1 x PCR buffer (50mmol l^{-1} KCl, 10mmol l^{-1} Tris-HCl[pH 8.3]), 2 mmol l^{-1} $MgCl_2$, 100 µmol l^{-1} deoxynucleotide triphosphates, 1 U *Taq* polymerase (Promega), 1µl primer PT69 (50 pmol) and 1µl of primer mixture PT70/PT181 (50 pmol). Thermal cycling was performed using a PE GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems) with the following cycling program: 95°C for 10 minutes, followed by 30 step cycles of 95°C for 30 seconds and 63°C for 60 seconds, ending with 72°C for 7 minutes final elongation. Primer annealing temperature optimization was also performed by lowering the temperature from the initial 63°C to 55°C in 2°C increments. PCR products were visualized by electrophoresis on a 2% ethidium bromide agarose gel using a standard UV-transluminator.

3.2.5 PCR specificity and sensitivity

PCR specificity was determined after annealing temperature optimization using 27 non-*Legionella* and 3 *Legionella* species (Table 3.2)

Table 3.2. Organisms used in the study to evaluate specificity of the amplification assay (Obtained from the culture collection, Department of Microbiology and Plant Pathology, University of Pretoria)

Acinetobacter calcoaceticus	*Micrococcus luteus*
Bacillus cereus	*Proteus mirabilis*
Bacillus circulans	*Proteus vulgaris*
Bacillus firmis	*Pseudomonas cepacia*
Bacillus lichenformis	*Pseudomonas earuginosa*
Bacillus megaterium	*Pseudomonas maltophila*
Bacillus polymyxa	*Pseudomonas putida*
Bacillus stearothermophilus	*Pseudomonas stutzeri*
Bacillus subtilis	*Salmonella typhimurium*
Enterococcus faecalis	*Serratia marcecens*
Escherichia coli	*Serratia marcecens*
Klebsiella oxytoca	*Shigella sonnei*
Klebsiella pneumoniae	*Staphylococcus aureus*
Legionella dumoffi	*Streptococcus faecalis*
Legionella micdadei	*Vibrio cholerae* (toxic)
Legionella pneumophila	*Vibrio cholerae* (non-toxic)

In order to determine the minimum amount of cells required to produce a detectable amplification band on agarose gel (sensitivity), amplification was performed on a serial dilution series. Bacterial numbers reported as cfu, were

determined by plate count (5-7 days incubation at 30°C) on BCYE agar (without antibiotics).

3.2.6 Hemi-nested PCR

A hemi-nested PCR approach was followed to increase the sensitivity of the assay in inhibitory waters: a new internal 19-mer reverse primer (PT80: 5'-CGGTTAAAGCCAATTGAGC -3') was designed to facilitate amplification of a 117 bp region within the first amplification product. Primer design was performed using the computer-based program PRIMERS for Windows. The chosen primer sequence was blasted (BLAST 2.0, NCBI) against the GenBank gene sequences to determine the target specificity. Hemi-nested PCR was performed using the exact same reaction conditions together with 20 μl of a thousand-fold dilution of the first reaction product.

3.2.7 PitStop PCR

PitStop PCR was performed by setting up a standard PCR reaction (PT69, PT70 and PT181), only running 10 cycles for the first PCR. A subsequent second 20-cycle PCR reaction was set up (PT69,PT70 and PT181 or PT69 and PT80 respectively), and between 1 and 10 μl of the 10 cycle PCR reaction was used as template for amplification. The PitStop approach was also compared to a hemi-nested PitStop approach where primer pair PT70 and PT181 were substituted by the internal primer PT80. Detection sensitivity was determined using a standard dilution series and compared to that of a full 30-cycle PCR reaction.

3.2.8 Testing of seeded water samples

Application of the PitStop PCR was evaluated by using non-sterilized, *L.pneumophila* negative (by PitStop PCR and culturing) water samples. The

water samples (cooling tower, river water, tap water and double distilled) were seeded with pure culture *L.pneumophila* at an initial concentration of approximately 10^5 cfu/ml as determined by duplicate plate count on BCYE. A standard dilution series (to 10^{-3}) was set up followed by freeze-thaw lysis and PCR.

3.3 Results and discussion

3.3.1 PCR optimization

Optimization of the PCR reaction was performed by altering $MgCl_2$, dNTP's and primer concentrations, keeping all other reaction component concentrations constant. Condition number 5 (1.5mM $MgCl_2$, 100μM dNTP's each, 50pmol of each primer) was chosen as near optimum for the PCR assay, displaying strong amplification at low $MgCl_2$ concentration (Table 3.1, Figure 3.1). The reaction was further optimized by evaluating $MgCl_2$ concentrations ranging from 1.25 mM (millimolar) to 5 mM (increasing with 0.25 mM steps). It was found that a concentration of 2.00 mM allowed strong amplification of the target sequence. Higher concentrations resulted in negligible increase in amplification intensity (on agarose gel), but would result in a decrease in annealing specificity (Kidd and Ruano, 1995).

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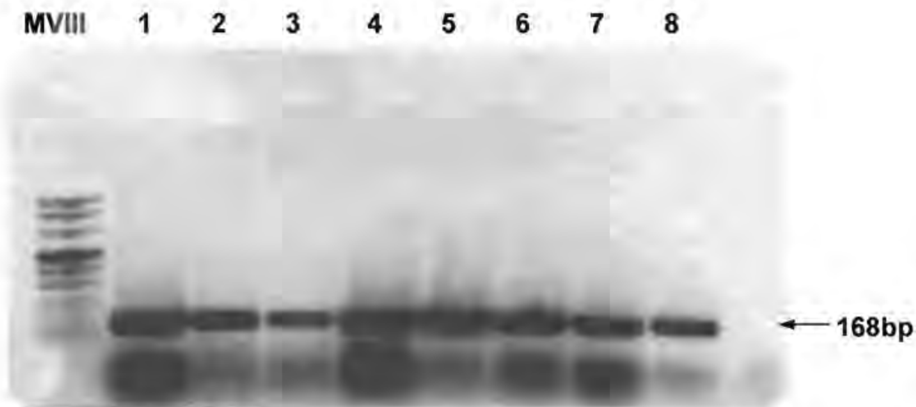

Figure 3.1. Agarose gel (2% w/v) of initial PCR optimization. Lane numbers 1 to 8 corresponds to the reaction conditions laid out in table 3.1

3.3.2 DNA extraction

The efficacy of the guanidinium thiocyanate based lysis method used in the EnviroAmp kit was compared to the well-known freeze-thaw lysis method, as previously described for *L.pneumophila* (Bej *et al.* 1991). Freezing with liquid nitrogen as well as -70°C deep freeze was applied during freezing steps, followed by normal thawing in a 35°C water bath. The three methods were evaluated by performing each separately on a *L.pneumophila* dilution series, followed by PCR amplification and agarose gel electrophoresis.

Higher amplification sensitivity was achieved by -70°C freeze-thaw method compared to the liquid nitrogen method (results not shown); this might be explained by the fact that efficient ice crystal formation was achieved using the slower freezing technique, which in return promoted higher cell rupturing and DNA release. The same was true when the chosen freeze-thaw method was compared to the chemical-based kit extraction method. Using the EnviroAmp DNA purification procedure, PCR product could be visualized up to the 10^{-5} dilution, whereas product visualization was possible up to the 10^{-7} dilution using freeze-thaw lysis prior to amplification (Figure 3.2). This experiment was

repeated and in each case the -70°C freeze-thaw method produced better results than the EnviroAmp extraction method. This extraction method was used for all further PCR studies described.

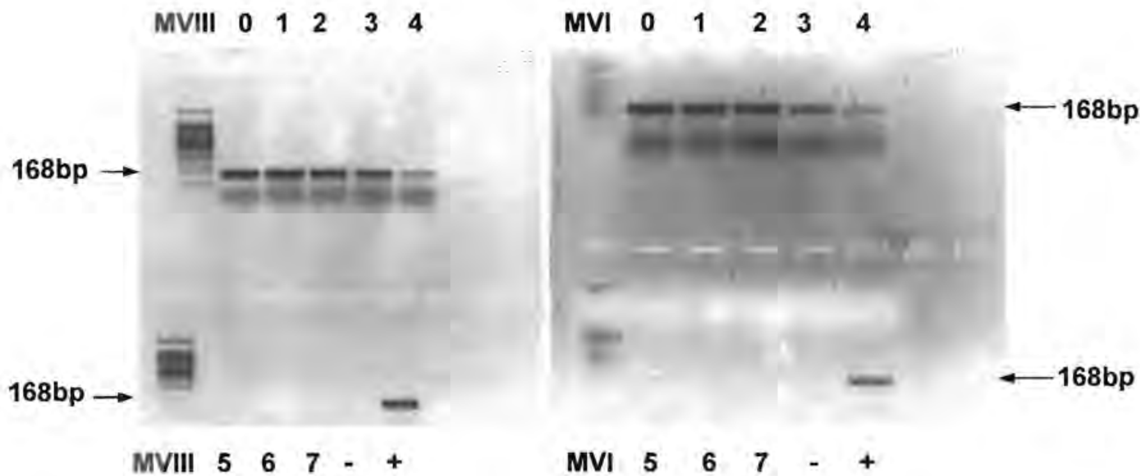

Figure 3.2. Comparison of the EnviroAmp DNA purification procedure (left gel) with the freeze-thaw lysis method (right gel). Lane numbers indicate the different dilutions in the series (0 indicates undiluted cell suspension).

3.3.3 PCR sensitivity and specificity

Standard culturing and 1-step 30 cycle PCR confirmed that a peak sensitivity of 1 to 10 cfu per ml seeded distilled water was obtainable (results not shown). However, reproducibility proved to be low, with lower sensitivity values reaching 1 x 10^3 cfu per ml. This might be ascribed to the efficacy of the amplification reaction as well as culturability of the cells leading to low cfu counts against presumable high amplification sensitivity and *vice versa*. Stepwise alteration of the primer annealing temperatures (Tm PT69 = 55°C, Tm PT70/PT181 = 56°C) from 63°C to a final 55°C lead to an improvement of 1 log in sensitivity in seeded distilled water (Figure 3.3). Specificity of amplification for the primer pair at the lowered annealing temperature (55°C) was determined, where no non-specific

amplification was observed in any of the non-*Legionella* species as well as two *Legionella* spp, *L.micdadei* and *L.dumoffi*, thus indicating target specific amplification.

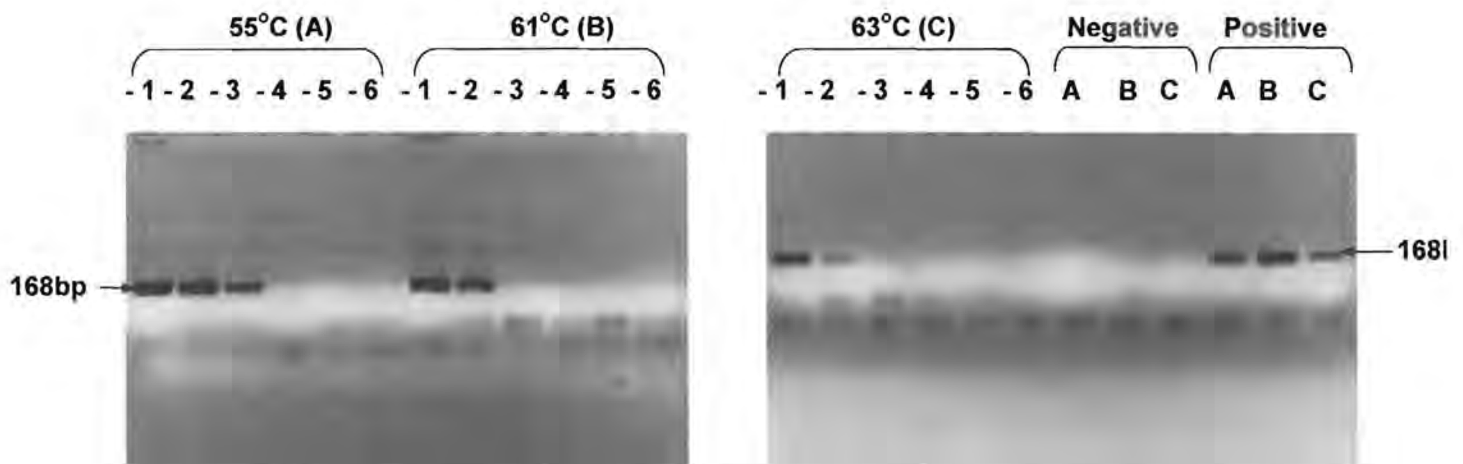

Figure 3.3. Optimization of primer annealing temperature. Alphabetical letters indicate negative and positive controls for temperatures 55, 61 and 63°C respectively.

3.3.4 Hemi-nested PCR

Although a hemi-nested PCR approach enhances the specificity as well as the sensitivity of the standard PCR assay, false-positive amplification may result from minute amounts of carry-over contamination. Using 20µl of a 10^{-3} dilution of the first reaction PCR, specific amplification of the 117bp product was obtained in both seeded as well as in negative control reactions, with the appearance of the 168bp product when 10-20µl of the first reaction was directly carried over. A thorough contamination elimination approach was followed, changing everything associated with the reaction. Although some differences in amplification intensity was noticed when using different PCR enzymes (Figure 3.4), complete negative reactions could not be achieved

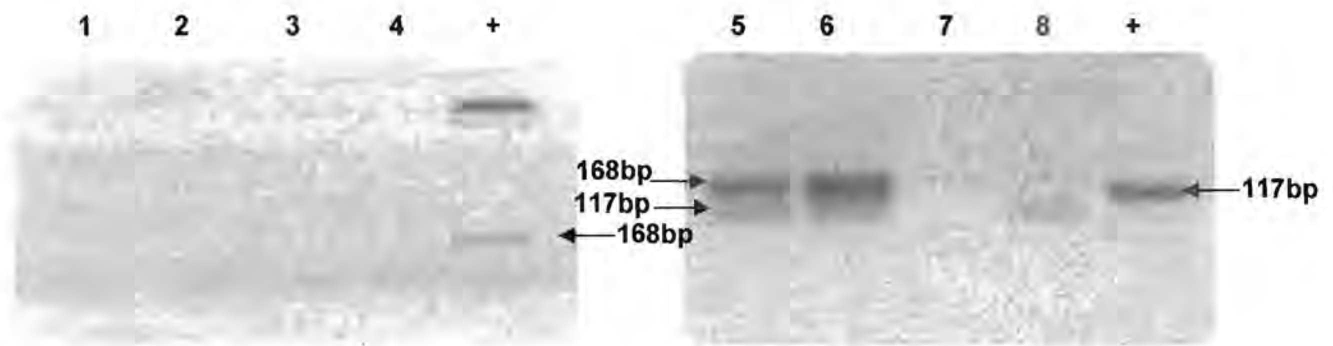

Figure 3.4. Evaluation of the possible contribution of different thermo stable DNA polymerases on the occurrence of false-positive amplification. Single step 30 cycle PCR (left gel) of 4 negative reactions (no DNA template), followed by 30 cycle hemi-nested PCR (right gel) was performed. Lanes 1 and 5, Taq polymerase (Promega Corp, U.S.A); Lanes 2 and 6, Southern Cross Taq (Southern Cross, R.S.A.); Lanes 3 and 7, AmpliTaq Gold (Perkin Elmer Corp, U.S.A.), Lanes 4, 8 and positives, Takara EX Taq (Takara, Japan); +, *L.pneumohila* template DNA.

Purification of the both the 117 and 168bp products by gel extraction (QIAEX®II DNA extraction kit, QIAGEN, Hilden, Germany) was followed by automated sequencing using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) and Applied Biosystems 377 DNA sequencer. Automated sequencing produced useable sequences of 58 and 120bp (respectively) in length. When these sequences were compared with sequences in the Genbank database (BLAST 2.0, NCBI) a 98 and 99% similarity (respectively) was found with *L.pneumophila*, thus indicating positive target DNA amplification. Sterile distilled water from various sources as well as UV irradiated water (30 minutes on a standard UV transluminator, 260nm) was evaluated for their possible contribution to this phenomena, however, they produced only slightly weaker amplification results. Filter sterilization of the water through a 0.22μm filter had, however no effect on the outcome of the results. The phenomenon of false-positives from seemingly

negative (no DNA template added) reactions have been described before by Oshiro *et al.* 1994: this was associated with an increase in the number of amplification cycles from 30 to 40 using the same PCR conditions as described above. No definite explanation was given in this regard, and it was hypothesized from the data that low concentrations contaminating template DNA was present in all of the control waters used, thus rendering any amplification experiments based on these controls non-valid. For this reason, a PitStop PCR approach was investigated which required less amplification cycles.

3.3.5 PitStop PCR

In order to overcome false-positive PCR results as described in the previous section, a 10/20 cycle PitStop PCR approach was followed. Using this approach, no false positive amplification were observed, even when 20μl of the 10 cycle PCR was carried over (Figure 3.5). Increased sensitivity of amplification was also observed over the standard 30-cycle PCR in double distilled water (Figure 3.6). The application of PitStop PCR was also tested in cooling water where single log higher amplification sensitivity was achieved over standard PCR (Figure 3.7). A modification of the PitSttop PCR, hemi-nested PitStop PCR (using primers PT69 and PT80), was compared to the standard PitStop approach. As in the case with normal 30-cycle PCR, the modified PitStop reaction produced 1 log higher amplification sensitivity over the hemi-nested PitStop approach (Figure 3.8).

Carry-over of reverse primer (PT70/PT181) produced the additional 168bp product in the hemi-nested reaction, and out competence of the 117bp product by the 168bp product produced this single band at lower target DNA concentrations (Figure 3.8). Higher amplification sensitivity was also observed when using 10μl of carry-over from the 10 cycle PCR compared to 1μl, and this was true for both seeded pure and cooling tower water.

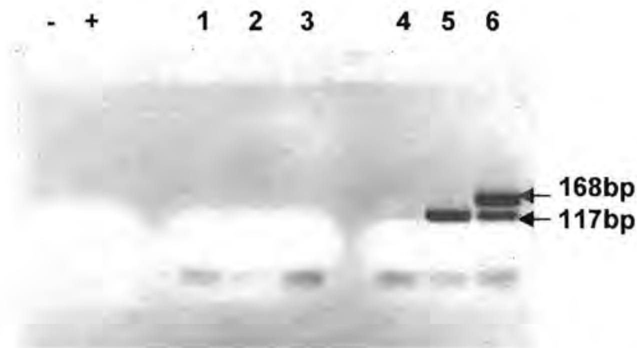

Figure 3.5. Pitstop PCR of a thousand, fifty (1µl) and 2.5 (20µl) times dilutions (respectively) of 10 cycle reaction products. Lanes 1-3, negative reactions (no DNA template); Lanes 4-6, positive PCR reactions.

Figure 3.6. Sensitivity of detection of the standard PCR method (lanes 1-3) compared to the PitStop approach (lanes 5-7) using a standard dilution series in distilled water (10^0 to 10^{-2} respectively; (-) negative reaction). MVI, molecular weight marker VI (Boehringer Mannheim, Germany)

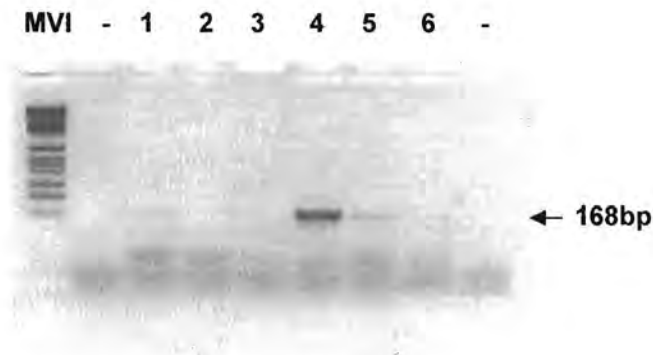

Figure 3.7. Detection of *L.pneumophila* in seeded cooling water dilutions (10^{-2} to 10^{-4}) by normal 30 cycle PCR (lanes 1,2 and 3) and PitStop PCR (lanes 4,5 and 6). A single log higher sensitivity was observed using the latter approach. (-) Negative reactions (no DNA template). MVI, molecular weight marker VI (Boehringer Mannheim, Germany)

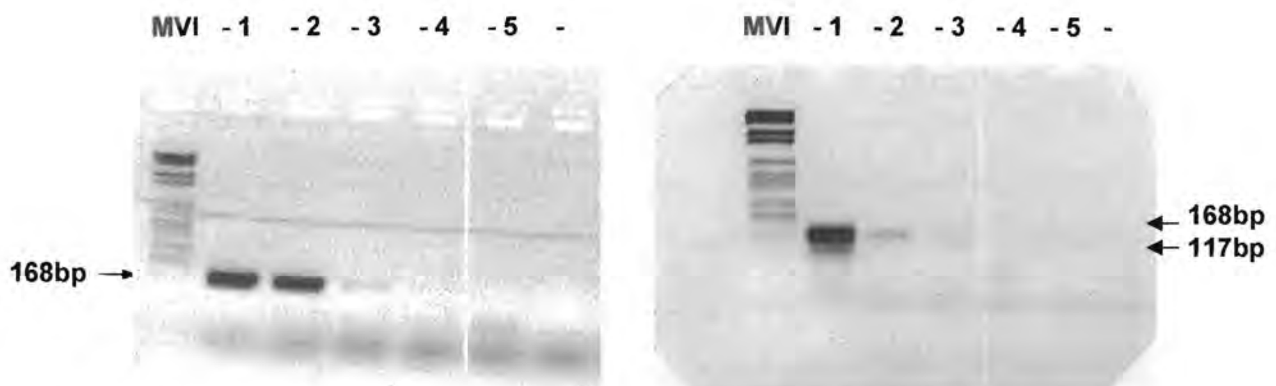

Figure 3.8. Sensitivity of detection of the PitStop PCR method (left gel) compared to a hemi-nested PitStop approach (right gel) using a standard dilution series (10^{-1} to 10^{-5}).

3.3.6 Testing of seeded water samples

PitStop PCR was evaluated by amplification from *L.pneumophila* seeded industrial, environmental and potable waters following DNA release by freeze-thaw lysis. PCR inhibitory substances present in both environmental as well as industrial waters proved to decrease amplification sensitivity, even producing false-negative results. Visible amplification was observed in the 10^{-1} to 10^{-3} (10^2 cells/ml) dilutions performed in cooling tower and tap waters, with lower detection sensitivity in seeded distilled water (Figure 3.9). This phenomenon have been described earlier (Hermans *et al.* 1990; Shawar *et al.* 1993) when testing seeded clinical and environmental samples, and could be contributed to low numbers of bacterial cells or even target DNA present in these waters. This was however difficult to confirm, where the absence of growth on media could be ascribed to either the absence of cells or the presence of cells in a non-cultureable state.

Negative results *via* the PitStop approach might be caused by PCR inhibition, where seeding of the sample could have contributed to a cumulative effect leading to higher detection sensitivity opposed to seeded distilled water. No visible amplification was observed from the environmental water sample, indicating high levels of PCR inhibition.

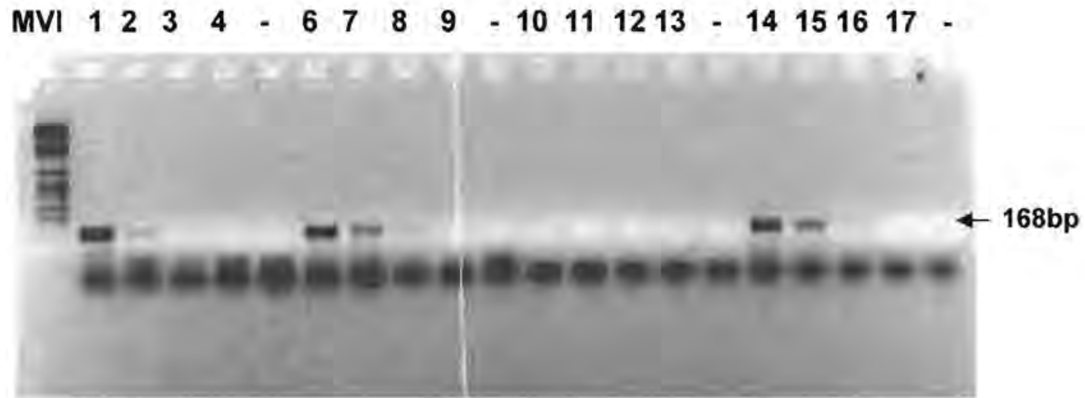

Figure 3.9. Detection of *L.pneumophila* in different seeded water dilutions. Lanes 1-4, distilled; lanes 6-9, cooling tower; lanes 10-13, river; lanes 14-17, tap water. (-) Negative reactions (no added DNA template). MVI, molecular weight marker VI (Boehringer Mannheim, Germany)

3.4 Conclusion

Cultivation of *L.pneumophila* from various water sources has been considered the standard against which other detection methods are compared. These methods, however, are time consuming and require specialized identification reagents and culture media, as well as a high degree of technical skill. Advances in molecular biotechnology and development of diagnostic applications of PCR in particular, enabled rapid and sensitive detection of these bacteria, specifically with regards to clinical samples (Kessler *et al*, 1993, Lindsay *et al*, 1994). Numerous groups have studied the detection of *L.pneumophila* in environmental water samples by PCR; however, the presence of inhibitory substances in these samples limited the applicability of PCR as a sole detection strategy.

Inhibition of DNA amplification from environmental water samples have been reported previously (Catalan *et al*, 1994, Maiwald *et al*, 1994, Oshiro *et al*, 1994) and the presence if inhibitory substances have also been shown during this study. Inhibition would lead to reduced sensitivity of detection or even false-

negative results, which might only be determined after prolonged standard culturing. For this reason, further modifications of the standard PCR detection assay were performed. By optimization of the PCR reaction as well as the use of an alternative and rapid DNA extraction procedure (freeze-thaw lysis), increased sensitivity was obtained, as compared with the standard EnviroAmp PCR procedure.

Development of a hemi-nested PCR assay was carried out to enable sensitive detection in waters inhibitory to the standard one-cycle PCR approach. Hemi-nested PCR enable sensitive and specific detection of target DNA, but lead to the occurrence of false-positive results possibly due to minute amounts of carry-over contamination combined with a large number of amplification cycles. The source of contamination is sometimes difficult or even impossible to trace (as discussed above), and may be associated with the presence of target DNA in water used for research applications. In a previous study, the application of a devised amplification approach (PitStop PCR) has proven useful in the detection of *Vibrio cholerae* in environmental water samples, where increased sensitivity of detection over standard 30-cycle PCR was achieved (Theron *et al*, 2000). This method relied on a two step amplification approach consisting of an initial 10 cycle amplification using the forward and reverse primers, followed by 20 cycles with the forward and an internal reverse primer. Using only a fraction of the initial amplification product as template for the second amplification cycle ensured dilution of any inhibitory substances still present in the first cycle PCR. This approach also enhances the specificity of the assay by amplification from within the first cycle amplicons. In the study discussed in this chapter, a modification of this approach was followed: the second cycle of amplification was performed by using the exact same primer pair as for the first cycle amplification. In this study, the modified PitStop PCR approach enabled a ten times increase in detection sensitivity compared to the PitStop PCR employing the internal primer. Application of modified PitStop PCR approach to *Legionella* detection delivered increased sensitivity over standard 30-cycle amplification (1 log higher in double

distilled water) without the occurrence of false-positives, even when using larger volumes (10μl) of first reaction carry-over. PitStop PCR, however, requires more hands-on laboratory time as well as PCR consumables, but can prove useful in the confirmation of PCR results when working with inhibitory water samples. When working with highly PCR inhibiting samples, however, confirmation of negative results should be validated by using a second detection method, e.g. culturing.

In conclusion, the development of a PCR method for the specific detection of *L.pneumophila* has been discussed in this chapter. This method enabled increased detection sensitivity over standard 30-cycle PCR, without the occurrence of false positive results or non-specific amplification associated with a higher number of amplification cycles. This approach, however, was still sensitive towards inhibition from the sample, and it is thus concluded that negative amplification results should thus be confirmed by standard culturing methodology. Isolation of the target organisms from these samples prior to amplification could enable sensitive detection and possibly overcome false-negative results. In chapters following, the applicability of such methods are discussed with regards to DNA-based amplification.

CHAPTER 4

Immunomagnetic-Based Detection of *L.pneumophila* Serogroup 1

4.1 Introduction

Sensitive and specific detection of pathogenic organisms, especially in industrial and potable water systems remains a challenge to the workers in the public health sector. The revolution in the development of PCR as a diagnostic tool opened several doors to workers in this field. However, due to its sensitive nature, the enzymatic polymerization is often inhibited in complex samples, usually by samples containing compounds such as humic acids, metal ions or organic matter (Rossen *et al*, 1992). For this reason, alternative strategies are required to improve the sample to allow high levels of sensitivity in detection. In this chapter the focus is placed on the applicability of immunomagnetic separation (IMS) towards the isolation of *L.pneumophila* from complex environmental water samples in order to increase the power of subsequent detection strategies.

Immunomagnetic separation relies on the concentration of target organisms from a heterogeneous sample using paramagnetic particles coated with a primary and/or secondary antibody. The former is usually directed towards an epitope or immunogenic surface marker on the surface of the organism, i.e. an exposed trans-membrane protein or surface lipopolysaccharides. The bead-cell complexes are then separated from the sample by using an exogenous magnetic field. In order to set up a successful isolation assay, antibodies specifically directed to the organism of interest must be available. These antibodies could be either mono-or polyclonal, and should easily be attached to the polymerized bead surface without steric hindrance between them. Primary antibodies can be linked

to these beads directly or via a secondary antibody, mostly anti-mouse or anti-rabbit (Olsvik *et al*, 1994).

For the purpose of the study, focus was placed on the development of a novel immunomagnetic-based assay for selective concentration of *L.pneumophila* serogroup 1 from complex environmental water samples. Emphasis was placed on the selection of an appropriate bead and antibody system followed by optimization of bead coating and extraction methods. The efficacy of the optimized method was assessed by means of fluorescence- and electron microscopy as well as direct visualization of amplified product using *L.pneumophila* specific PCR.

4.2 Materials and methods

4.2.1 Magnetic bead preparation

Dynabeads® M-280 (2.8 μm +/- 0.2 μm diameter, DYNAL A.S, Oslo, Norway) and sheep anti-mouse IgG was used in all the immuno-magnetic based experiments. The beads were pre-coated with affinity purified sheep anti-mouse IgG, and suspended in phosphate buffered saline (PBS) pH 7.4 containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3) at a concentration of 6-7 x 10^8 beads/ml (10 mg/ml). Stored beads (2-8°C) were washed before coating using a PBS + BSA buffer (0.1% or 0.5g/500ml BSA fraction V, Boehringer Mannnheim GmbH) at pH 7.4. For initial testing of beads, the manufacturers recommended 20μl (1,2 x 10^7) beads per 1ml reaction volume. Beads were vortexed to resuspend any sedimented beads, followed by magnetic extraction for 60 seconds using an Eppendorf tube type extractor (DYNAL® MPC®-E-1). The supernatant was discarded and the beads resuspendend in approximately double the volume PBS/BSA at room temperature. Gentle mixing was performed (60 seconds) followed by a second washing step. The beads

were resuspended in the original volume (20μl) using PBS/BSA. Washed beads could be stored at 4°C for up to 14 days (manufacturer's recommendation) before re-washing would be required.

4.2.2 Coating of magnetic beads

Washed beads were resuspended by gentle mixing, and 4μl (approximately 2μg) of the primary mouse anti-*L.pneumophila* IgG_{3k} (MAB830 – CHEMICON International Inc.) were added (manufacturer's recommendation: 0.8 -3μg primary antibody per 1 x 10^7 beads). The volume was adjusted to 100μl using the sterile PBS/BSA (pH 7.4), and the mixture was incubated overnight (approximately 16-18 hours) at 4°C with gentle mixing (15 rpm) using a rotary mixer (ROTAMIX, Heto-Holten, Denmark). Beads were collected using a magnetic particle concentrator, MPC®-E-1 (Dynal) (60 seconds) followed by 4 washing steps for 30 minutes each at 4°C. Coated beads were resuspended in the original volume (20μl) PBS/BSA and stored at 4°C before use.

4.2.3 Immunomagnetic separation and detection *L.pneumophila* serogroup 1

A cell suspension (1ml) was made by suspending a single colony of *L.pneumophila* serogroup 1 (ATCC 33152) from a BCYE (without antibiotics) culture plate that was incubated at 37°C for 4 days. One hundred μl of the suspension was dispensed in three separate 1.5ml Eppendorf-type tubes, and the volume was made up to 1ml using sterile double distilled water (working suspensions). Twenty μl of coated beads were added to each of two of the suspensions and gently mixed. The bead-cell suspension was then incubated at room temperature for 1 hour with gentle mixing using a rotary mixer. After incubation, the bead-cell complexes were washed 3 times as described above, followed by suspension in 20μl and 100μl (for PCR and culturing purposes

respectively) of sterile double distilled water. For future PCR detection purposes, the bead-cell complexes could be stored at -20°C until needed.

PCR and culturing were performed to test cell recovery. One hundred microlitres of the crude cell suspension and recovered cells were directly plated on BCYE (without antibiotics), using standard spread plate procedures. The plates were incubated at 37°C for 4 to 7 days and checked for colony formation. The duplicate IMS reaction was subjected to the five-cycle freeze-thaw lysis procedure described earlier, and the subsequent lysate was used as template for PCR (primers PT69, PT70 and PT 181, Chapter 3). Positive and negative reactions containing 20µl pure culture lysate (working suspension) and sterile double distilled water respectively, were included. The amplicons were resolved on a 2% (wt/vol) agarose gel in 1 × TAE (40 mM Tris-HCl, 20 mM sodium.acetate, 1 mM EDTA, pH 8.5) and visualized by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. Electrophoresis was carried out at 60mA/100V for 30 minutes using a Hoefer Mightyslim SX-250 electrophoresis apparatus.

4.2.4 Optimization of antibody and bead concentrations

Bead coating was carried out according to the technique described in section 4.2.2. A constant amount of beads, namely 1 x 10^7 was used in each of the following coating reactions:

Beads	20µl (1 x 10^7 beads)
Mouse anti-*Legionella**	2, 4 or 6µl (1, 2 or 3µg respectively) of stock

* Antibody stock concentration: 0.5µg/µl

One colony of *L.pneumophila* serogroup 1 was suspended in 1ml of sterile, double distilled water (stock suspension). Two hundred microlitres of this suspension was diluted by adding water to a final volume of 1 ml (for each of the 3 experiments). IMS was performed as described earlier using the full 1 x 10^7 beads per reaction. Following the final washing steps, the beads were resuspended in 200μl of sterile water. One hundred microlitres of each of the different recoveries as well as the stock suspension were plated on BCYE agar and incubated at 37°C. Colony counts were performed after 4 days of incubation. The remaining 100μl of each reaction was subjected to 5 freeze-thaw cycles, and 20μl of the lysates were used for subsequent PCR amplification (first reaction PCR).

In a second experiment, two separate bead coating experiments were performed, where 80μl of uncoated beads (4 x 10^7 beads) were coated using 16μl (2μg/10^7 beads) and 32μl (4μg/10^7 beads) respectively of mouse anti-*Legionella* antibody. 400μl PBS/BSA buffer was added to each tube, and coating was performed for 1 hour. Optical density of the antibody suspension was measured before and after coating at 280nm using a Shimadzu UV-160A spectrophotometer. A standard stock suspension was again prepared, where after the suspension was aliquated into two Eppendorf-type tubes (500μl each) and diluted by filling up the volume to 1ml using sterile water. IMS was performed on each of these tubes using 40μl of either of the different coated bead suspensions, and the washed bead-cell complexes were resuspended in 80μl sterile water. Twenty microlitres of these suspensions was used for standard plate count, and another 20μl of each was subjected to freeze-thaw lysis followed by PCR.

A stock suspension of *L.pneumophila* serogroup 1 was prepared as described above followed by a ten times dilution using sterile water (10^{-1} dilution).

Four 10^{-2} dilutions (working suspensions, three for IMS and one positive control) were made using the 10^{-1} dilution, followed by IMS and PCR.

For IMS:

Beads: 10, 20 and 40µl of the coated beads
(5×10^6, 1×10^7 and 2×10^7 beads respectively)

IMS was performed as described previously, and bead-cell complexes were resuspended in 1ml of sterile distilled water. One hundred microlitres of each reaction (three IMS reactions, one positive reaction, and sterile water negative) was subjected to the five cycle freeze-thaw lysis procedure, followed by PCR amplification (as described above).

4.2.5 Electron microscopic analysis

For electron microscopic analysis, approximately 5µl of the bead-cell suspension was filtered using a 0.22µm filter, and washed once using a 0.075M phosphate buffer (pH7.4 to 7.6). The beads were fixated in a 2.5% gluteraldehyde in 0.075M phosphate buffer. Three washing steps were carried out using the phosphate buffer, and the sample was fixated for a second time using 0.25% watery osmiumtetroxide. Three washing steps using double distilled water was carried out, and the sample was dehydrated using single steps of 70, 90% ethanol, followed by three consecutive 96% ethanol steps. The sample was air-dried followed by critical point drying in liquid CO_2 and coating with gold. Bead-cell complexes were observed using a JEOL 840 scanning electron microscope (JEOL Ltd, Japan).

4.2.6 Fluorescent microscopic analysis

For fluorescence microscopy standard stock and working suspensions were prepared as described above. A second working suspension was set up, where the cells were diluted from the stock suspension using a 1% formalin (1% w/v formalin, 0.85% w/v NaCl) solution. 10µl of the working suspension were spotted onto 3 glass microscope slides (6mm diameter, 8-well, STERILAB), and the suspensions were left to air-dry at room temperature for 30 minutes. The air dried spots were dehydrated by submerging the slides in 50, 70 and 90% ethanol solutions for 5 minutes each, followed by air-drying for 5 minutes. Two slides (one untreated and one formalin treated) were submerged in a Triton/EDTA solution [1 x PBS (150mM, pH 8.0), 1% w/v Triton X-100, 100mM EDTA] for 20 minutes, and washed twice for 5 minutes in a 90% ethanol solution. The remaining untreated slide was used as a positive control, where two clear slide wells were used as negative controls to determine possible background fluorescence. Ten microlitres of a 40 times dilution (0.125µg) of the stock antibody MAB830 was added to each well, and the slides were incubated for 30 minutes in humid chambers at room temperature. The wells were washed once with sterile PBS/BSA buffer, and 10µl of 500 times dilution (0.150µg) of the stock rat anti-mouse fluorecein isothiocyanate (FITC) (polyclonal IgG, Cappel Research Reagents, USA) was added to each well. Two additional negative control reactions was also set up, where only rat anti-mouse FITC were added to the cell spot, as well as a cell only spot without the addition of antibody. After 30 minutes of incubation in humid chambers, the wells were washed again with PBS/BSA buffer, air dried for 5 minutes and overlaid with the fluorescence enhancing agent CITIFLUOR (Glycerol/PBS solution – UKC Chem. Lab., Canterbury, U.K.) and cover slides. Fluorescence microscopy was carried out using a ZEISS Axioskop (Filter 09: 450-490nm excitation/510,520nm emission) and intensity of fluorescence was visually compared between the three slides. To evaluate antibody coating on beads, the whole experimental procedure was repeated using primary pre-coated beads as well uncoated beads (negative

control). 5 µl of coated and uncoated beads were respectively spotted on two 8-well microscope slides. FITC conjugated rat anti-mouse IgG was added to each well as described above.

4.2.7 Determining sensitivity of separation

Sensitivity of detection was determined by setting up a standard dilution series (as described above) and comparing results obtained from standard culturing, direct amplification from lysed cell suspensions as well as immunomagnetic-based concentration followed by DNA amplification. Dilutions 10^0 to 10^{-5} was used as the working suspensions. Twenty microlitres of each of the working suspensions was plated out on BCYE using standard spread plate procedures. An equal volume was subjected to five-cycle freeze-thaw lysis, followed by PCR, electrophoresis and UV-visualization. To determine IMS sensitivity of recovery, 20µl of the working suspension was diluted into 1 ml by adding 980µl of sterile, double distilled water, and 20µl of pre-coated beads (as described above) was added. IMS, freeze-thaw lysis as well as PCR were performed according to previously described procedures.

4.2.8 Testing beads for possible PCR inhibition

The possibility of PCR inhibition originating from free iron in the uncoated bead suspension was determined. By comparing the intensity of amplification from a diluted cell suspension in either sterile double distilled water or an equal volume of uncoated bead suspension, the effect on efficacy of amplification could be determined. A stock culture was set up as described above. One hundred microlitres was aliquated into two 1.5 ml Eppendorf-type tubes, followed by centrifugation for 7 minutes at 12 000 rpm. After centrifugation, the supernatant was discarded and the cell pellets were resuspended in either 20µl of sterile

water or water suspended uncoated beads (1 x 10^7 beads). Five freeze-thaw cycles was carried out, followed by PCR, electrophoresis and visualization.

A second inhibition experiment was carried out, with the following reactions:

- Dynabeads added to the sample prior to freeze-thaw lysis, followed by PCR
- Dynabeads added to the sample after freeze-thaw lysis, followed by PCR
- Negative: PCR without Target DNA template
- Positive: 20µl of stock suspension, freeze-thaw lysis and PCR

4.2.9 Determining percentage recovery

A standard stock suspension was prepared as described above. From this stock suspension, a serial dilution series was set up to 10^{-2} (working suspension). 10µl of this suspension was used for culturing (using standard spread plate methods on BCYE without antibiotics), direct microscopic counts (using DAPI - 4`,6-Diamine-2`-phenylindole dihydrochloride; Boehringer Mannheim biochemicals, Germany) and IMS followed by culturing (BCYE) and direct microscopic counts (DAPI). For the IMS assay, three separate reactions was set up, two of which were used for culturing (duplicate cfu counts) and one for direct microscopic counts. After IMS washing, the bead-cell complexes were resuspended in 100µl of sterile, double distilled water. 10µl of these suspensions were used for subsequent direct fluorescent microscopic counts in duplicate using a ZEISS Axioskop fluorescence microscope (filter 02: 365/395/420nm). DAPI stock and working solutions were all prepared according to manufacturer's instructions: shortly, a stock solution was prepared at 1mg/ml in sterile double distilled water, from which a working solution was prepared by dilution in filtered absolute methanol to a final concentration of 1µg/ml. Stock solutions were stored at -20°C, where working solutions were prepared freshly before each experiment.

4.2.10 The effect of buffers on recovery efficacy of IMS

A standard stock suspension of the pure culture was set up as described previously. Using the stock suspension, a working suspension (10^{-2}) was set up by serial dilution (100μl carry over). 100μl each of this suspension was aliquated into 4 Eppendorf-type tubes, and the following reactions were set up:

- One hundred microlitres suspension + 860μl sterile water + 40μl coated Dynabeads (Normal IMS)
- One hundred microlitres suspension, centrifuge to pellet (5 minutes, 12000 rpm), resuspend in 860μl sterile PBS/BSA, add 40μl coated Dynabeads (PBS/BSA buffer)
- One hundred microlitres suspension + 660μl sterile water + 100μl SL-A buffer + 100μl SL-B buffer (*Cryptosporidium* IMS system, Dynal, Oslo, Norway)
- One hundred microlitres suspension (positive control)
- One hundred microlitres sterile water (negative control)

Five freeze-thaw cycles were performed on each reaction following IMS, washing and resuspension. Twenty microlitre aliquots of each reaction lysate were used for subsequent PCR amplification (section 4.2.3). The experiment was repeated without including the SL-A/SL-B buffers, and a serial dilution series (10^{-1} to 10^{-6}) was set up of the freeze-thaw lysis products after IMS.

4.3 Results and Discussion

4.3.1 Immunomagnetic separation and detection using pure culture

Following coating of the magnetic beads, they were tested on their ability to recover viable and detectable *L.pneumophila* cells by means of culturing and PCR. Using IMS, positive amplification was achieved from recovered cells, and

the correct product size (168bp) was produced. Isolation of target *L.pneumophila* serogroup 1 cells from suspension was confirmed by culturing. BCYE plates displayed in excess of 300 colony forming units (cfu) from both the cells suspension as well as the IMS recovered cells; these results indicated that PCR amplification as well as recovery of viable and cultureable cells was possible from water samples containing a single culture of *L.pneumophila*.

Because of the fastidious nature of this bacterium, and long incubation periods needed on artificial culture media, problems are usually experienced with direct isolation from highly contaminated samples. Faster-growing heterotrophic organisms often cause overgrowth of the growth media leading to negative isolation of *L.pneumophila* (Ng *et al*, 1997). Target specific isolation *via* IMS could overcome this problem by isolating the target bacterium form the sample, and thus from substrate and space competitive conditions (Olsvik *et al*, 1994).

4.3.2 Optimization of antibody and bead concentrations

Optimization of IMS was performed by using different primary antibody (MAB830) and bead concentrations to isolate *L.pneumophila* from sterile distilled water, followed by PCR amplification. Optimum antibody concentration was determined at a concentration of 2μg MAB830 per 10^7 beads, where 1μg MAB830 per 10^7 beads displayed lower band intensity after amplification. 3μg MAB830 per 10^7 beads were chosen as the upper limit (in accordance to the bead manufacturers recommendations), and did not display higher band intensity after amplification than 2μg antibody at the same bead concentration. Coating efficiency is influenced by the binding efficiency of the mouse antibody, and in the case of the M-280 sheep anti-mouse Dynabeads the secondary bead surface antibody binds both the heavy and the light chain of the antibody. The secondary surface antibody reacts strongly with all mouse antibodies, but displayed lowered binding efficiency with subclass IgG_3 (manufacturers data). Because of the scarcity of non-conjugated mouse anti-*Legionella* antibodies, the only product readily

available at the time was a monoclonal antibody (MAB830) from CHEMICON International (California, U.S.A.). This antibody is from the subclass IgG_{3K}, and this might explain some variances and low isolation efficiencies found throughout this study, especially when compared to standard PCR amplification directly from the samples. No alternative antibody preparations were used in the study.

The optimum amount of beads to be used per ml sample was determined at 2×10^7 beads. The manufacturer claim a theoretical 95% recovery efficiency when using 1×10^7 beads per sample, and 95%+ efficiency when using double the amount of beads. The strongest band intensity was observed after amplification at double the normal beads concentration compared to using 5×10^6 and 1×10^7 beads (results not shown), and it was decided to use this amount for all further studies conducted.

4.3.3 Fluorescent and electron microscopic analysis

In order to evaluate bead and primary antibody binding to *L.pneumophila*, bead-cell complexes were studied by means of fluorescence- and electron microscopy. The results obtained from electron microscopic analysis of the beads are depicted in Figure 4.1. Most beads observed during scanning electron microscopy were found to be naked with little or none of the target cells bound to the bead surface. Binding and object preparation procedures were repeated with little or no increase in the amount of observable complexes. Standard sample preparation procedures might have influenced the binding capacity (chemically or mechanically) between beads and cells, and could thus have lead to large numbers of unbound cells.

Figure 4.1. Scanning electron microscopic (SEM) analysis of bead-cell complexes after recovery. Hair-like structures are *L.pneumophila* cells in chain. Scale: 1μm. Magnification: 5 000x.

Bacteria of the genus *Legionella* typically display chain-like structures, especially when cultivated on artificial media. These structures could reach 20μm or more in length and are usually directly associated to the age of the culture, i.e. older cultures typically display chain-like structures (Brenner *et al*, 1984). These structures could play a role in recovery by IMS where they might influence the physical binding and extraction procedures. The uses of younger, single cell cultures (4 to 5 days on BCYE, study observations) for further development of this technology are recommended.

In order to determine the possible binding location of the MAB830 antibody on the cell, formalin and Triton/EDTA treatment was used. Formalin fixates any surface proteins and could thus interfere with the availability of antibody epitopes, where Triton/EDTA will disrupt surface structures such as lipopolysachharides, which might display antigenic characteristics. No differences in fluorescence

intensity could be observed between the untreated, formalin, Triton/EDTA and formalin-Triton/EDTA reactions, indicating probable insufficient treatment conditions or resistance of the epitope to chemical modification. Only MAB830 coated cells and beads displayed a strong fluorescence at the given wavelength (Figure 4.2), with a total absence of fluorescence from the uncoated beads and cells. No auto fluorescence was observed from the cells at the given wavelength, and the FITC rat anti-mouse did not display any binding to the cells in the absence of MAB830 on the cell surface.

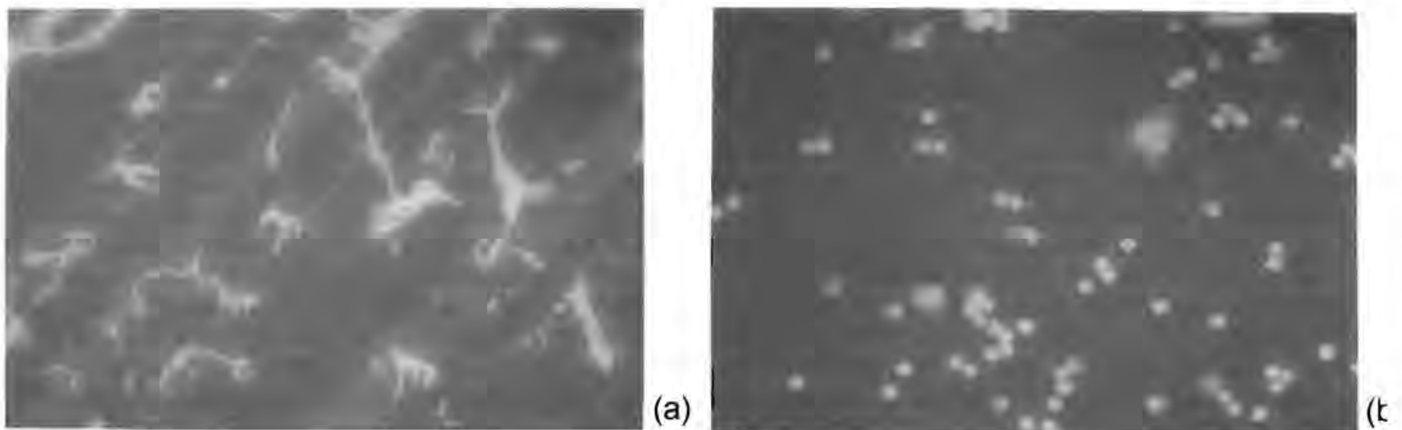

Figure 4.2. Immuno-fluorescence of MAB 830 coated cells (a) and magnetic beads (b) using FITC-conjugated rat anti-mouse IgG.

4.3.4 Determining sensitivity of separation

Sensitivity of recovery was determined by means of a standard dilution series of *L.pneumophila* cells, followed by IMS, fluorescent microscopic observation, culturing and PCR. Using IMS, a single log higher sensitivity could be achieved over standard PCR, with a peak sensitivity of 4 cfu by standard BCYE culturing (results not shown); however, direct counts using DAPI displayed cell counts ranging anywhere between 1 x 10^3 to 1 x 10^4 cells in the same sample volume. This variation might be explained on the basis of poor cell viability and cultureability, which was observed after continuous long term culturing.

Reproducibility of results was low, where variations in sensitivity were observed for both PCR and IMS-PCR. Low capturing sensitivity by IMS could be ascribed to a range of factors including variation in the efficacy of antibody binding to the bead (low efficacy binding of subclass IgG_{3k} antibodies), quality of antibody preparations, continuous thawing and freezing of antibody aliquots, binding conditions and settling of beads during binding (Olsvik *et al*, 1994). These factors could lead to insufficient bead coating, which in turn could lead to lower detection sensitivity by PCR. In order to circumvent the impact of these factors, great care was taken with regards to the use of single-use antibody aliquots, the use of different binding buffers and conditions (Section 4.3.6), and the continuous mixing of samples during coating and capturing to prevent bead settling.

4.3.5 Testing beads for possible PCR inhibition

The possibility of inhibition of amplification by iron leaking from the magnetic beads was determined by adding beads prior to and after freeze-thaw lysis. No visible differences in amplification could be observed when comparing PCR reactions performed with beads added before or after freeze-thaw lysis (Figure 4.3). Washed, uncoated beads were also added to the PCR reaction mixture as top up liquid instead of water, and again no visible inhibition was present. These results indicate that the beads did not produce visible inhibitory effects on DNA amplification at the given concentrations used in this assay.

4.3.6 The effect of buffers on recovery efficacy by IMS

Using different buffer systems compared to water for isolation did not produce any significant increase in amplification sensitivity, thus in efficacy of capturing. In order to compare efficacy of amplification, a standard dilution (to 10^{-6}) of freeze-thaw products from the captured cells were used in 6 reactions. No difference between the sensitivity of detection was observed for these reactions (Figure 4.4), indicating that the buffer systems used here did not significantly increase the efficacy of recovery.

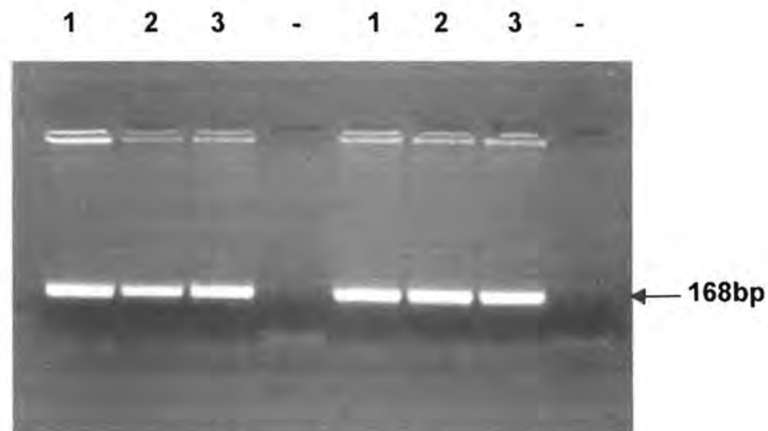

Figure 4.3. The effect of beads on amplification efficacy. Duplicate gel lanes were loaded. Lane 1, beads added prior cell lysis; lane 2, beads added after cell lysis; lane 3, standard PCR positive control; (-) negative PCR control (no DNA template).

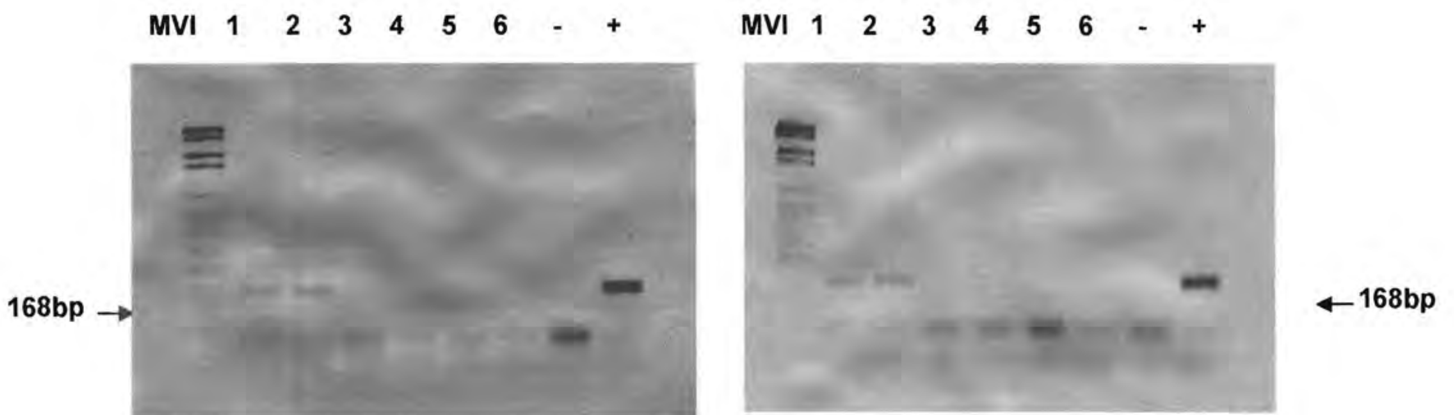

Figure 4.4. The effect of different buffers on recovery efficacy. Standard distilled water extraction (left gel) and PBS/BSA binding buffer suspended cells. Numbers 1 to 6 corresponds to cell dilutions 10^{-1} to 10^{-6} respectively.

4.4 Conclusion

Concentration of target organisms specifically from biological and chemical complex samples is one of the corner-stones of a successful detection assay, and greatly enhances the efficacy of such an assay. Although selective enrichment of organisms have been applied in various studies (Luk and Lindberg, 1991; Widjojoatmodjo *et al*, 1992; Lang *et al*, 1994; Pyle *et al*, 1999), it still poses a problem when dealing with members of the group *Legionella*. These organisms are easily displaced in media by faster growing types, and specialized media as well as long incubation periods are a prerequisite in cultivation-based assays. IMS provides an alternative to concentration of these organisms, and have successfully been applied to detection of Salmonellae (Luk and Lindberg, 1991, Widjojoatmodjo *et al*, 1992; Cudjoe *et al*, 1994), *Escherichia coli* (Bennet *et al*, 1996; Pyle *et al*, 1999) and *Listeria monocytogenes* (Uyttendaele *et al*, 2000). The technique provides clear advantages over standard enrichment assays, e.g. isolation is specific and could be altered depending on the choice of antibody, it requires less sample processing time, no incubation time is needed and the cells remain viable and unaltered for further studies.

This study focussed on the development of an IMS assay for *L.pneumophila* serogroup 1 to enable fast and further specific detection via PCR. Optimum antibody (2μg MAB 830 per 10^7 beads) and bead concentrations ($2x10^7$ beads/ml sample) were determined for the system, and binding of the antibody to the target organism and the beads was proven using scanning electron and fluorescent microscopy. The precise surface epitope recognized by the monoclonal antibody is still unknown, and no detailed information was given by the manufacturer (CHEMICON International). Treatment of the bacterial surface with formalin and Triton/EDTA did not produce any visual differences in binding of the antibody, indicating insufficient treatment conditions or resistance of the epitope to substantial modification.

Although detection was possible using IMS, problems were experienced during optimization of the assay. Reproducibility of results was low (see Chapter 3, section 3.3.3), where variations in sensitivity were observed for both PCR and IMS-PCR. Low capturing sensitivity by IMS could be ascribed to a range of factors including variation in the efficacy of antibody binding to the bead, the quality of antibody preparations, continuous thawing and freezing of antibody aliquots as well as settling of beads during binding (Olsvik *et al*, 1994). Availability and cost of monoclonal antibodies against *L.pneumophila* was also a problem, where only specific groups could be used with the available DYNAL bead systems.

Apart from the problems described above, IMS still provides a valuable tool for target organism isolation from complex and sometimes PCR inhibiting samples. Future research should focus on the use of different antibody preparations, i.e. comparing IgG antibodies from different sub-classes with multivalent IgM antibodies. Methods of sufficient mixing of preparations to overcome settling of beads, and the applicability of different bead systems (ranging in physical size as well as binding characteristics) should be investigated in future studies in this field of research.

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CHAPTER 5

Detection of *L pneumophila* in Water Using Solid Phase Sequence Capture and the Polymerase Chain Reaction

5.1 Introduction

The use of molecular-based techniques for the sensitive and specific detection of *Legionella pneumophila* have been reported in recent years (Koide *et al*, 1993; Catalan *et al*, 1994; Maiwald *et al*, 1994; Ng *et al*, 1997). However, these techniques proved to be specific towards their goal, but lacked reproducibility in terms of sensitivity of detection (see Chapters 3 and 4). With consideration of the various problems associated with inhibition of PCR when working with environmental and industrial water samples (Oshiro *et al*, 1994), an effective target isolation procedure is essential.

Immunomagnetic separation (IMS) focus on the isolation of target organisms from the sample, leaving behind inhibitory substances. This technique is however dependant on the commercial availability of non-conjugated subclass- specific antibodies against the target cell, where the latter could also prove to be expensive as well as sensitive towards loss of activity when applied in a routine setup. Thus, when focussing on target isolation and concentration, attention was drawn towards nucleic acids.

The use of magnetic-based capturing of nucleic acids has found a range of applications in various fields of molecular research (Hultman *et al*, 1989; Rimstad *et al*, 1990, Olsvik *et al*, 1994). For bacterial pathogen detection, positive detection of small numbers of *Mycobacterium tuberculosis* could be achieved (Mangiapan *et al*, 1996). In this approach, released target DNA is captured by a sequence-specific probe (direct-immobilized or indirect as in the

case of IMS), thus enabling separation from inhibitory conditions to increase the performance of amplification via a specific PCR.

In this chapter emphasis is placed on solid phase sequence capture assay to enable specific detection of *L.pneumophila* in seeded industrial and environmental waters. The study focused on the use of existing primer sequences and amplification assays and their applicability in the development of a magnetic-based target DNA recovery. The sensitivity of the technique was also compared to that of the developed PitStop PCR (Chapter 3) and IMS assays (Chapter 4).

5.2 Materials and methods

5.2.1 Bacterial strain applied in the study

L.pneumophila serogroup 1 (ATCC 33152) was used as test organism in all studies. The strain was cultured on buffered charcoal yeast extract agar (without antibiotics) at 37°C for 4-7 days.

5.2.2 Magnetic bead preparation

The beads were supplied as a suspension containing 6.7 x 10^8 Dynabeads per ml (10mg/ml), dissolved in phosphate buffered saline (pH 7.4), containing 0.1% bovine serum albumin and 0.02% NaN_3. Beads were prepared as described by the manufacturer. Briefly, beads were resuspended by gentle mixing, after which 50µl (500µg) of beads was washed twice by magnetic extraction using an eppendorf tube type extractor (DYNAL® MPC®-E-1). The supernatant was discarded and the beads resuspendend in 50µl binding and washing buffer (B&W, 10mM Tris-HCl, pH 7.5, 1mM EDTA, 1M NaCl). 1 µl (100 pmol) of biotinylated probe PTB69 (5'-Biotin-GCATTGGTGCCGATTTGG -3', MWG, Germany) complimentary to positions 948 to 965 of the *L.pneumophila* specific *mip* gene (Engleberg *et al*, 1989) was added, and the volume was made up to a total of 100µl. Bead coating

was performed at room temperature for 10 minutes with gentle mixing every 2 minutes. Three washing steps was carried out, and the coated beads were resuspended in 50µl B&W. Bead coating was determined by performing PCR amplification of target DNA (*L.pneumophila* ATCC 33125) in the absence of added primer PT69.

5.2.3 Target isolation using pure culture *L.pneumophila*

Target capturing was assessed using purified 168 bp PCR product (QIAX III system, Qiagen, Germany), followed by chromosomal DNA capturing. A stock suspension of cells were made by suspending 1 cfu from solid media in hybridization buffer [HB, 0.075M Na citrate, 0.75M NaCl, pH7.0, 0.1% (w/v) N-laurolylsarcosine, 0.02% (w/v) sodium dodecyl sulphate (SDS)]. DNA was released by 5 consecutive freeze-thaw (F/T) cycles (-70°C for 5 minutes, 37°C for 3 minutes) or purified for initial experiments using the CTAB method (Wilson, 1990). Released DNA was denatured in a 95°C water bath for 10 minutes. Probe-coated beads (50µl/500µg) were added to the sample, followed by probe-target DNA hybridization in a 40°C water bath for 1 hour. Gentle mixing was performed every 10 minutes to prevent bead settling. This was followed by three washing steps (two steps using 1 x B&W followed by a third step using sterile double distilled water) and beads were resuspended in 20µl sterile double distilled water. PitStop PCR was performed as described in Chapter 3, without the addition of additional forward primer in the reaction mix (PTB69 sequence identical to PT69, and would thus enable amplification) using a Perkin-Elmer 2400 thermal cycler (PE Applied Biosystems, USA) . The amplicons were resolved on a 2% (wt/vol) agarose gel in 1 × TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) and visualized by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. Electrophoresis was carried out at 60mA/100V for 30 minutes using a Hoefer Mightyslim SX-250 electrophoresis apparatus.

5.2.4 Optimization of assay

Target DNA isolation was performed by using both the direct (pre-coated beads added to sample) and indirect (probe added after denaturation) approaches. For the direct approach two single (50μl/500μg), one double (100μl/1mg) and one triple (150μl/1.5mg) coated bead reactions were prepared. In each case, 100pmol probe per 50μl beads were used for coating (maximum binding capacity per bead surface, as per manufacturer's recommendation). One of the single volume reactions (50μl pre-coated beads) was added to the sample prior to denaturation to determine possible template renaturation without probe hybridization. In the indirect approach 100pmol (1μl) and 150pmol (1.5μl) (respectively) of probe PTB69 was added after denaturation, followed by hybridization as in the case of the direct approach. Washed beads (50μl) were added, mixed gently, and binding was allowed to occur at room temperature for 30 minutes with occasional mixing (every 5 minutes) to prevent bead settling.

5.2.5 The effect of buffers on recovery efficacy

The effect of different buffers on isolation efficacy was also assessed: hybridization was performed in both HB and B&W buffers using the direct (50μl coated beads) as well as the indirect (100pmol PTB69) technique. The effect of adding additional 50pmol forward primer (PT69) to the PCR reaction was compared to that of the standard approach (PTB69 as captured forward primer). Standard PitStop PCR conditions (as described in Chapter 3) were followed for target amplification.

5.2.6 Target DNA isolation from cell-seeded environmental water

The detection limit of the optimized capturing approach was determined by standard culturing of a *L.pneumophila* dilution series on BCYE agar (without antibiotics). A standard dilution series was set up from a stock culture (1cfu/ml water), and a 10 fold dilution was made in double distilled, cooling (cooling tower) as well as environmental water (surface water). One hundred

microlitres and 500 µl was aliquatted in separate sterile 1.5ml Eppendorf type tubes (for PitStop PCR and magnetic separation respectively). Suspended cells in the 500µl aliquats were pelleted by centrifugation (12000 rpm for 10 minutes) using a standard benchtop centrifuge (Hermle, Germany). Cell pellets were resuspended in 450µl HB, followed by F/T lysis and capturing as described above. To determine whether PCR inhibition occurs from the environmental water, 19µl crude *L.pneumophila* DNA (freeze-thaw lysate) instead of make-up water was added to one of the duplicates of PitStop environmental PCR and PCR from bead captured cells.

5.3 Results and discussion

5.3.1 Target isolation

In order to determine if successful coating of beads were achieved, PitStop PCR was performed on purified target DNA without the addition of the forward primer PT69. Successful amplification could be achieved when adding PTB69 pre-coated beads to a PCR reaction containing template DNA and only the reverse primer PT70/PT181, thus indicating positively coated beads. To capture purified 168bp PCR product (approximately 500ng as determined by gel electrophoresis) and chromosomal DNA (crude lysate and CTAB purified), hybridization was performed at an initial 55°C for 1 hour. These conditions delivered weak amplification intensity, indicating sub-optimal conditions. Hybridization time was extended to 16 hours overnight, and the effect of immediate cool-down after denaturation as well as the addition of DNA denaturing agents dimethylsulfoxide (DMSO, 2.5 and 5% final), formamide (3.5 and 7% final; Merck, Germany) and sonication to fragmentize the DNA (20 kHz, 5 minutes) were evaluated. Immediate cooldown to room temperature following denaturation proved to be the most effective for capturing the purified 168bp amplification product opposed to overnight incubation at 55°C or the addition of denaturing agents (Figure 5.1).

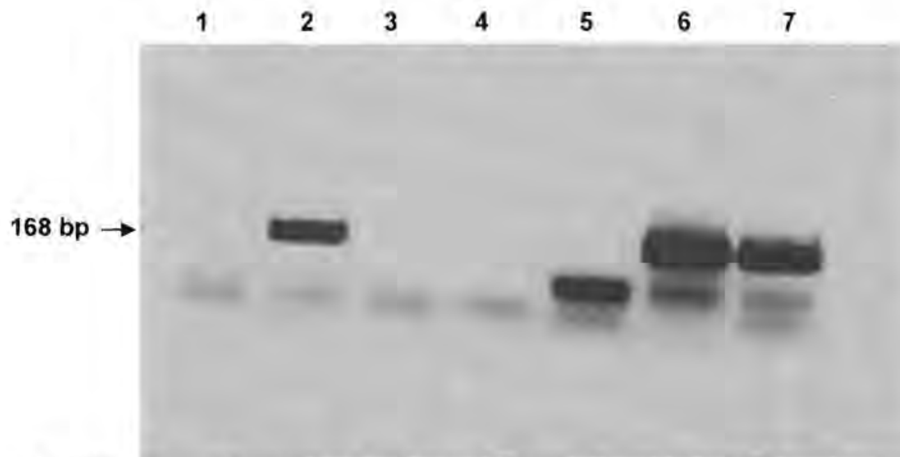

Figure 5.1. Recovery of 168bp PCR product by solid phase sequence capture. Lane 1, overnight hybridization at 55°C; Lane 2, immediate cooldown after denaturation; Lane 3, 5% DMSO (v/v) and overnight incubation; Lane 4, 7% formamide (v/v) and overnight incubation; Lane 5, PCR negative (no template DNA); Lane 6, 5μl of purified 168bp product (standard PCR positive 1) and Lane 7, 20μl of crude chromosomal DNA (standard PCR positive 2).

No detectable recovery of purified chromosomal DNA was observed using the above conditions (Figure 5.2) and it was decided to follow a target fragmentation approach in order to facilitate capturing. A restriction map of the 702bp *mip* (Genbank accession number AF023173) gene of *L.pneumophila* [National Center for Biotechnology Information (NCBI), 2000] was compiled using the internet- based program Web-Cutter (Web-Cutter, 2000). Double restriction of purified chromosomal DNA was performed using 5U each of *Alu*I [Boehringer Mannheim, Germany] (recognition: 27 bases upstream of the primer PT69 annealing site) and *Eco*RI [Boehringer Mannheim, Germany] (recognition: 13 bases downstream of the primer PT70/PT181 annealing site). The resulting target fragment would be in the range of 208 bp. Restriction was observed by gel electrophoresis and

restriction endonucleases were inactivated during denaturation (95°C for 10 minutes) prior to capturing.

Figure 5.2. Denaturing conditions to enable chromosomal capturing. Lane 1, 5% DMSO (v/v); Lane 2, sonication of crude DNA; Lane 3, 5% formamide (v/v); Lane 4, 12% formamide (v/v); Lane 5, captured 168bp product (positive 1); Lane 5, PCR negative (no target template); Lane 7, equal amount of 168bp product in PCR positive (positive 2); Lane 8, equal amount of crude lysate as in 1 to 4 (positive 3). 100 bp marker (Promega) from 100bp to 1,5kb.

Capturing of double restricted DNA was observed only after a 30 minute hybridization at 50°C followed by a 30 minute cool-down step to room temperature (Figure 5.3). Increased capturing was observed at 40°C hybridization, where effective capturing of non-restricted purified chromosomal DNA was observed (Figure 5.4). No further modification to the hybridization temperature was performed, and this temperature was used for all further studies conducted.

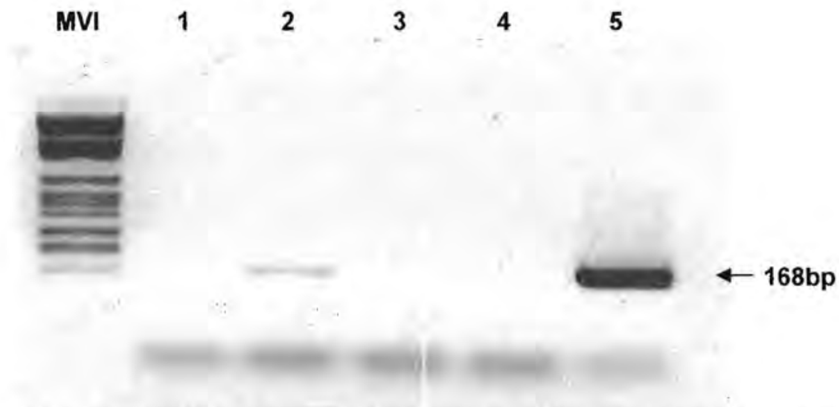

Figure 5.3 Optimization of hybridization temperature for capturing chromosomal DNA: Lane 1, non-restricted chromosomal DNA (no visible amplification); Lane 2, double restricted (*Eco*RI and *Alu*I, 208bp target fragment) chromosomal DNA, Lane 3, beads without target DNA (negative capturing and PCR), Lane 4, PCR negative (without target DNA); Lane 5, PCR positive (target template). Hybridization temperature: 50°C.

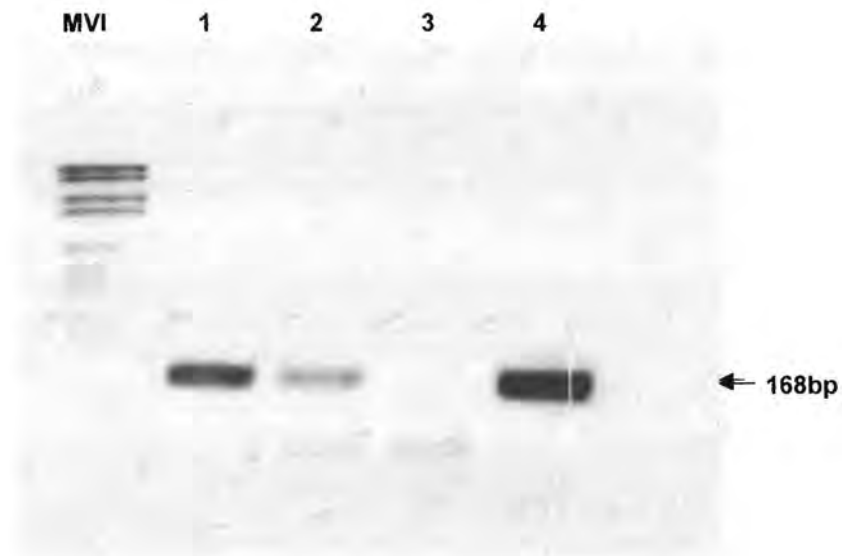

Figure 5.4. Capturing of double restricted (lane 1) and non-restricted (lane 2) chromosomal DNA using 40°C hybridization followed by cooldown to room temperature. Lane 3, PCR negative (no template DNA); Lane 4, PCR positive (*L.pneumophila* crude lysate).

5.3.2 Assay optimization

The efficacy of capturing was determined by altering bead to template DNA ratios during the direct capturing approach, after which this optimum bead concentration was compared to the efficacy of capturing via the indirect approach. A direct correlation was found between the volume of beads used for extraction and sensitivity of detection, where double the volume beads (100μl/1mg) displayed constantly higher levels of capturing of target DNA than that of 50μl beads (Figure 5.5). This was however not true for higher concentrations of beads (150μl/1.5mg), thus indicating a PCR inhibitory effect linked to bead concentration (probable physical inhibition, Figure 5.6). This phenomenon has been described previously (Mangiapan *et al*, 1996) when larger amounts of beads had progressively prominent inhibitory effects. Inhibition of amplification due to increased bead concentration was not observed previously during the study (IMS, chapter 4, section 4.3.5) and might be ascribed to lower bead concentrations used previously (200μg vs. 1.5mg per 50μl PCR reaction)

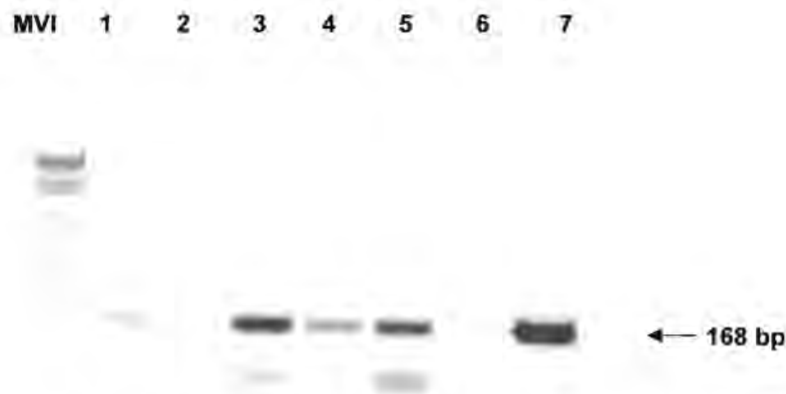

Figure 5.5. Optimization of bead concentration, bead addition time as well as comparing it to the indirect approach. Lanes 1 and 3, 50 and 100μl pre-coated beads respectively added before denaturation; Lanes 2 and 4, 50 and 100μl pre-coated beads respectively added after denaturation; Lane 5, indirect approach; Lane 6, PCR negative (no template DNA); Lane 7, PCR positive (*L.pneumophila* lysate).

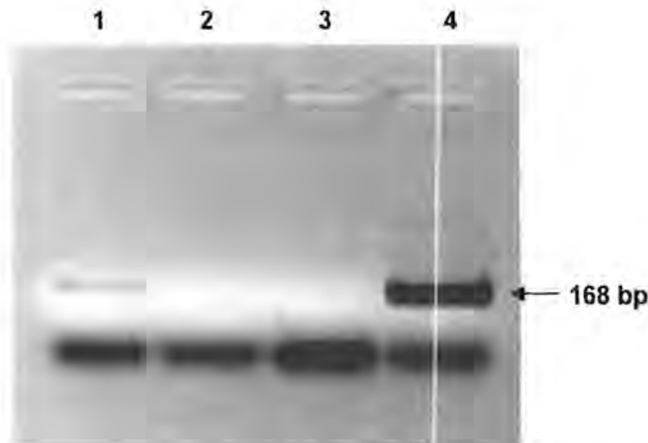

Figure 5.6. Indirect approach of capturing of *L.pneumophila* target DNA using 100pmol (lane 1) and 150pmol (lane 2) of biotinylated primer PTB69. Lane 3, PCR negative (no template DNA); Lane 4, PCR positive (*L.pneumophila* lysate).

When using the indirect approach, positive target capturing was achieved at 100pmol probe but not 150pmol added to the reaction. This might be explained at the hand of physical blocking of available strepavidin molecules on the bead surface by unhybridized biotinylated probe, thus inhibiting binding of target hybridized probe to these sites. Optimization of the exact ratio of probe to product concentration could be determined by means of competitive radio-labeled probe detection (Mangiapan *et al*, 1996). However, due to the high cost and dangers involved with radioactive work, optimal probe concentration was determined visually by comparing amplification intensities after target capture.

A constant level of target capturing was achieved using the direct approach, where fluctuations in amplification sensitivity was noted using the indirect approach (Figure 5.7). Self hybridization of target DNA stands might occur just before addition of biotinylated probe, when removing tubes from the water bath. Addition of probe before denaturation delivered satisfactory but inconsistent results; this might be ascribed to stability of the biotin-label at prolonged denaturing conditions in the water bath. It was thus decided to

follow the direct capturing approach for all future studies. In the light of raw material costs regarding strepavidin M-280 Dynabeads, it was decided to use 50μl instead of 100μl of beads for capturing, which increased the stock life of beads to 20 reactions per ml opposed to half that amount using the higher bead concentration.

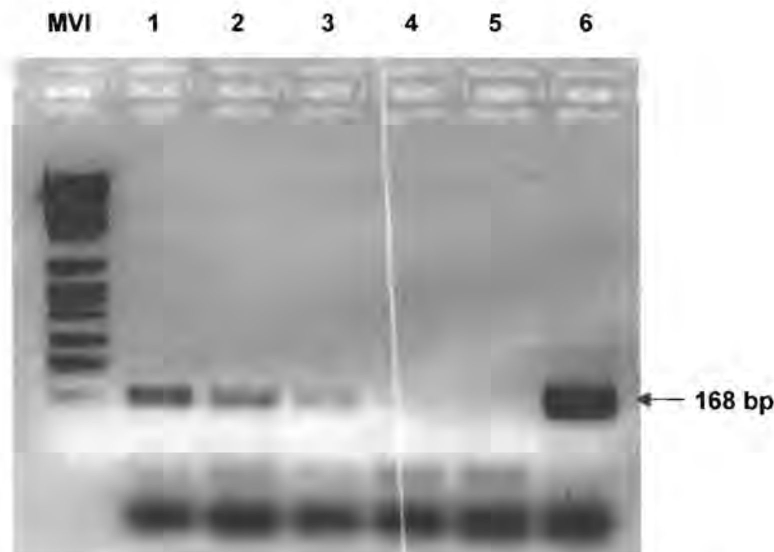

Figure 5.7. Comparison between the direct and indirect target DNA capturing approaches. Serial dilutions of cells (10^{-1} and 10^{-2}) was set up, and both approaches was used for capturing. Lanes 1 and 3, 10^{-1} and 10^{-2} respectively (direct approach); Lanes 2 and 4, 10^{-1} and 10^{-2} respectively (indirect approach); Lane 5, PCR negative (no template DNA); Lane 6, PCR positive (*L.pneumophila* lysate).

5.3.3 The effect of buffers on recovery efficacy

The possible contribution of different hybridization buffers on capturing efficacy was determined by PCR amplification of the target, following capturing. No visible differences in capturing efficacy were observed when hybridization was performed in an alternative buffer (B&W buffer) or using the two buffers at either 1M or 0.75M NaCl concentrations. This was true for both the indirect as well as the direct approaches (Figure 5.8). After several

repeats, no difference in amplification intensity was observed in the direct approach, were variable results were achieved using the indirect approach. Addition of primer PT69 to the PCR reaction mixture did not produce higher amplification efficacy, indicating sufficient forward primer availability in the form of captured PTB69 (Figure 5.8).

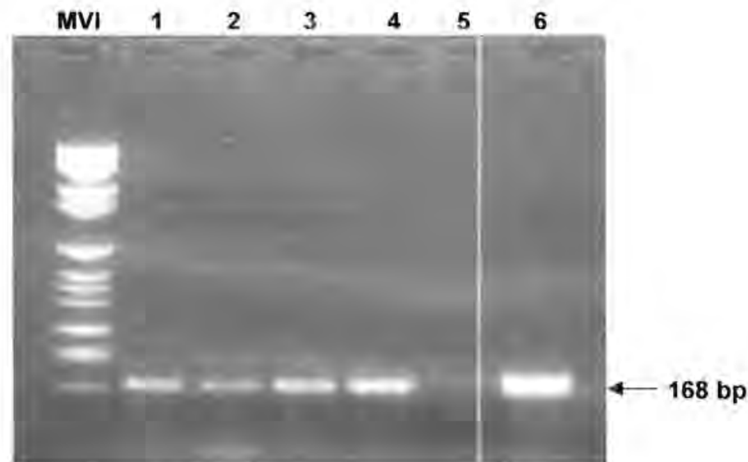

Figure 5.8. The effects of buffers on the efficacy of target DNA capturing. Lane 1, indirect approach in buffer HB; Lane 2, indirect approach in buffer B&W; Lane 3, direct approach in buffer HB; Lane 4, direct approach in buffer HB including 50pmol primer PT69 in PCR reaction; Lane 5, PCR negative (no DNA template); Lane 6, PCR positive (*L.pneumophila* lysate).

5.3.4 Target DNA isolation from cell-seeded environmental water

Sensitivity of capturing from different water samples was determined visually from PCR amplification product intensity on agarose gel after electrophoresis, as in the case of standard and PitStop PCR. Detection sensitivity of 4×10^2 cells was obtainable in both double distilled as well as cooling water (figure 5.9), however, inconsistent results was found upon repeating of the experiment, where inhibitory water samples appeared to influence efficiency of post-capture amplification more severely than in the case of PitStop PCR.

Figure 5.9 Capturing of target DNA from double distilled (lanes 1-5) and cooling water (lanes 6-9). Standard dilution of cells was prepared in sterile double distilled water, followed by carry-over in each of the two water types. Lane 10, PCR negative (no DNA template); Lane 11, PCR positive (*L.pneumophila*).

In order to assess the effect of inhibitory substances on the assay, the effect of addition of external DNA template prior to PCR amplification was determined. Low level of amplification was detectable on 2% agarose using both double distilled as well as environmental water. With the addition of template DNA just before amplification, a noticeable increase in amplification was observed. In the case of magnetic capturing, efficient amplification was observed when capturing from double-distilled water, but not from environmental water (as in the case of inhibitory water samples). Amplification was only visible after the addition of template DNA following capturing (Figure 5.10). This indicates possible inhibition of DNA capturing rather, but not exclusively, to the carry-over of inhibitory substances into the PCR reaction. Reduced efficacy of amplification could therefore be contributed by both inhibition of capturing (availability of template for amplification) as well as enzymatic reaction inhibition due to small amounts of inhibitory substance left behind following the multiple washing steps.

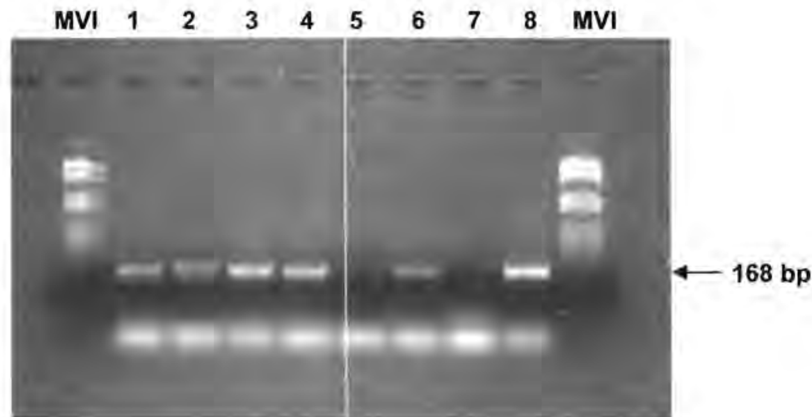

Figure 5.10. The effect of inhibitory substances on the efficacy of DNA capturing and amplification. Lane 1, PitStop PCR (double distilled water); Lane 2, PitStop PCR (environmental water); Lane 3, PitStop PCR (environmental water + DNA added to PCR); Lanes 4-6, magnetic based DNA capturing: double distilled water and environmental water with DNA added respectively. Lane 7, PCR negative (no template DNA); Lane 8, PCR positive (*L.pneumophila* crude lysate).

5.4 Conclusion

In this study development of a new PCR-based detection strategy for the detection of *Legionella pneumophila*, namely solid-phase sequence capture-PCR, has been discussed. This strategy enabled rapid concentration of *L.pneumophila* DNA present in water samples prior to amplification using an optimized PCR assay (chapter 3). The use of magnetic based separation of target DNA could, in theory, overcome some of the limitations of direct amplification from inhibitory water samples. Currently these techniques prove to be more useful and widely applied in clinical applications (Hultman *et al*, 1989; Rimstad *et al*, 1990; Olsvik *et al*, 1994, Mangiapan *et al*, 1996). Infected tissue normally bears vast numbers of organisms, where much lower numbers are usually associated with environmental and industrial water samples. Concentration of these organisms by filtration could also increase the concentration of inhibitory substances, thus producing reduced amplifications efficacy and therefore lowered sensitivity as well as possible

false-negative results. In order to eliminate these substances, tedious multi-step DNA purification procedures could be applied. Unfortunately, these processes are difficult to apply in routine practice when large volumes of samples are processed. Purification of specific target DNA before amplification could theoretically increase the sensitivity and specificity of an assay by elimination of inhibitory substances and non-target sequences from the sample.

Sequence capture PCR of target DNA could be applied using either a direct (pre-coated beads) or indirect (capturing of hybridized biotinylated probe) technique. When comparing the indirect with the direct approach in this study, the latter proved to deliver constant amplification from DNA seeded water, where the former displayed fluctuations in capturing efficacy. Self hybridization of target DNA might occur just before addition of the probe outside the heated water bath. When biotinylated probe was added prior to denaturation, inconsistent results were achieved; this might be ascribed to the stability of the biotin label at prolonged denaturing conditions in the water bath. It was therefore decided to follow a direct sequence-capture based amplification approach. Although a high sensitivity of detection in double-distilled water was obtainable ($4x10^2$ cfu), the assay was severely affected by inhibitory substances in the sample. Assessment of inhibition was done by means of addition of exogenous target template to the sample prior to capture; however, results obtained indicate inhibition at the capture level rather than carry-over of inhibitory substances (multiple washing steps should dilute these substances to a near undetectable level). Inhibition of capturing was also present with increased bead and biotinylated primer concentrations. This phenomenon has been described earlier (Mangiapan *et al*, 1996) and could respectively be explained on the basis of physical hindrance and blockage of available biotin binding sites on the bead surface by unhybridized probe.

Due to the nature of the research, it was also beyond the scope of the project to quantify probe binding by employing competitive non-labelled and radioactive labelled probe capture assays (as described by Mangiapan *et al*,

1996). Further research should focus on the possible contribution of each of these factors to amplification efficacy as well as the kinetics of the physical binding of probe and target DNA. Application of a DNA amplification-based assay of this type for the detection of *L. pneumophila* could enhance the field of routine bacteriological analysis of water without the need for time-consuming traditional culturing practices.

CHAPTER 6

Summary of Results and Conclusions

Detection of *Legionella pneumophila*, the causative agent for Legionnaires' disease, has been performed in the past by culture-based methods, and more recently, by means of target-specific DNA amplification by PCR. Although these techniques proved to be rapid and sensitive compared to culture-based assays, they are prone to inhibition from complex environmental samples. This may lead to a reduction in the sensitivity of detection as well as false negative results, as been shown during this study. Multiple template purification steps are tedious in a diagnostic setup, and could lead to valuable template loss. For this reason, further development on the standard PCR assay was performed, which included : (1) Optimization of the previously described amplification assay, (2) use of a rapid freeze-thaw lysis procedure to increase the detection sensitivity of the assay, (3) development of a hemi-nested PCR to increase both specificity and specificity of amplification and (4) application of PitStop PCR (2 step PCR procedure) to achieve increased amplification sensitivity over standard 1 step 30-cycle PCR.

Although hemi-nested PCR display certain advantages over 30-cycle PCR, increased number of amplification cycles could lead to the occurrence of false positive results due to minute amounts of carry-over contamination. During this study, the origin of these false-positive results could not be determined. The source of the target DNA could possibly originate from the water used for research applications. In order to circumvent the use of a large number of amplification cycles associated with nested and hemi-nested PCR assays, PitStop PCR was adapted. Application of this approach delivered increased sensitivity over standard amplification (1 log in seeded distilled water) without the occurrence of false positive results. This assay has also proven useful in PCR inhibiting samples, however, when working with highly inhibiting samples, DNA should be isolated and purified, and the occurrence of negative results should be validated by means of semi-selective culturing.

Although PCR technology could potentially be applied towards *L.pneumohila* detection, the need exist for rapid and selective concentration methods for this pathogen to further improve the sensitivity of the assay. In this study focus was placed on two of these methods: Immunomagnetic separation (IMS) of target bacteria and solid-phase sequence-capture of target DNA. IMS is based on the separation of target organisms from the sample using para-magnetic beads coated with specific antibodies against cell surface antigens. Although positive isolation of viable cells was achieved, some problems were experienced during the study. Reproducibility of the assay was low, and thus the efficacy of capturing was difficult to quantify. This technique is also limited by the commercial availability and quality of target-specific antibody preparations.

Developments in the area of specific DNA isolation by means of solid-phase magnetic particles provided an opportunity with regards to detection of bacterial pathogens, such as *L.pneumophila*, without the need for specific antibodies. The technique is based on the capturing of freeze-thaw released chromosomal DNA by means of hybridization to biotinylated sequence specific probes immobilized on para-magnetic beads via a biotin-strepavidin interaction. Pre-immobilized probes (direct approach) was compared to free probes added prior or just after DNA denaturation; comparison of the two approaches confirmed that the direct approach delivered constantly better results, possibly due to protection of the probe's biotin label by selective binding to strepavidin. Solid-phase sequence capture combined with pitstop PCR obtained a sensitivity of detection of 4 x 10^2 cfu, but as in the case of IMS, the assay was severely affected by inhibitory substances in the sample. Assessment of inhibition indicated that it occurred on the level of capturing rather than post-capturing amplification. These results render the need for more in depth research on the precise mode of inhibition as well as binding kinetics.

In conclusion, PCR, IMS and solid-phase sequence capture have proven to be applicable in the field of *L.pneumohila* detection in environmental and industrial waters. These methods are, however, severely affected by unknown

inhibitory substances present in these waters. Although standard semi-selective culturing of *L.pneumophila* is time-consuming, it could be applied in the confirmation of results achieved by the above-mentioned methods. Further research is however needed to facilitate the use of these methods as industry standards in high sample volume throughput laboratories.

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SUMMARY

Development of PCR-based detection assays for *Legionella pneumophila* in water

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A modified polymerase chain reaction (PitStop PCR) based detection procedure was developed for *Legionella pneumophila* and compared to standard culturing and DNA amplification methodology. PitStop PCR is based on a two step PCR procedure consisting of a total of 30 cycles of amplification of which the first step comprises of 10 cycles followed by product carry-over into a subsequent 20 cycle second step. Using this approach, a ten-fold increase in detection sensitivity could be obtained over standard PCR, without the occurrence of false-positive results usually associated with a large number of amplification cycles. The assay has also proven useful in industrial and environmental samples; however, PCR inhibiting samples could lead to the occurrence of false-negative results. For this reason, two separate target concentration procedures, specific for *L.pneumophila*, were developed: Immunomagnetic separation (IMS) of *L.pneumophila* cells and solid-phase

sequence capture of *L.pneumophila* chromosomal DNA. These techniques proved to be useful in concentration of target cells and DNA respectively, where a peak detection sensitivity of 4×10^2 cells were obtainable; however, as in he case of PCR, these assays displayed low reproducibility and where severely affected by inhibitory water samples. In conclusion, a second procedure, such as standard culturing, should be applied in confirmation of results achieved by PCR-based methods.