UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Evaluation of detection methods for *Legionella* in industrial cooling water systems

Catheleen Bartie

Promotor: Professor LH Nel

A thesis submitted to the Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences of the University of Pretoria, Pretoria, in fulfilment of the Degree of Doctor in Philosophy.

June 2002

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DEDICATION

In memory of my late husband Cecil and with love to Neill, Etienne and Mom

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DECLARATION

This is to certify that this dissertation, submitted for the degree of Doctor of Philosophy at the University of Pretoria, is my own work. It has not been presented before for any degree or examination at any other University.

Catheleen Bartie

ACKNOWLEDGEMENTS

It is with appreciation that the following persons are acknowledged

Professor LH Nel, who supervised this project;

Professor D Rees, the Director of the National Centre for Occupational Health for affording me the opportunity to do this study;

My colleagues and friends in the Immunology/Microbiology Section of the NCOH for their support;

The management and staff of the various industries who provided samples for the study and did the sampling on various occasions;

The Water Research Commission for funding the project;

The members of the Legionella Action Group, for their valuable input and ideas.

A very special word of thanks to Tanusha Soogreem for her support and friendship throughout this study.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

BACKGROUND

Legionella (plural: legionellae) is a type of bacteria that is found primarily in warm water environments. They are present in most natural water environments and are often present in man-made water environments, like cooling towers, humidification, air-conditioning and ventilation systems and other humid environments.

Legionella was first identified in 1976 when a over 200 of veterans attending an American Legionnaires' Convention in Philadelphia, contracted a serious type of pneumonia. Thirty-four people died before the cause of the disease was identified by scientists of the Centres of Disease Control and Prevention. Today, serious outbreaks of the disease are still reported from all over the world. In fact, even more outbreaks are reported now than in the past.

Legionellosis is any form of illness caused by exposure to legionellae and include mainly two types of infections: Legionnaires' disease and Pontiac fever. Legionnaires' disease is a very serious multisystem disease with a potentially fatal pneumonia as the main symptom. Pontiac fever, on the other hand, is a mild disease, with flu-like symptoms, that mostly do not require medication. Many legionellosis cases go unreported because most people are not familiar with the symptoms. Although these diseases can be fatal, they can be treated successfully if treatment with the appropriate antibiotics is started early enough. Many health care professionals are under the false impression that legionellosis is rare, but the disease is actually quite common.

It is difficult to isolate legionellae from water samples and in South Africa, the methods used are not standardised. As a result, the prevalence of legionellosis in the country is not known. A Legionella Action Group has been formed to assist in the standardisation of the laboratory methods. One of their main functions is to create an awareness of the disease in the medical profession to reduce the risk of legionellosis and to assist water treatment companies with the treatment of water distribution systems to decrease the risk of the disease for workers and for the public. This study was undertaken in part to assist the Legionella Action Group with this, very difficult, task.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

TABLE OF CONTENTS

PAGE

1. LITERATURE REVIEW

1.1 Introduction 1

1.2 Classification 4

1.3 Morphology 4

1.4 Cell envelope 5

1.4.1 Cell membrane 6
1.4.2 Peptidoglycan 6
1.4.3 Cell wall 7

1.5 Capsular envelope material 9

1.6 Flagella and fimbriae 9

1.7 Chemical composition 10

1.7.1 Enzymes and toxins 10
1.7.2 Fatty acids 11
1.7.3 Outer membrane proteins 12

1.8 Antigenic composition 13

1.9 Genetic composition 15

1.10 Clinical significance 15

1.10.1 Legionnaires' disease 17
1.10.2 Pontiac fever 17
1.10.3 Incidence 18

1.11 Ecology 19

1.11.1 Non-potable water sources 20
1.11.2 Potable water sources 21
1.11.3 Biofilms 22
1.11.4 Interactions with protozoa 23

1.12 Transmission 24

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1.12.1 Aerosolisation 25
1.12.2 Direct installation 26
1.12.3 Aspiration 26
1.12.4 Ingestion 27
1.12.5 Other potential routes of entry 27

1.13 Prevention 27

1.13.1 Non-potable water systems 27
1.13.2 Potable systems 28

1.14 Disinfection and control 29

1.15 Nutritional requirements 31

1.16 Detection methods 33

1.16.1 Sampling 34
1.16.2 Sample transportation and storage 36
1.16.3 Sample concentration 37
1.16.4 Sample resuspension 39
1.16.5 Sample pretreatment 40
1.16.6 Culture media 40
1.16.7 Standardised culture methods 41
1.16.8 Colony morphology 43
1.16.9 Cysteine dependence 44
1.16.10 Direct immunofluorescence 44
1.16.11 Agglutination tests 45
1.16.12 Biochemical tests 45
1.16.13 Molecular methods 46
1.16.14 Typing methods 48

2. EVALUATION OF DETECTION METHODS USING SEEDED SAMPLES

2.1 Summary 50

2.2 Introduction 51

2.3 Materials and methods 53

2.3.1 Seeding of samples 53
2.3.2 Samples evaluated 53
2.3.3 Sample inoculation 55
2.3.4 Sample concentration 55
2.3.5 Sample resuspension 56
2.3.6 Sample pretreatment 56
2.3.7 Evaluation of culture media 57
2.3.8 Comparison of culture methods 57
2.3.9 Cysteine dependence 57

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

PAGE

2.3.10 Direct immunofluorescence 58
2.3.11 Latex agglutination 58
2.3.12 Reporting and interpretation 58
2.3.13 Calculations 59

2.4 Results and discussion 59

2.4.1 Effect of sample concentration on recovery 59
2.4.2 Comparison of culture media 61
2.4.3 Comparison of culture methods 62

3. EVALUATION OF DETECTION METHODS USING INDUSTRIAL SAMPLES 64

3.1 Summary 64

3.2 Introduction 65

3.3 Materials and Methods 67

3.3.1 Samples evaluated 67
3.3.2 Sample concentration and resuspension 69
3.3.3 Sample screening 69
3.3.4 Sample pretreatment 70
3.3.5 Agar inoculation 70
3.3.6 Cysteine dependence 71
3.3.7 Direct immunofluorescence 71
3.3.8 Latex agglutination 71
3.3.9 Recording and interpretation of results 72
3.3.10 Sample re-incubation with amoebae 72

3.4 Results 72

3.4.1 Screening for legionellae and amoebae 72
3.4.2 Evaluation of culture media 73
3.4.3 Evaluation of pretreatment methods 74
3.4.4 Comparison of detection methods 75
3.4.5 Legionella prevalence 76
3.4.6 Effect or re-incubation on culture outcome 77

3.5 Discussion 77

3.6 Conclusions 81

4. CONCLUDING REMARKS 82

4.1 Introduction 82

4.2 Emerging pathogens 83

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.3 Surveillance 84

4.4 Prevalence 87

4.5 Resource development 89

4.5.1 Improvement of laboratory services 89
4.5.2 Development of training programs 90

4.6 Guidelines for prevention and control 91

4.7 Summary of major results and conclusions 91

4.7.1 Seeded samples 91
4.7.2 Environmental samples 93
4.7.3 Most probable number (MPN) method 93

4.8 Guidelines for development of a standard detection method 95

4.8.1 Definition 95
4.8.2 Principle 95
4.8.3 Quality control of culture media 96
4.8.4 Concentration and resuspension 96
4.8.5 Sample pretreatment 97
4.8.6 Sample dilution 97
4.8.7 Direct immunofluorescence 97
4.8.8 Testing for the presence of amoebae 97
4.8.9 Agar inoculation 98
4.8.10 Incubation 98
4.8.11 Presumptive identification 98
4.8.12 Cysteine dependence 98
4.8.13 Confirmation 98
4.8.14 Expression of results 99

4.9 The way forward 99

LIST OF REFERENCES 102

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF TABLES

PAGE

1. LITERATURE REVIEW

1.1 Currently known Legionella species 2
1.2 Currently known Legionella-like amoebal pathogens 3
1.3 Recent Legionnaires' disease outbreaks 3
1.4 Major fatty acids of the genus Legionella 12
1.5 Major fatty acids of individual Legionella species 13
1.6 Antigenic composition of Legionella species 14
1.7 Genetic composition of Legionella species 16
1.8 Basic supplements in Legionella media 34
1.9 Supplements in Legionella selective media 41

2. EVALUATION OF DETECTION METHODS FOR LEGIONELLA USING SEEDED SAMPLES

2.1 Seeded samples evaluated 53
2.2 Effect of sample concentration on organism recovery 60
2.3 Effect of concentration method on organism recovery 60
2.4 Recovery (%) of *L pneumophila* from seeded samples: comparison of culture media and methods 62

3. EVALUATION OF DETECTION METHODS USING INDUSTRIAL SAMPLES

3.1 Industrial samples evaluated 67
3.2 Comparison of culture media and pretreatment methods for Legionella detection 75
3.3 Comparison of Legionella prevalence 76
3.4 Effect of re-incubation of culture unconfirmed, amoebae positive industrial samples 77

4. CONCLUDING REMARKS

4.1 Prevalence of Legionella in hospitals 87
4.2 Legionellosis cases notified in South Africa 88

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF FIGURES

PAGE

Figure 2.1 General procedure for evaluation of detection methods using seeded samples 54

Figure 3.1 General procedure for evaluation of detection methods using environmental samples 68

Figure 4.1 General procedure for proposed method 90

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

PUBLICATIONS AND PRESENTATIONS EMANATING FROM THIS THESIS

PUBLICATIONS

1. Bartie C (1999). Legionella in South Africa: background, diagnosis, treatment and prevention. *Occup Health SA* 4 (6): 8-13.
2. Bartie C and Soogreem T (2001). Legionellosis. *NCOH Occupational Disease Series Information Booklet* 1 (1): 1-23.
3. Nel LH, Venter SN, Bartie D and Goosen C (2001). Detection methods for Legionella in cooling water systems. *WRC Report* No 827/1/01.
4. Bartie C, Venter SN and Nel LH (2001). Evaluation of detection methods for Legionella species using seeded samples. *Water SA* 27 (4): 523-528.
5. Bartie C, Venter SN and Nel LH (2001). *Chapter 56: Legionella detection from South African cooling water systems. In: Legionella: Proceedings of the 5th Legionella Conference, 2000.* ASM Press, Washington DC.
6. Bartie C, Venter SN and Nel LH (2002). Identification methods for Legionella from environmental samples. *Water Research* (in press).

PRESENTATIONS

1. Identification methods for Legionella in industrial cooling water systems. *Annual microbiologists meeting, Eskom.* (25 July 1996).
2. Identification methods for Legionella. *University of Pretoria.* (1996).
3. Legionella identification from environmental samples. *Second South African Legionella seminar.* (16 February 1998, Johannesburg).
4. Legionella identification from environmental samples: a progress report. *FuturePath '98 Conference.* (3 July 1998, Pretoria).
5. Legionella detection from South African cooling water systems. *5th International Legionella Conference.* (26-29 September 2000, Neu-Ulm, Germany).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.8.11 Presumptive identification

If single colonies are present, test all the characteristic colonies for cysteine dependence. In cases where no single colonies can be distinguished in any of the dilutions, stain a representative smear from each dilution by DFA. If positive, report as presumptive legionellae.

4.8.12 Cysteine dependence

Inoculate single colonies onto BCYE and nutrient- or blood agar and incubate as usual. Colonies that grow on the BCYE but not on the other media are considered cysteine dependent.

4.8.13 Confirmation

Cysteine dependent colonies are confirmed by DFA and/or latex agglutination.

4.8.14 Expression of results

Results are expressed as an estimated number of cfu/ml of original water sample of weight of original solid sample, taking the dilution factor into account. The highest dilutions yielding confirmed legionellae is reported.

4.9 THE WAY FORWARD

4.9.1 Surveillance and notification

South Africa has a well developed disease surveillance network. The prevalence of disease like tuberculosis, HIV/AIDS, malaria and cholera is well known. Unfortunately, the surveillance of other respiratory infections, including Legionnaires' disease and legionellosis has been neglected in the past, with the result that their prevalence is not known. The NCOH has embarked on a program of assistance in the reporting of legionellosis cases to the Gauteng Provincial Gavernment. This program also includes a

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

continued effort to increase the awareness of this disease, not only in medical professionals, but also in workers, management and the general public.

Only one Legionnaires' disease outbreak has been reported in South Africa to date. However, there is always a possibility of outbreaks occurring in the country; in order to recognise such outbreaks and be able to respond timeously and efficiently to these threats, cases have to be reported to the proper officials. This program is designed to assist in this process.

4.9.3 Standardisation of detection methods

There is no standard method for the detection of legionellae from environmental samples in South Africa. This project served to point out some of the problems regarding legionella detection and propose a standard method for development and discussion. This method will be subjected to extensive interlaboratory testing before being standardised and used routinely.

4.9.4 Research

- The presence of legionellae in dental unit water lines

  The NCOH is currently involved in a research project, studying the presence of Legionella organisms in dental unit water lines at the Dental School, University of the Witwatersrand. This project will be expanded during 2002 to study the prevalence of antibodies in students and personnel at the Dental School. Preliminary results have shown a high proportion of dental unit water lines to contain legionellae. The findings from this study will be used to initiate appropriate prevention and control measures and will be of great value to increase the awareness of the risk of legionellosis in these systems.

- Legionella infections in HIV positive mine workers

  Pneumonia is one of the most frequent complications of HIV/AIDS patients. Apparently *Pneumocystis carinii* is the leading cause. The prevalence of *L pneumophila, C pneumoniae, C trachomatis* and *M pneumoniae* in this population is unknown. The reason for this may be the currently used diagnostic methods that make use of the measurement of antibody levels which is of limited value in HIV/AIDS patients due to their compromised immune response.

- Legionella infections in miners with tuberculosis

  Similarly, the high number of mine workers with tuberculosis increases the immunocompromised population in this sector. In order to provide the best primary and occupational health care, these patients should be tested for the presence of antibodies to respiratory pathogens like *C pneumoniae, L pneumophila* and other pathogens. To this effect, a research project is planned by the NCOH for the near future.

- Legionella-like amoebal pathogens (LLAPs)

  Intracellular replication within protozoa plays a major role in the ecology and pathogenesis of Legionella species. The recent emphasis on the presence of pathogenic free-living amoebae in water distribution systems, legionella-like amoebal pathogens and their potentially serious health risks in both the occupational and public health sectors have received much attention in scientific literature. A research project in collaboration with the Water Research Centre at the Tennessee Technological University is in the planning stage. A visit to the Centre has provided excellent information and training into the methodologies to be developed for this work, which will be a first in South Africa.

- Molecular methods

  Polymerase chain reaction (PCR)-based methods have been used fairly successful in detecting legionellae in clinical specimens, but problems remain in environmental samples. Although an effective PCR methods for Legionella has been developed in a recent South African study, the results obtained from using this method for Legionella detection from the samples evaluated in this study did not correlate with those obtained by culture. This was especially true for the samples from the underground areas of the mine (results not shown). Further research is therefore needed on this area of Legionella detection. Molecular typing methods should be developed for use in outbreak situations where it is essential to link isolates from patients and environments.

- Immunomagnetic separation techniques

  Immunomagnetic separation has been shown to have potential as a simple way of extracting bacteria from environmental samples prior to detection by other methods, by workers at the University of Pretoria. Research into this aspect of Legionella detection may prove useful.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Legionnaires' disease was first described after a pneumonia outbreak at an American Legion Convention held in Philadelphia during 1976. In total, 182 delegates were affected and 29 died before workers at the Centres for Disease Control and Prevention (CDC) in Atlanta, GA, isolated the causative organism during January 1977. The organism was placed in the family *Legionellaceae*, genus *Legionella* (Brenner *et al.* 1979), to commemorate the first victims of the disease and the first species was named *pneumophila*, Greek for 'lung loving'. It soon became clear that these organisms were not new: retrospective studies showed that an organism isolated by Tatlock in 1944 and called *Tatlockia micdadei*, actually belonged to the genus Legionella. The organism was subsequently renamed *Legionella micdadei* (strain TATLOCK) (Pasculle 1992). The first strain of *L pneumophila*, (OLDA), was isolated in 1947 from a guinea pig that had previously been inoculated with blood from a patient with 'unknown febrile disease' (Bozeman 1968). Another historical isolate, WIGA, was isolated in 1980 by Bozeman and coworkers and named *L bozemanii*.

The two decades following the discovery of the family Legionellaceae was marked by rapid developments in Legionella detection and identification of numerous new species. In fact, some workers dispute the creation of a single genus containing such a large number of species (Pasculle 1992). Twenty-eight new Legionella species and two Legionella-like amoebal pathogens (LLAPs) (LLAP-1 and LLAP-6) were isolated during the 1980s, mostly from sources in the USA. The 1990s were marked by an increase in Legionella isolation from countries in Europe and Australia with fifteen new Legionella species and LLAP-10 were described. LLAP-1, LLAP-6 and LLAP-10 are now known as *L drozanskii*, *L rowbothamii* and *L fallonii* respectively (Adeleke *et al.* 2001). The addition of these three organisms to the genus now brings the total number of species to 48. *L lytica* was previously known as *Sarcobium lyticum* while the TATLOCK strain of *L micdadei* is referred to as *L pittsburghensis* in some of the older literature. The currently known Legionella species are listed in Table 1.1 and LLAPs in Table 1.2.

Table 1.1 Currently known Legionella species

ORGANISM	SGs	AUTHOR	YEAR	SOURCE	PATHOGEN
L adelaidensis	1	Benson et al.	1991	Cooling water (Adelaide Australia)	Unknown
L anisa	1	Gorman et al.	1985	Faucet (Chicago), tap water (LA)	Yes
L beliardensis	1	*	2002	Water, France	Unknown
L birminghamensis	1	Wilkinson et al.	1987	Lung biopsy (Alabama)	Yes
L bozemanii	2	Brenner et al.	1980	Lung aspirate (Toronto)	Yes
L brunensis	1	Wilkinson et al.	1989	Cooling tower water (Czechoslovakia)	No
L cherrii	1	Brenner et al.	1985	Thermally altered water (Minnesota)	Yes
L cincinnatiensis	1	Thacker et al.	1988	Lung tissue (Cincinnatti)	Yes
L donaldsonii*	*	*	*	*	*
L drozanskii (LLAP-1)	1	Adeleke et al.	2001	Tank of well water (Leeds 1981)	Yes
L dumoffii	1	Brenner et al.	1980	Lung tissue	Yes
L erythra	2	Brenner et al.	1985	Cooling tower water (Seattle)	No
L fairfieldensis	1	Thacker et al.	1991	Cooling tower water (Fairfield Australia)	Unknown
L fallonii (LLAP-10)	1	Adeleke et al.	2001	Ship airconditioner (1994)	Yes
L feeleii	2	Herwaldt et al.	1984	Grinding machine coolant fluid	Yes
L geestiana	1	Dennis et al.	1993	Hot water tap, office building (London)	Unknown
L gormanii	1	Morris et al.	1980	Bronchial wash of pneumonia patient	Yes
L gratiana	1	Bornstein et al.	1991	Thermal spa water (France)	No
L gresilensis	1	*	2002	Water, France	Unknown
L hackeliae	2	Brenner et al.	1985	Bronchial biopsy (Ann Arbour)	Yes
L israelensis	1	Bercovier et al.	1985	Water (Israel)	No
L jamestowniensis	1	Brenner et al.	1985	Wet soil (New York)	No
L jordanis	1	Cherry et al.	1982	Water and sewage (Israel)	Yes
L lansingensis	1	Thacker et al.	1994	Bronchial washing, leukemia patient	Yes
L londiniensis	1	Dennis et al.	1993	Office building cooling tower (London)	Unknown
L longbeacheae	2	McKinney et al.	1982	Human lung (Longbeach Australia)	Yes
L lytica	1	Hookey et al.	1996	Previously Sarcobium lyticum	Yes
L maceachernii	1	Brenner et al.	1985	Water (Phoenix)	Yes
L micdadei	1	Hebert et al.	1980	Human blood via yolk sac	Yes
L moravica	1	Wilkinson et al.	1989	Cooling tower water (Czechoslovakia)	No
L nautarum	1	Dennis et al.	1993	Hot water tap (London)	Unknown
L oakridgensis	1	Orrison et al.	1983	Cooling tower water (Pennsylvania)	Yes
L parisiensis	1	Brenner et al.	1985	Cooling tower water (Paris)	Yes
L pittsburghensis	1	Pasculle et al.	1980	Synonym for L micdadei, strain TATLOCK	Yes
L pneumophila	15	Brenner et al.	1979	Water (Pennsylvania)	Yes
L quateirensis	1	Dennis et al.	1993	Shower in hotel bathroom (Portugal)	Unknown
L quinlivanii	2	Benson et al.	1990	Water in bus airconditioner (Australia)	No
L rowbothamii (LLAP-6)	1	Adeleke et al.	2001	Water and sludge, industrial liquefier	Yes
L rubrilucens	1	Brenner et al.	1985	Tap water (Los Angeles)	Yes
L sainthelensi	2	Campbell et al.	1984	Spring water (Washington)	Yes
L santicrucis	1	Brenner et al.	1985	Tap water (Virgin Islands)	No
L shakespeari	1	Verma et al.	1992	Cooling tower water (England)	Unknown
L spiritensis	1	Brenner et al.	1985	Lake water (Washington)	No
L steigerwaltii	1	Brenner et al.	1985	Tap water (Virgin Islands)	No
L taurinensis	1	Lo Presti et al.	1999	Water, hospital humidifier (Italy)	Unknown
L tusconensis	1	Thacker et al.	1990	Pleural fluid, transplant patient (Arizona)	Yes
L wadsworthii	1	Edelstein et al.	1983	Sputum	Yes
L waltersii	1	Benson et al.	1996	Potable water system (Australia)	Unknown
L worsleiensis	1	Dennis et al.	1993	Industrial cooling water (England)	Unknown

*Sources: ATCC website, NCTC website, L donaldsonii mentioned once only in the literature (Adeleke 1996). Information regarding pathogenesis: De Gheldre et al. (2001); * no information available at time of print.*

The first Legionella-like amoebal pathogen (LLAP) was isolated from soil in Poland in 1954 and was named *Sarcobium lyticum* (Adeleke 1996). The next isolation of an LLAP was in England more than 20 years later. Since then, LLAPs have been isolated from various sources and found to infect a variety of amoebae (Table 1.2). Except for *S lyticum*, LLAP-1, LLAP-3 and LLAP-5, all LLAPs were originally isolated

Table 1.2 Currently known Legionella-like amoebal pathogens (LLAPs)

STRAIN	HOSTS	YEAR	ORIGINAL SOURCE	PATHOGENIC
Sarcobium lyticum	A polyphaga H vermiformis	1954	Soil	Yes
LLAP-1	A polyphaga	1981	Tank of portable water well	Yes
LLAP-2	A polyphaga H vermiformis	1986	Garage steam cleaning pit	Yes
LLAP-3	A polyphaga	1986	Sputum from pneumonia patient	Yes
LLAP-4	A polyphaga	1986	Hospital whirlpool bath	Yes
LLAP-5	A polyphaga	1988	Nursing home plant spray	Yes
LLAP-6	A polyphaga H vermiformis	1988	Factory liquefier tower	Yes
LLAP-7	A polyphaga H vermiformis	1991	Hotel whirlpool spa	Yes
LLAP-8	H vermiformis	1990	Hospital shower	Yes
LLAP-9	A polyphaga H vermiformis	1992	Factory cooling tower	Yes
LLAP-10	A polyphaga	1994	Ship air-conditioning system	Yes
LLAP-11	A polyphaga	1993	Furnace cooling system	Yes
LLAP-12	A polyphaga	1994	Furnace cooling system	Yes

Adapted from Adeleke 1996

from sources associated with confirmed cases or outbreaks of Legionnaires' disease (Adeleke 1996).

Legionnaires' disease outbreaks have been documented from as early as 1957 in Philadelphia (Terranova *et al.* 1978), Wadsworth (Haley *et al.* 1979), Washington DC (England *et al.* 1981) and Minnesota (Osterholm *et al.* 1983). The first outbreak of Pontiac fever was reported in Pontiac, Michigan, in 1968 (Glick *et al.* 1978). Today serious Legionnaires' disease outbreaks are still regularly reported (Table 1.3).

Table 1.3 Recent Legionnaires' disease outbreaks (1999-2001)

DATE	SITE AND SOURCE	CASES	DEATHS
March 1999	Flower show, Netherlands: spa bath	242	28
June 1999	Hotel, Belgium: hot water system	4	1
February 2000	Hotel, South Wales: food display humidifier	5	3
March 2000	Office building, Melbourne: cooling tower	5	0
April 2000	Aquarium, Melbourne: cooling towers	110	2
June 2000	Public bath, Japan: hot water system	14	1
June 2000	Hospital, Maryland: hot water system	5	3
March 2001	Office building, Melbourne: cooling tower	5	2
March-April 2001	Ford plant, Ohio: burst pipe	6	2
March-June 2001	Hospital, Melbourne: source unknown	7	2
May-June 2001	Central London building: hot water system	3	0
February-June 2001	Las Vegas: source unknown	3	0
July 2001	Hospital, Paris: hot water system	12	6
July 2001	Murcia, Spain: cooling towers	315	2
August 2001	Hospital, Spain: hot water system	18	3
October 2001	Murcia Spain	U	U
October 2001	Melbourne Australia	U	U

Sources: http://www.q-net.net.au/~legion/Legionnaires_Disease_2001.html,
http://www.hcinfo.com/outbreaks-news.htm#0107Spain

1.2 CLASSIFICATION

The family Legionellaceae contains one genus (Legionella) and 46 species, together consisting of 58 serogroups (SGs) (Table 1.1). *L pneumophila* contains 15, and *L bozemanii, L feeleii, L hackeliae, L sainthelensi* and *L longbeacheae* each contain two serogroups. Legionellae are distantly related to the purple sulfur bacteria and their non-photosynthetic relatives. Hybridisation studies demonstrated that the legionellae were closely related to common members of the Enterobacteriaceae and to Pseudomonas, all of which are also related at the ribosomal level to the purple photosynthetic bacteria. The guanine plus cytosine (G+C) content of the DNA of Legionella species ranges between 38 and 52 mol% (Brenner *et al.* 1985). The different species in the genus Legionella were created on the basis of their DNA relatedness. The various Legionella species share up to 67% DNA relatedness at optimal hybridization temperature while strains within a given species have 70% or greater relatedness and <5% deviation in related sequences.

1.3 MORPHOLOGY

Legionella species are thin, obligatory aerobic, faintly staining gram-negative bacilli that are non-spore forming and contain no capsules. Although legionellae are generally non acid-fast, partial acid-fastness has been described in *L micdadei* and one strain of *L pneumophila* SG 6. All legionellae except *L oakridgensis* are motile (Brenner 1986, Winn 1984).

Legionellae are typically between 0.3-0.9 μm wide and 2-20 μm long. However, coccobacillary forms measuring 1-2 μm may be observed in clinical specimens and under conditions of iron deprivation (Ristroph *et al.* 1981) whereas long, filamentous organisms may result from growth on certain culture media (Yu 1990). The organisms often appear tapered at one or both ends but without the needle-shaped appearance of Fusobacterium species (Winn *et al.* 1984).

Although physically hardy and heat tolerant in nature, legionellae require special laboratory media and techniques for isolation (Brenner *et al.* 1984). Under laboratory conditions, they can survive in distilled water for long periods of time (up to 139 days) and in tap water for more than a year when kept at room temperature. Although they grow and multiply in temperatures between 25-42°C, with an optimum of 35-37°C, they cannot survive for long periods at temperatures above 50°C.

Legionella-like amoebal pathogens are nonculturable, protozoonotic, gram-negative bacilli. They were named LLAPs because of their ability to infect amoebae and replicate

intracellularly, in the same way that legionellae infect protozoa and human macrophages. They differ from legionellae in that they cannot be cultured on laboratory media (Adeleke 1996).

1.4 CELL ENVELOPE

The typical gram-negative cell envelope is a multi-layered structure composed of two trilaminar membranes, the outer membrane (cell wall) situated outside a thin peptidoglycan layer which is separated from the cytoplasmic (inner) membrane by a periplasmic space. The cell envelope contains approximately 10-20% peptidoglycan and polysaccharides, proteins and a glycolipid known as lipopolysaccharide (LPS). Toxins and enzymes are located in the periplasmic space and are present in concentrations sufficient to assist in the destruction of substances that may harm the organism (Craeger *et al.* 1990).

The cell envelope of Legionella species is similar to that of other gram-negative bacteria consisting of two trilaminar membranes, each 75Å wide (Chandler *et al.* 1979, Neblett *et al.* 1979). Between the two membranes a fine peptidoglycan layer is visible by electron microscopy after plasmolysis or papain treatment. The two layers of the cell envelope can be separated by sucrose density gradient centrifugation but not by treatment with detergents (Gabay *et al.* 1985).

The outer and cytoplasmic membranes of legonellae have very distinct protein profiles, but their enzymatic activity and 2-keto-3-deoxyoctonate (KDO) content is similar (Winn 1984). *L micdadei* contains an unusual, electron-dense layer between the trilaminar membranes, visible after cultivation in eggs or cell culture, but not after culture on agar media. The significance of this layer is unknown, but may be related to the partial acid fastness of the organism. Complex surface structures, resembling capsules and containing mainly polysaccharides, have been demonstrated in *L pneumophila*. However, the superficial antigens of these structures are different from those normally found in capsules; they migrate in the opposite direction from most polysaccharides when subjected to counter-current electrophoresis. In addition, membrane-bound granules that resemble beta-3-hydroxylbutarate granules have been demonstrated by freeze-fracture methods (Winn 1984).

1.4.1 Cell membrane

The main function of the cell membrane is to regulate the movement of materials into and out of the cell by various transport mechanisms. The membrane also synthesises cell wall (outer membrane) components, assists with DNA replication, secretes proteins, carries on respiration and captures energy in ATP. Furthermore, it contains the bases of flagella and responds to chemical substances in the environment. The typical cell membrane consists of about 70% protein and 30% phospholipid by weight and contains small amounts of carbohydrate. Two forms of cell membrane (CM-1 and CM-2) were detected in *L pneumophila* (Hindahl and Iglewski 1984). The CM-1 fraction was the purest form of cell membrane while CM-2 was consisted mainly of cell membrane associated with small amounts of peptidoglycan.

The phospholipids in the membrane form a bilayer and are in a fluid state with protein molecules scattered among, and embedded in, the lipid molecules in the membrane, forming a mosaic pattern. Some of the protein molecules extend through the entire membrane and act as carriers or form pores or channels through which materials enter and leave the cell. Others are embedded in or loosely attached to the inner or outer surface of the membrane. Proteins on the inner surface are usually enzymes; those on the outside include proteins that make the cell identifiable as a particular organism (Craeger *et al.* 1992).The phospholipid composition of legionellae species is different from that of other gram-negative bacteria in that they possess an unusually high content of phosphatidylcholine. However, there are no marked differences in the phospholipid composition of the different Legionella species.

1.4.2 Peptidoglycan

Peptidoglycan, a macromolecule unique to prokaryotes, is synthesised in the cell cytoplasm and comprises 10-20% of the cell envelope. It surrounds the cell membrane, providing rigidity, shape and mechanical protection to the cell. Peptidoglycan consists of a glycan (polysaccharide) backbone containing mainly N-acetyl-muramic acid (murNAc) and N-acetyl-glucosamine (gluNAc), which can be cleaved by the bacteriolytic enzyme lysozyme. Peptide side-chains containing amino acids and in some instances diaminopimelic acid are attached to the backbone and are cross-linked by peptide bridges that vary in structure among bacterial species. The peptidoglycan layer is attached to the outer membrane by a small, 75 kilodalton (kDa) lipoprotein, which assists in anchoring the

peptidoglycan to the outer membrane and in maintaining the structure and organisation of the cell. The peptidoglycan layer of a gram-negative cell differs from that of a gram-positive cell in that it is thinner and more flexible although sufficiently strong to protect the cell against osmotic lysis.

The *L pneumophila* peptidoglycan layer was demonstrated for the first time in 1979 by electron microscopy, following plasmolysis or papain treatment (Chandler *et al.* 1979, Neblett *et al.* 1979). It is similar in composition to that found in most gram-negative bacteria, except for the absence of diaminopimelic acid in most species. Diaminopimelic acid has been demonstrated in some isolates of *L pneumophila* SG1. The peptidoglycan of *L pneumophila* is composed of muramic acid, glucosamine, glutamic acid, alanine and meso-diaminopimelic acid with a molar ratio of 0.8:0.8:1.1:1.7:1, sensitive to lysozyme hydrolysis and insensitive to alkali dissolution (Amano and Williams 1983).

1.4.3 Cell wall (outer membrane)

The cell wall, situated outside the peptidoglycan layer, is a porous structure containing mainly proteins and lipopolysaccharide that performs two main functions. It maintains the shape of the cell and prevents it from bursting when fluids flow into it by osmosis. The phospholipids in the cell wall are similar to those in the cell membrane, being primarily phosphatidyl ethanolamine and phosphatidyl glycerol. The remainder of the cell wall comprises proteins.

Lipopolysaccharide (LPS) or endotoxin is a complex glycolipid present in the outer membrane of all gram-negative bacteria. LPS is essential for bacterial growth and viability. It contributes to the low membrane permeability and participates in physiological functions of the membrane, increases the resistance of the cell to hydrophobic agents and is the primary target for the attack of antibacterial drugs and proteins such as components of the host's immune response (Wiese 1999). LPS consists of three distinct, covalently bound regions: lipid A, core polysaccharide and O-specific side chains.

Lipid A, the biologically active part of the LPS molecule in which the toxicity of the LPS resides, is embedded in the outer part of the cell wall. It consists of a carbohydrate backbone of alternating pyrophosphate units and glucosamine and resembles a glycerol-based phospholipid but is larger and more rigid. Long fatty acid side chains, usually containing 10-18 carbons, are attached to the backbone. The core polysaccharide chain contains mainly two sugars, 2-keto-3-deoxyoctulosonic acid (KDO) and heptose and is attached to the inner portion of the carbohydrate backbone via the KDO. Other sugars usually present in the core polysaccharide are glucose, glucosamine, galactose, rhamnose

and mannose (Sonesson *et al.* 1989, Petitjean *et al.* 1990). O-specific side chains are attached to the terminal sugar of the core polysaccharide and extend outward into the medium surrounding the cell. They consist of variable numbers (usually 10-50) of repeating oligosaccharide units of 3-5, often very unusual, sugars each. The O-specific side chains enhance the toxicity of the cell by keeping the hydrophobic lipid A portion water-soluble (Rietschel *et al.* 1985, Wiese *et al.* 1999). The major serologic determinants of most gram-negative bacteria reside in the O-specific side chains; organisms lacking these chains (rough or 'R' type) are less virulent than their smooth ('O' type) counterparts.

The LPS of Legionella species has a complex, unusual structure and chemical features that may account for its importance as a bacterial virulence factor (Gabay *et al.* 1985, Otten *et al.* 1986, Sonesson *et al.* 1989, Zähringer *et al.* 1995, Yu 1990). All the legionellae tested to date, except *L longbeacheae* and *L dumoffii*, produce a smooth form of LPS (Sonesson *et al.* 1994). Carbohydrates represent 10-20% of the total weight of the *L pneumophila* LPS; 1-13% of the carbohydrate backbone consists of KDO (Ciecielski *et al.* 1986).

The carbohydrate backbone of the Legionella lipid A is antigenically related to that of Enterobacteriaceae but possesses some unique structural features (Ciecielski *et al.* 1986). It consists of glucosamine, quinovosamine and glycerol, in the molar ratio 3.9:1.0:3.4 with glycerol as a phosphorylated moiety and contains phosphatidylcholine (Lambert *et al.* 1989). The major constituent of *L longbeacheae, L bozemanii, L israelensis, L maceachernii* and *L micdadei* lipid A is 2,3-dideoxy-2,3-diamino-d-glucose. *L israelensis* contains a substantial amount of d-glucosamine while *L bozemanii* and *L longbeacheae* SG 1 lipid A contains d-glucosamine and glycerol as minor constituents of the backbone structure (Sonesson *et al.* 1989)

The core polysaccharide of *L pneumophila* contains rhamnose, mannose, glucose, quinovosamine, glucosamine and KDO in the molar proportion 1.6:1.8:1.0:1.5:4.1:2.7. Heptose is absent and glucose is mainly phosphorylated (Sonesson *et al.* 1989). The outer core polysaccharide of *L pneumophila* exhibits hydrophobic properties due to the presence of N- and O-acetyl groups and 6-deoxy sugars (Zähringer *et al.* 1995). The inner core contains KDO but lacks heptose and phosphate groups. Sonesson's group (1994) demonstrated the presence of d-glucosamine, d-mannose, d-glucose, l-rhamnose, d-glycero-d-manno-heptose, l-glycero-d-manno-heptose, KDO and glycerol in the core polysaccharides of *L bozemanii* and *L longbeacheae*. In their study, the core polysaccharide from *L longbeacheae* appeared shorter, but composed of the same sugars as *L bozemanii*, except for the absence of l-fucosamine. Glycerol- and glucosamine

phosphate was present in both organisms and *L longbeacheae* contained glucose phosphate as well. The d-quinovosamine and l-fucosamine constituted 80mol% of the *L bozemanii* core polysaccharide.

The core polysaccharides of *L maceachernii and L micdadei* both contain mainly l-rhamnose, l-fucose, d-mannose, d-glucose, l-fucosamine, d-glucosamine, KDO and the rare octose yeersiniose A. The composition of the *L israelensis* core is simpler and consists of mainly l-rhamnose and 3-amino-3,6-dideoxy-D-mannose. In *L israelensis* and *L micdadei* the 2-keto-octonic acid is linked to the KDO (Sonesson *et al.* 1994). Phosphorylated sugars have been demonstrated in *L israelensis, L micdadei* and *L maceachernii*, whereas ethanolamine is only present in *L maceachernii*. This group also demonstrated that *L micdadei* contains a higher proportion of the low molecular weight constituents than *L maceachernii* and *L israelensis*.

The O-chain of *L pneumophila* SG 1 constitutes a homopolymer of an unusual sugar, legionaminic acid, of which approximately 10-75 residues are present (Zähringer *et al.* 1995, Knirel *et al.* 1996). The lack of free hydroxyl groups of this homopolymer renders the cell surface highly hydrophobic, thereby supporting adherence to the membranes of target cells. Epidemiologic studies have shown an epitope situated in the environment of the *L pneumophila* SG 1 legionaminic acid to be associated with virulence (Zähringer *et al.* 1995). The O-chain is linked to position 3 of the terminal rhamnosyl group that, together with the occurrence of the reducing KDO residue in multiple forms, contributes to the heterogeneity of the core oligosaccharide. This highly O-acetylated core oligosaccharide is unique to legionellae (Knirel *et al.* 1996).

1.5 CAPSULAR-LIKE ENVELOPE MATERIAL

Legionella species do not contain capsules but complex surface structures, resembling capsules and containing mainly polysaccharides, have been demonstrated in *L pneumophila, L micdadei* and *L dumoffii* (Hébert *et al.* 1984). The capsular-like envelope contains approximately 35% carbohydrate, 2.6% protein, 1.8% phospholipid and 1% KDO and has been a variable finding in more recent studies (Yu 1990).

1.6 FLAGELLA AND FIMBRIAE

Flagella (H-antigens) are long, hollow, helical filaments, usually several times the length of the cell and 10-20nm in diameter that are involved in cell movement. The number and location of flagella are distinctive for each genus. Fimbriae (pili) are straight filaments

composed of protein subunits called pilin, are thinner and shorter than flagella, extend out from the surface of the cell and are not associated with cell movement. They are present only on gram-negative cells and are an example of a class of surface structures termed adhesins that allow attachment of bacterial cells to other cell surfaces. As such they are extremely important for bacterial survival in an animal host. Most fimbriae are lectins, able to bind to specific sugar residues on glycoproteins and other surface components on host cells.

Most Legionella species and serogroups possess single polar flagellae and fimbriae on primary isolation (Hébert 1981, Yu 1990). The *L pneumophila* flagellum is composed of one major subunit, the FlaA protein (Dietrich *et al.* 2001). Recent studies have indicated that expression of this flagellum is temperature regulated and influenced by the growth phase of the organism, the viscosity and the osmolarity of the growth medium and by amino acids (Heuner *et al.* 1999). Flagellated legionellae are usually short forms (Chandler *et al.* 1980) whereas dividing organisms are seldom flagellated.

Fimbriae, approximately 10-11 nm in diameter and coiled and bent at irregular intervals, similar to the fimbriae found on *Pseudomonas aeroginosa*, have been demonstrated on *L pneumophila.* The presence and expression of a type IV pilin gene may provide an advantage for colonisation of lung tissues during infections and invasion of amoebae in the environment (Stone and Abu Kwaik 1998).

1.7 CHEMICAL COMPOSITION

1.7.1 Enzymes and toxins

Legionellae produce a number of enzymes and extracellular products (potential toxins). These products are usually linked to virulence although their exact role in pathogenesis and tissue damage is still not clear, especially since some of these products are produced by avirulent strains. The products produced by legionellae include hemolysins, proteases, esterases, phosphatases, aminopeptidases and endonucleases (Yu 1990). Cianciotto (2000) reported the presence of acid phosphatase, zinc-metalloprotease, monoacylglycerol lipase, phospholipase A, lysophospholipase A, RNAse and pNPCC hydrolase in the type II *pilD*-dependent exoproteins of legionellae.

Under natural conditions aminopeptidases are necessary for the degradation of algal extracellular products in aquatic environments and are known to split the amino acids used as carbon and energy sources off from appropriate compounds. In legionellae

they may be involved in virulence together with esterases through their ability to degrade peptides and proteins of the infected host.

Phosphatases are important bacterial virulence factors. They are believed to be involved in pathogenesis of several intracellular bacteria, including *L micdadei*. Although *L pneumophila* possesses phosphatase activity, its exact role in pathogenesis is still unclear. However, *L pneumophila* produces an organic pyrophosphatase during intracellular survival but acid phosphatase is apparently not essential for intracellular infection (Aragon *et al.* 2000, Inglis *et al.* 2000). *L pneumophila* has been reported to secrete phospholipase A but not phospholipase C (Cianciotto *et al.* 2000).

Low catalase activity has been reported in *L pneumophila, L wadsworthii, L oakridgensis* and *L gormanii* whereas *L jordanis, L longbeacheae, L micdadei* and *L bozemanii* possess high catalase activity. Peroxidase activity has been demonstrated in *L pneumophila, L gormanii* and *L dumoffii* (Pine *et al.* 1984). *L pneumophila* contains two enzymes (KatA and KatB) with catalytic and peroxidatic activity (Bandyopadhyay and Steinman 2000). KatA is induced during exponential growth and is the predominant peroxidase in the stationary phase. When KatA is inactivated, the sensitivity of *L pneumophila* to exogenous hydrogen peroxidase is increased and its virulence to macrophages of the THP-1 cell line is decreased. KatB has similar characteristics, except that its inactivation had no effect on the stationary phase in this study.

Superoxide dismutase (SOD) is present in *L pneumophila, L wadsworthii, L oakridgensis, L gormanii, L jordanis, L longbeacheae, L micdadei* and *L bozemanii* at levels ranging between 8.2 and 30.5 U/mg protein. *L pneumophila* contains three types of SOD: iron-containing (Fe-SOD), iron,zinc-containing (Fe,Zn-SOD) and copper,zinc-containing (Cu,Zn-SOD). Cu,Zn-SOD is not often present in bacteria but is widespread in eukaryotes. St. John and Steinman (1996) reported an increase in the activity of Cu,Zn-SOD during the transition from the expoenetial to the stationary phase of *L pneumophila* growth, suggesting that this product probably contributes to the pathogenecity of the organism but is not essential for intracellular survival or macrophage killing.

1.7.2 Fatty acids

Bacterial cells usually contain fatty acids with 16-18 carbons that exist in saturated and monounsaturated forms. Small amounts of cyclopropane and branched fatty acids, the latter containing large amounts of ubiquinones with more than 10 isoprenes in the side chain, are often encountered. These fatty acids are relatively uncommon in gram-negative bacteria except in *Thermus aquaticus* and a thermophilic

flavobacterium but are common in gram-positive bacteria like Listeria, Propionibacterium, Bacillus and Staphylococcus species (Brenner 1986, Ehret *et al.* 1986, Moss *et al.* 1977).

The fatty acid composition of Legionella species has been studied extensively. The cell walls of Legionella species contain distinctive fatty acids, 80-90% of which are branched-chain, containing 14-17 carbons. Lambert and coworkers (1989) found only one type strain of *L erythra* that did not contain such a high percentage of branched-chain fatty acids. Trace amounts (<0.5-5%) of ester-linked hydroxy fatty acids are present in about 50% of Legionella species. The predominant fatty acids found in legionellae are C^{i}16:0, C^{a}15:0 and C^{a}17:0 (Ehret *et al.* 1986, Lambert *et al.* 1989). Ubiquinones Q11 and Q12 are major components in *L pneumophila* and Q13 is present in small quantities (Brenner *et al.* 1986, Ehret *et al.* 1986). The most recent findings regarding the fatty acid composition of Legionella species are summarised in Tables 1.4 and 1.5.

Table 1.4 Major fatty acids of the genus Legionella

MET-B	(%)	UNSAT	(%)	SAT	(%)	SAT-OH	(%)	BC-OH	(%)	CYCLO	(%)	UNSAT-OH	(%)
a11:0	(4)	a15:1A	(4)	14:0	(86)	10:0 3OH	(2)	i14:0 3OH	(58)	$_{cyc}$17:0	(77)	16:0 2OH	(5)
i12:0	(2)	a17:1A	(1)	15:0	(99)	11:0 3OH	(1)	i15:0 3OH	(1)				
i13:0	(4)	16:1H	(57)	16:0	(100)	12:0 3OH	(1)	i16:0 3OH	(24)				
a13:0	(10)	15:1$^{(n-6)}$	(100)	17:0	(93)	15:0 2OH	(16)						
i14:0	(100)	16:1$^{(n-5)}$	(51)	18:0	(79)	15:0 3OH	(8)						
i15:0	(84)	16:1$^{(n-7)}$	(100)	19:0	(54)								
a15:0	(100)	16:1$^{(n-11)}$	(3)	20:0	(74)								
i16:0	(100)	17:1$^{(n-8)}$	(4)										
16:0$^{10\text{-MET}}$	(1)	17:1$^{(n-9)}$	(16)										
i17:0	(73)	a17:1$^{(n-9)}$	(42)										
a17:0	(100)												
i18:0	(53)												
i19:0	(13)												
a19:0	(24)												
i20:0	(8)												

MET-B: methyl branched; UNSAT: unsaturated; SAT: saturated; SAT-OH: saturated hydroxy; BC-OH: branched chain hydroxy; CYCLO: cyclopropoane; UNSAT-OH: unsaturated hydroxy; (%): percentage of Legionella species that contain this fatty acid; Information adapted from Diogo et al. 1999.

1.4.4 Outer membrane proteins (OMPs)

Associated with the peptidoglycan layer, *L pneumophila* contains a major outer membrane protein (MOMP) that acts like a porin by forming ion-permeable channels in contact with lipid membranes (Gabay *et al.* 1985, Hindahl *et al.* 1986, Yu 1990). This MOMP is similar to the *E coli* K-12 OmpF, OmpD and LamB porins and can be partially dissociated from the peptidoglycan layer in the presence of high salt concentrations (Hindahl *et al.* 1986). The MOMP is similar in all Legionella species. The major protein in the *L pneumophila* MOMP has a molecular weight of 28kDa and is exposed on the cell surface (Kniros *et al.* 1999).

It has been suggested that the Legionella MOMP is composed of 28kDa and 31kDa subunits, crosslinked by interchain disulfide bonds. It is covalently bound to the underlying peptidoglycan via the 31kDa subunit (Butler *et al.* 1990, Hoffman *et al.* 1992a). Further studies however revealed that the 31kDa subunit is in fact a 28kDa subunit containing a bound fragment of peptidoglycan (Hoffman *et al.* 1992a). Butler et al (1990) demonstrated that the banding patterns ranging between 31-45kDa and between 55-60kDa resolved as a single 31kDa protein when the material was treated with N-acetylmuramidase.

Table 1.5 Major fatty acids in individual Legionella species

UNKNOWN	METHYL BRANCHED				UNSATURATED	
	15:0 (%)	i16:0 (%)	a17:0 (%)	i14:0 (%)	$16:1^{(n-7)}$(%)	$16:1^{(n-11)}$(%)
cincinnatiensis	anisa (24)	cherrii (30)	lansingensis(34)	waltersii (U)	feeleii* (24)	adelaidensis (27)
fallonii	birminghamensis(32)	erythra (30)	londiniensis (22)		geestiana (33)	
lytica	bozemanii (26)	fairfieldensis (38)			moravica (32)	
rowbothamii	brunensis (37)	gratiana (26)			quateirensis (32)	
taurinensis	dumoffii (37)	longbeacheae(23)			sainthelensii (29)	
	feeleii* (25)	oakridgensis (25)			worsleiensis (33)	
	gormanii (25)	pneumophila (33)				
	hackeliae (36)	rubrilucens (30)				
	israelensis (38)	santicrucis (27)				
	jamestowniensis(39)	shakespearii (37)				
	jordanis (54)	spiritensis (30)				
	maceachernii (32)					
	micdadei (39)					
	nautarum (38)					
	parisiensis (28)					
	pittsburghensis(39)					
	quinlivanii (26)					
	steigerwaltii (27)					
	tusconensis (35)					
	wadsworthii (48)					

*Adeleke et al. 2001, Diogo et al. 2001, Lo Presti et al. 1999, Woods et al. 1988. *Diogo et al. reported differences in fatty acid contents of L feeleii species tested. (%):percentage of total fatty acids of the particular species*

1.5 ANTIGENIC COMPOSITION

Legionella antigens are high molecular weight complexes with the chemical properties of a lipopolysaccharide (endotoxin) on the cell surface. Serogroup-spcecific antigens are high molecular weight, protein-carbohydrate-lipid complexes containing the major fatty acids and enzymes like hemolysins, proteases, esterases, phosphatases, amino-peptidases and endonucleases. The majority of these antigens are situated in the O-side chain of the LPS, contain small amounts of KDO, consist of lipid A and resemble the endotoxin of other gram negative bacteria like *S minnesota, C burnetti* and *C psittaci* (Ciecielski *et al.* 1986).

Table 1.6 Antigenic composition of Legionella species

MOL WT	CHARACTERISTICS	REFERENCES
11kDa	Species-specific for *L pneumophila*	Sampson et al. 1986
19kDa	Similar to the PplA in *E coli*. SG-specific for *L pneumophila* SGs 1-8 but not identical in all SGs. Exhibits sequences homologous to *E coli* and *H influenza* LPS. *L pneumophila* SG 1 LPS is tightly bound to this protein. Sensitive to formalin.	Ott et al. 1991 Hindahl et al. 1987 Boissinot et al. 1987 Sampson et al. 1986
24kDa	Mip (macrophage infectivity potentiator), surface antigen in *L pneumophila*. Prokaryotic homolog of FK506 protein needed for optimal infection of macrophages. Required for optimal infection of human macrophages but not for extracellular growth. Other legionellae express a 24-30kDa Mip-like protein. Mip-like analogs have also been described in other prokaryotes for example Chlamydia, Neisseria and Pseudomonas species. It is believed that Mip-related proteins may constitute a family of prokaryotic proteins.	Engleberg et al. 1993 Dumais-Pope et al. 1993 Hurley et al. 1993 Cianciotto et al. 1992 Hacker et al. 1993
25kDa	Adhesive molecule for host cells and plays a major role in Legionella virulence. Species-specific.	Kniros et al. 1999 Sampson et al. 1986
28kDa	Some workers regard this protein as the Legionella MOMP. It is covalently bound to the underlying peptidoglycan via a 31kDa subunit and is exosed on the cell surface.	
29kDa	Generally regarded as the Legionella MOMP. All legionellae have the same MOMP except for a few species with a 46-48kDa band. Present *in L pneumophila* SGs 1-8 but not identical among serogroups. Major component of the *L pneumophila* serogroup-specific antigen and is heat-stable. Only partly exposed on the cell surface; exposure can be increased by treatment with the detergent EDTA.	Boissinot et al. 1987 Gosting et al. 1984 Sampson et al. 1986 Ehret et al. 1985 Hindahl et al. 1986 Yu 1990 Ciecielski et al. 1986
30kDa	The molecular weight of the MOMP falls between 24-30kDa. Legionella species other than *L pneumophila* possess a Mip-like protein of 24-30kDa.	
31kDa	Part of MOMP, binding the 28kDa subunit to the peptidoglycan.	Butler et al. 1990
43kDa	Genus-specific.	
44kDa	Present in some legionellae. Most prominent 2-4 hours after infection of various cell lines. Synthesis not dependent on protein synthesis. Also known as 'Legionella intracellular growth antigen' (LIGA) since it is found exclusively in intracellularly grown *L pneumophila*.	Susa et al. 1996
45kDa	Present in *L pneumophila* SGs 1-8 but not identical in all serogroups.	Boissinot et al. 1987
46kDa	Present in some legionellae.	
47kDa	Present in some legionellae.	
48kDa	Present in some legionellae.	
55kDa	The banding pattern between 55-60kDa proteins resolves as a single 31kDa protein when treated with N-acetylmuramidase.	Butler et al. 1990.
58kDa	Genus-specific.	
61kDa	Genus-specific.	
65kDa	Single major proteint in the cell membrane of legionellae.	Gabay et al. 1985
66kDa	Identity unknown.	
68kDa	Genus-specific.	
88kDa	Species-specific for *L pneumophila*.	Sampson et al. 1986
97kDa	Present in some species.	
135kDa	Species-specific for L pneumophila; surface exposed; not peptidoglycan-associated	Lever 1993
154kDa	Genus-specific.	

The antigenic composition of Legionella species has been studied quite extensively since the late 1970s, when the presence of serogroup-specific antigens was first reported (Johnson *et al.* 1979). By the early 1980s, 85 different family-, genus-, species- and serogroup-specific antigens had been described (Collins *et al.* 1983). Soon thereafter, in 1984, *L pneumophila* alone was shown to possess at least 23 different antigens of which 11 were heat stable and presumably non-protein in composition (Winn 1984). The complete chemical structure of the species- and serogroup-specific antigens and the role of individual antigens in pathogenesis were unknown at the time. It was suggested that they play a role in attachment to mucosal surfaces, antibody binding and the coiling phagocytosis characteristic of legionellae (Pearlman *et al.* 1985, Ciecielski *et al.* 1986). The different antigens and their functions are summarised in Table 1.6.

1.6 GENETIC COMPOSITION

The Legionella genome is currently being sequenced in its entirety; to date approximately 80% of the genome has been sequenced. The guanine plus cytosine (GC) content ranges between 38 and 52mol% (Brenner *et al.* 1988). Legionella species share up to 67% DNA relatedness at optimal hybridisation temperature. Strains within one species share ≥70% DNA relatedness with <5% deviation in related sequences. Although not closely related the other groups of organisms, their closest relatives are the Enterobacteriaceae and Pseudomonas species. They are also distantly related to the purple sulfur bacteria. Plasmids of 85, 84, 80, 60 and 43Mdal (megadaltons) have been described in some Legionella species in the early 1980s. (including *L pneumophila* SGs 2, 3 and 4, *L bozemanii, L dumoffii, L micdadei* and *L gormanii* but not *L pneumophila* SGs 1, 5 and 6) (Aye *et al.*1981, Brown *et al.* 1982, Maher *et al.* 1982). Nolte *et al.* (1984) detected a 21-Mdal plasmid in clinical and environmental strains of *L pneumophila* SG 1. The genetic composition of legionellae is summarised in Table 1.7.

1.6 CLINICAL SIGNIFICANCE

Infections caused by legionellae are collectively known as legionellosis and include Legionnaires' disease and Pontiac fever. Subclinical infections have been reported. Legionella infections occur worldwide, in people of all ages and race groups with no evidence of person-to-person spread of infection (Kurtz 1988). The mode of transmission, inoculum size, particle size and host susceptibility appear to influence the severity of the

Table 1.7 Genetic composition of Legionella species

COMPONENT	CHARACTERISTICS AND FUNCTIONS	REFERENCES
enh	enh1, enh2 and enh3 loci involved host cell entry of *L pneumohila*.	Cirillo et al. 2001
icm	Icm locus prevents phagosome-lysosome fusion for intracellular multiplication and macrophage killing. Contains 10 genes: icmW, icmX, icmY, icmZ, icmT, icmS, icmR, icmQ, icmP and icmO. IcmQ-icmR-complex regulates formation of translocation channel that delivers proteins to host cells. IcmS-icmW-complex important in virulence. Icm system in *L pneumophila* transfers effector molecules to host cells.	Sadowski et al. 1993 Brand et al. 1994 Coers et al. 2000 Segal and Shuman 1997
dot	Dot virulence genes encode a large complex that functions as a secretion system and can transfer plasmid DNA from one cell to another. DotA is highly variable among *L pneumophila* strains, involved in macrophage killing and the lysis of red blood cells and engaged in organelle traficking during intracellular stage of life cycle. DotH, dotO and dotI apparently play a role in intracellular growth.	Kirby et al. 1998 Brand et al. 1998 Coers et al. 2000 Andrews et al. 1998
dot/icm	Encode a type IV-related transport apparatus. Subsets of genes are necessary for pore formation and modulation of phagosome trafficking. Most of the icm and dotA gene products act during the early stages of phagosome establishment.	Coers et al. 2000 Wiater et al. 1998
gsp	Encoding for the global stress proteins of *L pneumophila*, gspA, is induced upon intracellular infection in macrophages and exposure to in vitro stress stimuli.	Abu Kwaik et al. 1997
pts	Necessary for in vitro virulence in *L pneumophila* and apparently also for normal growth within macrophages.	Higa et al. 2000
nmv	Non-macrophage virulence gene. Nmv negative bacteria are complement resistant. Although the mechanisms of nmv virulence is still unknown, it may be related to the production of a toxin or to resistance of the organism to host factors important in early pathogenesis of disease, before macrophage ingestion.	Edelstein et al. 2000
hel	Three loci, helA, helB and helC, have been described. They are similar to the cnz and cnr loci of Alcaligenes xylooxidans, but their functions remain to be determined.	McClain et al. 1996
eml	This locus probably expressed in response to non-stress stimuli during the early stages of intracellular infection.	McClain et al. 1996
lly	The lly-locus (legiolysin) produces browning of culture medium during the late stationary growth phase. Lly negative strains have decreased resistance to ordinary light. This locus encodes for a 39kDa protein that is involved in hemolysis, pigment production and fluorescence in L pneumophila.	Steinert et al. 1995 Wintermeyer et al. 1994
mip	The gene coding for Mip, the macrophage infectivity potentiator protein. It is essential for optimal intracellular survival of L pneumophila. Mip-like genes have been described in other Legionella species.	Helbig et al. 2001 Cianciotto et al. 1990
msp	A set of genes in L pneumophila with homology to the protein of type II general secretion pathway of gram-negative bacteria. They are not necessary for growth in amoebae but other proteins that are important for growth in amoeba are likely to be secreted by this pathway.	Hales and Shuman 1999
lcy	This locus is also known as Legionella cyclophilin. It shows similarity to the cytoplasmic and perplasmic cyclophilins of E coli.	
fla	The flaA gene encodes for the flagellum subunit of L pneumophila SG1. Two strains of L pneumophila, L bozemanii and L feeleii SG 1 were shown to possess flaA-specific sequences but were not able to produce flagella. It has high sequence homology to the flil family of genes, responsible for the formation of flagellae.	Heuner et al. 1995
pil	The genes in the pil locus (pilB, pilC, pilD) are homologous to type IV pilin genes and may enhance macrophage colonization during legionella infections and amoeba invasion in the environment. PilD is involved in type IV pilus biogenesis and type II protein secretion.	Stone and Abu Kwaik 1998

infection (Girod *et al.* 1982, Yu 1990). A seasonal distribution of legionellosis has been reported, with the majority of cases being identified during summer to autumn months in most studies (Roig *et al.* 1991, Tobiansky *et al.* 1986). Approximately 50% of the currently known Legionella species have been implicated in human infections. However, all the legionellae should be regarded as potentially pathogenic under appropriate conditions.

1.6.1 Legionnaires' disease

Legionnaires' disease is a severe multisystem disease with pneumonia as the most predominant clinical finding. Symptoms may range from a mild cough and slight fever to a coma with widespread pulmonary infiltrates and multisystem failure. Clinical features of the disease are similar to those of other pneumonias and may lead to the formation of lung abscesses. Asymptomatic infections have been reported; they occur more frequently in *L micdadei* infections (also referred to as Pittsburgh pneumonia) than in those caused by *L pneumophila* (Finch 1988, Roig *et al.* 1991). Survivors usually recover completely although lung fibrosis and some neurological abnormalities may persist in some cases. Legionnaires' disease has a low attack rate with a ratio of 10-20% in sporadic cases. A mortality rate of 12% in Britain and 15% in South Africa has been reported but this figure may be as high as 50% in the absence of appropriate treatment (Zumla 1988). Legionnaires' disease may have a sudden or gradual onset and usually an incubation period of 2-10 days.

Erythromycin was historically the drug of choice for the treatment of Legionnaires' disease, but the newer macrolides, especially azithromycin, have superior in vitro activity and greater intracellular and lung-tissue penetration. Quinolones have even greater in vitro activity and better intracellular penetration than the macrolides and numerous successes have been reported with these drugs, especially with ciprofloxacin. Rifampin is highly active both n vitro and in vivo against Legionella and is recommended as part of combination therapy (with a macrolide or a quinolone) for patients who are severely ill. The total duration of therapy is usually 10-14 days; however a 21-day course may be needed for immunocompromised patients or those with extensive evidence of disease on chest radiographs.

1.6.2 Pontiac fever

Pontiac fever is an acute, self-limiting, flu-like illness without symptoms of pneumonia. It is characterised by high fever, chills, myalgias and malaise but without the pneumonia or cough typical of Legionnaires' disease. Some authors suggest that it is a hypersensitivity pneumonitis caused either by infection with Acanthamoeba filled with legionellae (Vandenesch 1990) or as a result of a toxic reaction to the organism (Muder *et al.* 1986). The incubation period is short, ranging from 3-5 days, and the attack rate high, exceeding 90% in some cases. The fatality rate is low. Pontiac fever symptoms usually resolve spontaneously within one week, only symptomatic treatment is needed and the chest radiograph is clear. There is no evidence of secondary spread of the infection in Pontiac fever. Diagnosis can only be made by seroconversion, which may be delayed for up to 6 weeks after onset of symptoms.

1.6.3 Incidence of legionellosis

The incidence of legionellosis varies from country to country and from region to region. In the United States, Legionnaires' disease is considered fairly common and serious and legionellae are among the top three causes of sporadic, community-acquired pneumonia. However, many cases are still not reported, as Legionnaires' disease is difficult to distinguish from other forms of pneumonia. Although only approximately 1,000 cases are reported to the Centers for Disease Control and Prevention (CDC), it is estimated that over 25,000 cases occur every year, causing more than 4,000 deaths.

The epidemiology of legionellosis in Australia is significantly different from that in other parts of the world, in that *L pneumophila* SG 1 infections are rare and usually associated with international travel, especially during winter months. The majority of infections in this region occurs during spring and summer, is caused by *L longbeacheae* and is usually associated with the use of contaminated potting soil mixes and other garden products.

Only one outbreak of Legionnaires disease has been reported in South Africa to date but the prevalence of antibodies in the general population is high. Ratshikhopha (1990) reported antibodies to *L pneumophila* SGs 1-6 in 65% of healthy blood donors from Gauteng. Furthermore, antibodies to *L pneumophila* SGs 1-4 were reported in 36% of health mineworkers, compared to 10% of healthy factory workers and 16% of pneumonia patients (Bartie and Klugman 1997). Maartens *et al.* (1994) reported

seroconverions to *L pneumophila* SGs 1-4 in 9% of patients hospitalised between 1987 and 1988 with symptoms of community-acquired pneumonia.

1.7 ECOLOGY

Legionellae are natural inhabitants of water, found in a wide range of habitats. They are ubiquitous in natural sources like streams, rivers and lakes, thermal ponds and thermally polluted waters (Dennis 1993). *L longbeacheae* is the only species that is more often isolated from potting soil than from water to such an extent that soil has been suggested as the natural habitat of the species (Steele *et al.* 1990).

Legionellae from these natural environments can enter man-made water systems by various means. The organisms can be transmitted from raw water collected directly from rivers and reservoirs, during water treatment, as part of post-treatment aftergrowths within water distribution systems or during building or plumbing repair and construction activities (Colbourne and Dennis 1985). Once established, they can persist in the water supply for long periods of time and are difficult to eradicate. Therefore, as it is impossible to remove legionellae from natural waters and to control their entrance into water distribution systems completely, their presence must be considered in the design, operation and maintenance of buildings. This makes collaboration between engineers, occupational health practitioners and microbiologists essential.

Water distribution systems of hotels, hospitals, ships, retail establishments, office blocks and public buildings, industrial premises, power stations and domestic premises are often colonised with legionellae. Misting machines in supermarkets, fountains, spas, whirlpools, ice machines and dental water lines (Williams *et al.* 1996) have also been implicated in legionellosis. Eyewash stations are often colonised by legionellae and amoebae (Paszko-Kolva *et al.* 1991).

Legionellae are usually present in low numbers in natural sources but certain factors present in man-made reservoirs may amplify their numbers significantly:

- Temperature Environmental legionellae can survive over a wide temperature range (20-43°C) with an optimum of 30°C (Hoffman 1984, Kusnetsov *et al.* 1996). Kusnetsov and coworkers (1996) showed that legionellae can survive at 50°C for up to one hour and at 55°C for up to 40 minutes. The temperature tolerance of legionellae is illustrated in Figure 1.2
- pH Legionellae can survive and multiply at a pH of 5.0-8.5.
- Stagnant water The presence of stagnant water in distribution systems may increase the risk of Legionella contamination.

- ⇨ Water treatment The type of water treatment used in the system may affect legionellae numbers
- ⇨ Disinfectants The concentration and persistence of residual disinfectant in the system affect
- ⇨ The chemical environment Turbidity and the presence of organic carbon and certain metals like zinc and copper have been implicated in an increased risk of contamination (Brundett *et al.* 1989, Marrie *et al.* 1992, States *et al.* 1987). Legionellae can store carbon and energy in the form of polyhydroxybutarate granules, allowing them to survive without substrates for long periods of time (http://www.e-tiology.com/legion.htm).
- ⇨ High relative humidity (RH) Legionellae survive best at 65% RH, they are least stable at ≤55% RH (Tiefenbrunner *et al.* 1993).
- ⇨ A build up of algae, slime and protozoa Amoebae, cyanobacteria and flavobacteria have been associated with the growth of *L pneumophila* (Colbourne and Dennis 1985). The organism was shown to adhere to the slime coat of Fisherella species (Bohach *et al.* 1983).
- ⇨ The presence of biofilm, scale and sediment In hot water systems, concentrations of the organism are usually highest in biofilms within the system and at the openings of water outlets.
- ⇨ The presence of corrosion products
- ⇨ Construction Major construction has been associated with numerous cases of legionellosis. It is believed that legionellae are released from soil during excavations. The organisms can then enter the cooling tower of the building, air intakes or water pipes, or may even be inhaled directly. Dust and dirt created during construction may also provide nutrients to legionellae already present in a cooling tower or potable water system (Freije 1996). New buildings may present legionellae problems if the plumbing is not flushed to remove dirt before occupation of the building (Frieje 1996). The water systems of renovated, idle buildings may contain stagnant water, a known source of legionellae.
- ⇨ Water pressure shock An increase in water pressure may send dirt into a system, providing a food source for the bacteria, or may dislodge scale and sediment, containing legionellae from pipes (Freije 1996).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1.7.1 Non-potable water sources

Heat rejection devices like cooling towers, evaporative condensers and HVAC systems are often implicated as sources of Legionella infections. They contain reservoirs filled with warm, recirculating water that makes them ideal for the growth, amplification and dissemination of microorganisms like legionellae. In a typical water-cooled condenser air is induced through, or blown over, packing of the cooling tower down which water, circulating from a pond beneath the packing, is allowed to fall by gravity, producing a large wetted surface that cools the descending water. The constant fall of water through the tower, the large area of the basin, fill, pipes and heat exchanger, the warm temperature of the water, the high relative humidity and high organic content within these devices provide conditions that favour contamination by legionellae, algae, protozoa, fungi and other bacteria. This risk is increased further by the open nature of the systems, excessive aeration and the constant addition of fresh water to make up that which is lost by evaporation increase the degree of biofouling in these systems.

Water is continually added to the ponds of cooling towers to make up for that lost by evaporation. Particles from the air drawn into the tower are captured in the water pumped down the structure and added to the pond. Algae and other organic substances released from construction, protective and plumbing materials can serve as nutrients (Fields et al. 1989, Muraca *et al.* 1988, Yamamoto *et al.* 1992). In towers that are not regularly cleaned, sludge accumulates in the reservoir and the slime adheres to the water covered surfaces, resulting in the presence of large concentrations of organisms including legionellae on these surfaces. In addition, water temperatures below 60°C, high relative humidity (Freije 1996), the age and configuration of the system, the pH of the water and the presence of certain metals may increase the risk of biofouling further (Yamamoto *et al.* 1992).

Water derived from municipal supplies but subsequently stored in cisterns, or conditioned prior to heating, is not considered potable, because of the deterioration in chemical and bacteriological quality during storage (Colbourne and Dennis 1985). The authors suggest that colonisation of such non-potable water sources inside large buildings, such as hotels, factories or hospitals, may be a major cause of legionellosis outbreaks.

i16022191
b15422793

1.7.2 Potable water systems

Legionellae are often present potable water supplies, especially in the hot water sections of these systems. The organisms may enter potable water supplies from the main water source, even from municipal water. Because most municipal water systems are not routinely screened for the presence of legionellae and the organisms are chlorine tolerant, they may survive many of the standard water treatment protocols. Once inside the system, they find a suitable environment to multiply and are usually very difficult to eradicate.

Legionella counts can rise from very low to very high within a short period of time but the factors that give rise to these changes in counts are not well understood and often very hard to determine (Joly 1993). The age and condition of the pipes, the degree of scaling and sediment and the potential for biofilm formation within the system increase the risk of contamination. Furthermore, water temperatures of 25-42°C, stagnation and the presence of certain free-living amoebae that are capable of supporting the intracellular growth of legionellae are amplifying factors often mentioned in published reports. Biofilm and scale that form in valves and fittings and on pipe walls not only feed legionellae, but also protect them from hot water and chemical disinfectants. Some materials used in the construction of plumbing systems, for example neoprene washers, are more readily colonised than others (Marrie *et al.* 1992). The location of the building may also play a role in legionellae contamination of potable water systems.

Hot-water tanks are often colonised with legionellae, especially at the bottom where a warm zone often develops and scale and sediment accumulate (Alary and Joly 1992, Freije 1996). However, hot water piping presents an even higher risk of contamination than tanks. Biofilm and scale that form in valves and fittings and on pipe walls feed legionellae and protect them from disinfectants. Rubber washers and gaskets in fittings also support and protect the organisms. Unused piping (dead-legs) creates additional problems because legionellae grow well in stagnant water.

1.7.3 Biofilms

Survival of microorganisms in low-nutrient environments such as man-made water systems regularly treated with biocides usually involves attachment to solid surfaces with subsequent formation of biofilms that are rich in nutrients and consist of a mixed population of organisms. Bacteria in industrial water systems therefore tend to grow in

biofilms where they have access to more nutrients, are in close proximity to other cells and are protected from various antimicrobial mechanisms like biocides, antibiotics and protozoan predators (Wireman *et al.* 1993, LeChevalier *et al.* 1998). It has been suggested that the slow growth rates within biofilms and the particular nutrient deficiencies under biofilm conditions may cause the expression of distinct phenotypes that are often resistant to chemical and antibiotic agents (Barker *et al.* 1992).

In these systems, legionellae may exist as planktonic cells, as free-living components of the biofilm ecosystem or in association with other organisms that colonise surfaces, like flavobacteria, cyanobacteria and amoebae that they may infect (Barker *et al.* 1993, Rogers *et al.* 1993, Wright *et al.* 1989). Wireman *et al.* (1993) suggested that legionellae are primarily biofilm organisms. Although their exact ecology in biofilm conditions is not yet known it is influenced by factors like contact time, Legionella concentration and surface type, but not by flow rate, temperature, water hardness or the frequency of contact with the surface. Recent studies have continued to demonstrate the importance of biofilms in Legionella persistence and growth in both natural and man-made water systems, even in the absence of eukaryotes like protozoa (Keevil 2000).

1.7.4 Intracellular replication

Legionellae are slow-growing organisms that require a combination of nutrients for growth. Due to their fastidious nature and lack of antibiotic activity, they may be replaced by faster growing organisms if they do not have an alternative means of survival in aquatic environments. The fact that legionellae are ubiquitous in these environments suggests that protozoa, especially amoebae, play a supportive role in their survival and multiplication and that they may in fact act as natural hosts and amplifiers for legionellae.

Rowbotham (1980) was the first to demonstrate interactions between legionellae and protozoa. To date, protozoa of the genera Acanthamoeba, Tetrahymena, Naegleria, Echinamoeba and Vanella species have been implicated in these interactions. Although little is known about the metabolic and physiological status of legionellae after passage through protozoa, in vitro studies have shown alterations in their physiological status resulting in iron deprivation, possibly changing the susceptibility of the released bacteria to chemical inactivation.

Amoeba trophozoites feed and multiply in water and biofilm. When conditions become unfavourable, these trophozoites are transformed into cysts with hard, impermeable outer walls that provides protection for ingested Legionella organisms. When conditions

become more favourable, the cysts change to trophozoites again and the bacteria are set free. Legionellae have been recovered from cysts treated with 50 ppm chlorine suggesting a high level of protection by the cysts (Kilvington and Price 1990). This high resistance of amoebal cysts to biocides may be the mechanism for the apparent reseeding of water systems by legionellae often experienced in the water treatment industry. However, recontamination may also occur via transmission of airborne cysts acting as carriers for the legionellae.

The intracellular replication of legionellae within free-living amoebae has recently been studied in vitro in detail using in vitro models/studies. McNealy *et al.* (2001) reported that a multiplication ratio as small as 0.001:1 (*L pneumophila* : amoebae) may establish a strong intracellular replication cycle in amoebae suspended in spring water. Their results indicated a 7,000-fold increase in *L pneumophila* colony forming units and lysis of all amoebae trophozoites. Furthermore, the addition of non-Legionella species did not decrease the replication cycle in amoebae but the addition of a ciliate (Tetrahymena) to amoebae and *L pneumophila* suspended in cooling tower water resulted in packaging of the legionellae into vesicles. This process was dependent on the presence of amoebae, which was necessary to initially amplify the low number of legionellae. Their results suggested that different protozoa probably function sequentially to increase the number and influence the distribution of legionellae in aquatic environments.

On the other hand, studies by Keevil *et al.* (2000) and Lüneberg (2000) suggested that intracellular growth is not essential for the proliferation of *L pneumohila* within mixed bacterial populations. They reported that, even when mixed populations were treated with cycloheximide to eradicate all eukaryotes, the numbers of *L pneumophila* actually increased >50-fold and represented a significant fraction of the total biofilm flora (Keevil 2000). It is therefore unsafe to assume that the absence of protozoal hosts within water systems precludes the survival and growth of legionellae; as long as there are other bacterial species present appropriate measures should be taken to prevent Legionella proliferation.

Legionellae can tolerate a broad range of physical parameters, since pH, temperature and conductivity have no significant influence on the frequency of detection in a natural aquatic environment (Grimm *et al.* 2000).

1.8 TRANSMISSION

The mere presence of legionellae in a water distriubtion system does not necessarily imply a human health risk. For human infection to occur certain conditions are necessary

(Colbourne and Dennis 1989, Breiman 1993). Interfering with any link in this 'chain of infection' will decrease the occurrence of disease significantly. The links in the chain of infection are as follows (Figure 1.3):

- ⇨ Reservoir Water is the major environmental reservoir for legionellae but other sources have been recognised.
- ⇨ Amplification A better understanding of the amplification process may lead to the development of methods to limit the multiplication of legionellae and to interrupt the chain of events leading to organism transmission with subsequent infection (Breiman 1993).
- ⇨ Dissemination The primary mechanism of Legionella dissemination is inhalation of droplets of respirable size. However, other routes have been described. In most cases of Legionnaires' disease, airborne spread [from cooling towers] has been implicated.
- ⇨ Virulence The species disseminated must be virulent enough to cause infection. In nature legionellae can survive and possibly replicate in free suspension and grow in biofilms and protozoa, thus leading to diverse phenotypes, potentially increasing their virulence and susceptibility properties (Barker and Brown 1995).
- ⇨ Susceptibility The organism must be disseminated to a susceptible person; this is a key factor in the chain of causation.
- ⇨ Inoculation site The organism must be inoculated at a site on the person where it can cause infection.

1.8.1 Aerosolisation

Aerosolisation is generally recognised as the major mechanism of Legionella transmission. The survival and viability of legionellae in aerosols are influenced by a number of factors (Colbourne and Dennis 1989, Tiefenbrunner 1993).

- ⇨ Relative humidity (RH) The survival time of legionellae in aerosols increases from 3 minutes at RH 30% to 15 minutes at RH 80% under laboratory conditions. Some authors suggest that the organisms will survive best at RH 65%.
- ⇨ Metabolic state Water is a low nutrient growth medium, therefore legionellae in an aerosol will be in a low metabolic state and relatively stable when compared to the same organism in a high metabolic state when grown in a nutrient-rich medium.
- ⇨ Aerosol age affects the viability of legionellae by allowing longer action of degrading factors such as high temperatures, biocides and ultraviolet light.
- ⇨ Aerosol composition influences the survival of aquatic bacteria. This applies particularly to legionellae as it is suggested that other aquatic organisms may

secrete protective substances which, when coating *L pneumophila*, may enhance its survival in an aerosol.

Apart from affecting persons outside a building, aerosols can be disseminated to people inside a building via contaminated ventilation and dehumidification systems. The distance legionellae can drift from a cooling tower and still be viable depends on humidity, sunlight and wind conditions. In most cases the distance will be less than 200 metres but some workers suggest that viable legionellae can travel up to 1-3 km in an aerosol. It is therefore no surprise that aerosols have been implicated in numerous outbreaks and sporadic cases (Freije 1996, Joly 1993). Aerosols may also transmit legionellae via showerheads and tap faucets as well as respiratory care equipment, whirlpool baths, humidifiers, decorative fountains, mist machines as these devices can cause droplets of respirable size (Breiman 1993). The rate of particle deposition from an aerosol depends on the particle size. Small particles (<5 μm in diameter) will remain in suspension thereby increasing their potential to cause infections. However larger particles may also cause infection in conditions of low relative humidity when they may evaporate before deposition, thereby becoming smaller and more respirable and thus capable of reaching the smaller airways of the lung.

1.8.2 Direct installation

Respiratory therapy devices have the potential to disseminate pathogenic microorganisms by delivering gases at high pressures and volumes and by generating intensive aerosols. These devices may thus be able to bypass the normal defence mechanisms of the respiratory tree. Numerous cases of nosocomial Legionnaires' disease have been traced to the use of respiratory therapy devices (Muder *et al.* 1986).

1.8.3 Aspiration

Aspiration as a mode of transmission has been largely overlooked because of the popularity of the aerosolization theory, but warrants further investigation. Pneumonia can develop in humans via two mechanisms. Those associated with aerosolization (for example tuberculosis) typically have a high attack rate while pneumonias caused by of aspiration typically have a low attack rate as infection depends mainly on the susceptibility of the individual (Muder *et al.* 1986). There are a number of reasons for suggesting aspiration as a possible mode of transmission. Legionnaires' disease has a low attack rate; the attack rate in Pontiac fever is high. Also, bacteria normally associated with aspiration pneumonia

tend to be normal flora possessing pili to mediate adherence to pharyngeal epithelial cells; legionellae also possess pili. Symbiosis between selected oropharyngeal flora and legionellae has been reported (Muder *et al.* 1986, Roig *et al.* 1991). Muder *et al.* (1986) also suggested that, given previously overlooked information, the only parameter that could be linked statistically to acquisition of the disease during the first outbreak was the ingestion of water and not as previously thought, the inhalation of contaminated aerosols.

1.8.4 Ingestion

A potential mechanism by which legionellae might be transmitted via ingestion is that of bacteremic spread after penetration of the intestinal tract. It is well known that *L pneumophila* can disseminate to extrathoracic organs (Muder *et al.* 1986). The fact that diarrhoea is one of the symptoms of Legionnaires' disease, further suggests the possibility that the gastrointestinal tract may be the portal of entry in some cases. However there is not much evidence to support this theory.

1.8.5 Other potential routes of entry

There have been reports of pericarditis followed by seroconversion to L pneumophila in the absence of pneumonia. In addition, one case of culture-proven *L pneumophila* prosthetic valve endocarditis has been reported (Muder *et al.* 1986).

1.9 PREVENTION

1.9.1 Non-potable water systems

The key factors to reduce the risk of Legionella contamination of non-potable water sources are to keep the Legionella counts as low as possible and to prevent transmission from the tower to people. In order to prevent Legionella contamination in these sources, the following are recommended (Freije1996):

- ⇨ Physical cleaning Physical cleaning alone has little effect on the risk of Legionella contamination, but it makes water treatment more effective by reducing any build-up of slime, dirt, biofilm, algae, fungi and amoebae, placing a greater demand on the biocide by preventing proper contact with microorganisms.
- ⇨ Water treatment Non-potable water sources should be treated regularly to control scale, corrosion and the build-up of microorganisms. Because biocides are designed

only to control slime and algae for optimum system efficiency and not to eliminate all bacteria in a system, water treatment programs should be evaluated by checking the total bacteria count (TBC) to indicate the general cleanliness of the system. A high TBC count in a cooling tower generally indicates the presence of a food source for legionellae, although there is no reliable correlation between TBC counts and Legionella counts (Freije 1996).

- ⇨ Periodic disinfection A regular water treatment routine is believed to be more effective than periodic disinfection. Broadbent (1987) suggested that hyperchlorination with cleaning usually had only a short-term effect on legionellae counts. The counts usually rise again within one month after treatment. However, periodic disinfection may be useful for some institutions in some instances.
- ⇨ Maintenance Overall good maintenance is widely recommended for the control of legionellae in cooling towers. Maintenance plans should be based on manufacturers' instructions and regulations published in formal guidelines. Cooling towers should be inspected regularly for leaks, corrosion and blockages and fans, motors and pumps should be operating properly. All operating and maintenance procedures should be documented and inspection reports kept for future reference. Cold water humidifiers should be provided with a domestic water source rather than using recirculated water. However, replace cold water humidifiers wherever possible with steam humidifiers because steam units operate at ≥70°C. Humidifiers should be kept clean and leak free at all times.

1.9.2 Potable water sources

Freije (1996) recommends the following preventive measures for potable water systems:

- ⇨ Water lines and fixtures Removing deadlegs to reduce the risk of stagnant water is critical to legionellae control. Natural rubber in plumbing fixtures provides nutrients for Legionella growth and protects the organisms from disinfectants. Washers and gaskets should preferably be made of neoprene or other synthetics. It is believed that copper is the best material to use in plumbing fixtures because it resists scale and biofilm formation. Steel is reasonably good while plastics are not recommended. Heavily scaled faucets and showerheads should be replaced. Faucet aerators and shock absorbers should be avoided as legionellae grow well in these fixtures. Water should be treated to avoid the formation of scale. Recirculation lines should be extended to the point furthest from the supply, lines should be run at a slight fall to simplify draining of the system and hot water piping should be run above cold water

piping to prevent warming of cold water. Freije also recommends the reaming of pipe ends to remove parts that can trap sediment and cause corrosion.

- ⇨ Hot water generators and cold water storage tanks The typical hot water tank keeps approximately 65% of the tank at the set temperature; the other 35% will be cooler. Some water heaters develop a warm zone at the bottom of the tank where scale and sediment can accumulate and provide a suitable habitat for legionellae. This problem can be overcome by installing thermometers at various locations of the tank. If possible, high-temperature semi-instantaneous heaters should be installed, as they are not conducive to legionellae growth. It is also important to keep in mind that horizontal tanks are less prone to Legionella growth than vertical tanks. When new tanks are purchased, it is recommended to consider a tank that recirculates the water to keep the temperature constant. The tank should also be capable of producing water at 70°C in case high temperatures are needed for disinfection.
- ⇨ Regular operation and maintenance Water temperatures at or above 60°C in tanks and 50°C at taps will control legionellae while cold water should be stored and distributed at ≤ 20°C. The optimum temperature will depend on the physical and mental conditions of the users and may therefore vary among institutions. In addition, hot water tanks should be drained at least once a year to remove scale and sludge and flushed with chlorine, and should be operated so that water is stored in the tank for one day or less. Coldwater storage tanks should be cleaned approximately once a year and preferably also flushed with chlorine. New systems should be flushed with chlorine prior to start-up, while idle systems require periodical running of water to all outlets to avoid stagnation.

1.10 DISINFECTION AND CONTROL

In order to reduce or eliminate the risk of legionellosis, the concentration of legionellae in the affected system should be minimised and transmission of the organisms to susceptible persons should be prevented. This can be achieved in a number of ways.

- ⇨ Superheating is the most common way of decontaminating a water distribution system contaminated by legionellae. The water temperature is raised to ≥70°C for 24 hours and each outlet is flushed for 20 minutes. The water temperature at each outlet should reach 60°C. Prior to superheating, dead legs should be removed from the system to prevent reseeding of the system. Sediment and scale can act as insulators, thus protecting legionellae from the effects of superheating. These layers

can be removed by opening each outlet slightly for about 8 hours. This should allow enough time so that the entire scale layer can be heated to ≥60°C.

⇨ Periodic chlorination is another method of controlling legionellae in hot water systems. After removing scale and sediment, sodium hypochlorite is added until approximately 50 parts per million (ppm) of free residual chlorine is produced. The pH of the water should be maintained between 7.0 and 7.6 to maintain the biocidal activity of the chlorine. All taps are subsequently flushed until the odour of chlorine is evident and the water allowed to stand for one hour prior to draining and refilling the tank and flushing each outlet to remove the chlorine from the system (Springston 2000). There are some disadvantages to chlorine shock treatment as compared to superheating in hot water systems. Firstly, chlorine is very corrosive and will shorten the service life of metal plumbing materials. Secondly, chlorine will not penetrate layers of scale, resulting in legionellae contained in such layers will recontaminate the system soon after hyperchlorination. The best way of overcoming this problem is continuous chlorination with 2ppm free chlorine following superchlorination (Springston 2000).

⇨ Alternative methods to control legionellae growth include the use of metal ions (copper and silver), in-line ultraviolet systems and ozone. Cooling towers can also be treated with algaecides and bactericides (biocides) such as gluteraldehyde to assist in controlling the multiplication of bacteria, protozoa and algae. Biocides should be added continuously to maintain constant concentrations in the system. Cleaning of the system is also important in the control of legionellae. A buildup of protozoa, algae, fungi, dirt and biofilm can prevent a biocide from making contact with microorganisms. The correct type and concentration of biocide used is therefore very important.

For cooling towers that have been implicated in outbreaks or are highly contaminated, emergency treatment is necessary. Springston (2000) recommends chlorination of the system with 50ppm free residual chlorine, together with a dispersant. This is followed by 10ppm free residual chlorine for 24 hours, taking into account the correct pH for the chlorine to be active. The entire system is then drained and the chlorination process repeated. Some workers recommend the use of bromine (that is less affected by pH) instead of chlorine. In order to prevent transmission of legionellae to susceptible persons, by using drift eliminators and by locating cooling towers as far as possible away from windows, air intakes and outdoor areas frequented by people. In addition, prevailing wind directions should be taken into account with regard to cooling towers and air intakes. [http://www.esmagazine.com/es/cda/articleinformation/features/bnp_features_item/0,2503,3615,00.html]

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1.11 NUTRITIONAL REQUIREMENTS

Legionellae are chemo-organotrophic and use amino acids, catabolized through the Krebs cycle, as their main source of carbon and energy. *L pneumophila* can synthesise all the other chemical components *de novo* and apparently has no vitamin requirements. However, the exact nutritional requirements for legionellae are not clear as they appear to be influenced by variations in culture conditions. It has been suggested that the addition or deletion of certain nutrients from culture media stimulate the induction of enzymes that bypass certain metabolic steps in order to use only the materials available in the medium.

Early studies suggested that the amino acids needed for growth include arginine, isoleucine, methionine, threonine, valine, phenylalanine, tyrosine. alanine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan and tyrosine. Threonine and tyrosine stimulate oxygen uptake of legionellae, depending on the medium used for culture. Most strains require serine that can be used as the sole source of carbon and energy. Some strains also require proline (Rowbotham 1980, Muller 1981, Tesh *et al.* 1983). However, Wadowski *et al.* (1985) suggested that legionellae can meet all their carbon and energy needs with nine amino acids. This need for amino acids implies that legionellae can use these products from other organisms that produce them in excess or from decaying matter.

Extensive differences in the sensitivity of legionellae to chelators have been reported. In a study by Reeves *et al.* (1981) Legionella growth was completely inhibited by ethylene-diamine-tetra-acetic acid (EDTA), citrate, acetate and malate and partly by lactate. He also reported that the strains that were the least sensitive to the chelators appeared to need the smallest amount of metals for optimal growth and survived longer. In addition, the strains of *L pneumophila* tested by this group needed a greater variety of metals and were more sensitive to the loss of metals by chelation than *P aeroginosa*. It is however important to bear in mind that the salt content or the pH of a medium can also alter the specificity of chelators (Reeves *et al.* 1981). L-cysteine is one of the most commonly used chelating agents. Although normally added to culture media to act as a chelating agent, it acts as a required amino acid and not as a chelating or reducing agent in media used for Legionella culture.

The addition of trace metals to culture media stimulates heme proteins in aerobic organisms and non-heme enzymes. Most organisms need boron, calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, vanadium and zinc for

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

growth. Calcium and magnesium are cofactors in many bacterial enzymes and are important components in cell walls and membranes. Iron, manganese and zinc are important cofactors for some enzymes and are important in oxidation-reduction systems. Molybdenum plays and essential role in nitrogen metabolism. Although cobalt, copper and nickel are less frequently required, they are essential cofactors in certain enzymes. Vanadium is not a common bacterial nutrient but stimulates growth in some strains of *L pneumophila* and can substitute for molybdenum in some bacterial strains and for iron in some strains of lactic acid streptococci (Reeves *et al.* 1981, Hutner 1972). Iron is present in ferredoxin and cytochrome and is an important cofactor for growth. It also plays a regulatory role in some fermentation processes. Iron salts are essential for the growth of all legionellae, although *L pneumophila* can grow over a wide range of iron concentrations. Under conditions of iron deprivation the lag phase of the organism is increased and cellular morphology is affected, giving rise to short, coccobacillary forms. Long slender rods are formed when the iron concentration is high (Ristroph *et al.* 1981). Legionellae will not grow on media deficient in all trace metals (Winn 1984).

It has been suggested that the difficulties in the isolation of legionellae from the environment may be explained by their sensitivity to relatively low levels of hydrogen peroxide and superoxide that are generated in culture media exposed to light. Although legionellae grow in the absence of charcoal in filter sterilised broth media, no growth occurs in broth media when sterilised by autoclaving. It is believed that toxic products might be released into the broth medium during autoclaving thereby inhibiting growth. The addition of charcoal to culture media can enhance Legionella growth by preventing this photochemical oxidation reaction and by detoxifying media of reduced forms of oxygen.

Yeast extract is rich in nucleotides and provides vitamins and coenzymes to culture media. The addition of alpha-ketoglutarate to agar media increases the isolation rate and the quantity of viable bacteria detected but may inhibit growth in broth media. Alpha-ketoglutarate is added to the media used for Legionella culture to stimulate the production of oxygen-scavenging enzymes in order to promote growth (Pine *et al.* 1986, Winn 1984). Potassium, a cofactor for some enzymes, is required for carbohydrate metabolism. It is used mainly for ribosomal function: when the protein content of bacterial cells bacterial cells is lowered, protein synthesis stops while glycolysis continues.

When exposed to light, yeast extract contains substantial levels of hydrogen peroxide and superoxide radicals. Cysteine is also known to form hydrogen peroxide upon auto-oxidation and when present in solutions of amino acids, increases the production of peroxide. Photo-oxidation or free radical-mediated oxidation of membrane lipids produces

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

toxic lipid peroxides and hydroperoxides. Hydrogen peroxide may therefore form in the medium used for Legionella culture.

The charcoal added to buffered charcoal yeast extract (BCYE) medium generally used for Legionella culture consumes oxygen but this effect decreases with time. Activated charcoal in suspension decomposes hydrogen peroxide and superoxide radicals, decrease their accumulation in complex media exposed to light and prevents yeast extract medium from light-accelerated oxidation of cysteine. According to Hoffman *et al.* 1983) this can be overcome by adding peroxide and superoxide radical scavengers (for example catalase and superoxide dismutase) to culture media that do not contain charcoal.

The presence of even low levels of hydrogen peroxide has a substantial effect on *L pneumophila* growth but apparently not on *L micdadei* and *L bozemanii* (Hoffman *et al.* 1983). This can probably be explained by the fact that, although it contains considerable amounts of superoxide dismutase (SOD), *L pneumophila* contains no catalase. Of the legionellae tested, *L micdadei* contains the highest amount of SOD. Autooxidation and/or photochemical oxidation of complex culture media, solutions of amino acids and vitamins may generate considerable levels of hydrogen.

1.12 DETECTION METHODS

Classical detection methods for Legionella species from the environment relied on the inoculation of susceptible guinea pig hosts (McDade *et al.* 1977). Although selective, these methods were expensive and time consuming and were soon replaced by isolation by culture on agar media. To improve the recovery of legionellae by culture, the use of certain selective media and pretreatment steps were introduced to minimise contamination by non-legionellae (Bopp *et al.* 1981, Groothuis *et al.* 1983). In attempts to simplify Legionella identification, radioimmunoassays (RIAs), enzyme linked immunosorbent assays (ELISAs), agglutination tests and nucleic acid probes and polymerase chain reaction (PCR)-based assays have since been developed and tested (Bej *et al.* 1991, Lye *et al.* 1997, Mahbubani *et al.* 1990). Although the majority of these new methods are fairly successful for the detection of environmental legionellae, culture remains the method of choice. However, no single culture method has so far proven to be ideal for all samples in all given circumstances and environments. Even in the absence of contaminating bacteria or other inhibitory substances, the detection of small numbers of legionellae from environmental samples remains difficult. This, together with the lack of standardisation of methods, complicates the interpretation of culture results and comparisons of results

from different institutions (Kusnetsov *et al.* 1995, Ta *et al.* 1995). Variations in bacterial numbers in different areas within a water distribution system and the sampling method used often complicate the interpretation of culture results even further.

1.12.1 Sampling

Numerous guidelines have been developed for environmental sampling for Legionella detection. The purposes of sampling for legionellae are firstly to evaluate the possibility of legionellae amplifying in building water systems that generate aerosols to which building occupants and passers-by can be exposed. Secondly, to determine the efficacy of water treatment programmes in preventing Legionella amplification and thirdly to determine whether legionellae may be causing disease in building occupants (Shelton *et al.* 2000). The appropriate sampling strategy should be based on the specific conditions of the building and the specific objectives of the investigation.

When deciding on the frequency of sampling for legionellae, one has to remember that Legionella testing will only be applicable to the area of the system sampled, at the time of sampling. The sampling frequency should therefore be based on factors such as

Table 1.8 Basic supplements in Legionella media

SUPPLEMENT	FUNCTION
Charcoal	Legionellae are sensitive to relatively low levels of peroxides and superoxides that are generated by media exposed to light. Activated charcoal prevents these reactions and detoxifies media from reduced forms of oxygen. It also prevents free radical-associated oxidation of cysteine, as essential growth factor for legionellae.
L-cysteine	L-cysteine is normally added to agar media as a chelating agent but in the case of legionellae it acts as a required amino acid and not a chelating or reducing agent.
Potassium hydroxide	Potassium hydroxide is necessary for protein synthesis. Because the addition of sodium, even in the small quantities used for pH adjustment can reduce growth of legionellae, KOH should also be used for this purpose.
Alpha-ketoglutarate	In general, alpha-ketoglutarate increases the isolation rate and viability of organisms on agar media but it may have an inhibitory effect in broth media.
Iron	A trace element that plays a regulatory role in various fermentation processes.
ACES buffer	Addition of a buffer with a pK at optimal pH for legionellae growth, ACES buffer, is normally added to culture media but any other suitable buffer can be used instead.

the potential for amplification of legionellae in the system, the suspected concentration and the need to verify the efficacy of treatment programmes (Shelton *et al.* 2000). The number of samples to collect will depend on the size of the facility and the number of systems in the facility that may be potential sources for legionellae (ASTM Guidelines D5952-96).

Legionellae can be isolated from a wide variety of environmental samples including water, biofilm, sediment and scale, as well as soil and dust. Water and biofilm samples are usually the first to be tested when contamination is suspected. However, aerosol samples may be useful in some investigations, for example to indicate how many legionellae organisms are passing from a water source into the air or the distance the legionellae are being transmitted. Samples should be collected from areas in the system where legionellae are most likely to be detected. Areas most likely to contain legionellae are (ASTM Guidelines D5952-96) incoming water supplies, water storage tanks and hot water heaters, hot and cold water faucets and shower heads, water-cooled heat-transfer equipment and humidifiers, spas, decorative fountains and other water systems suspected of containing legionellae.

The volume of water collected for legionella detection usually depends upon the nature of the system and the purpose of the examination. In general samples of one litre are collected in sterile glass, polyethylene or similar containers. Smaller sterile containers with screw caps are recommended for collecting biofilm, scale and sediment samples. Previously used sampling containers should be sterilised or pasteurised before use. Biocides can be inactivated by adding an inactivating agent to the sampling container before or during sampling. Chlorine and other oxidising biocides are inactivated by the addition of potassium thiosulphate or sodium thiosulphate at a concentration of 1 ml of a 30% solution per litre of sample. For other biocides adding a universal neutralising agent is not practicable.

In some instances swabs are more appropriate than water samples. Although experts agree that water rather than swab samples should preferably be collected from cooling towers, water tanks and sources like decorative fountains, some workers regard swab samples from biofilms critical for the evaluation of Legionella colonisation (Stout 1998, Ta 1995). For some sources, for example showerheads, some workers prefer swab samples, others prefer water samples and some prefer both. Water samples will provide a more accurate comparison of legionellae numbers in different water outlets in a building than swabs and will provide a total picture of the bacteria present in the system whereas swabs will only provide information on the particular area sampled. However swab samples

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

will provide more information on the presence of legionellae in a biofilm and are more convenient than water samples to collect. If both water and swab samples are collected, the water sample should be collected first and contain a minimum of 200ml (Stout 1998). Barbaree *et al.* (1987) recommended that swabs be dipped in sterile water before use.

Aerosol sampling for legionellae is usually done with Andersen air samplers or liquid impingers (Breiman *et al.* 1990, Ishimatsu *et al.* 2001). Barbaree *et al.* (1987) suggested sampling with a six-stage Andersen sampler containing a selective medium at a flow rate of 0.0283m^3/minute. Although selective media are not recommended for use in this type of sampling under normal circumstances it is suggested for legionellae to control the number of non-legionellae on the plates. Alternatively, an all-glass impinger containing 0.25% yeast extract can be used at a flow rate of 0.0108m^3/minute.

Despite the reasonable success in detecting legionellae from aerosols reported by some workers, water sampling has several advantages over aerosol sampling. Because legionellae live in water and on surfaces these samples check the actual source of contamination. Legionella counts will be more consistent in water than in air because the amount of contaminated water droplets entering the air will vary significantly over time; a water test will therefore be more accurate in indicating a potential health risk. Detection methods relying on culturing legionellae may fail to isolate legionellae from aerosol samples if the bacteria lose their culturability while being airborne, during the collection procedure, during sample storage or during the culturing process. However, methods not based on bacterial multiplication, may be useful for detection of legionellae from air samples that test negative by the culture method (ASTM Guidelines D5952-96)

A very important aspect of sampling for legionellae is personal protection during sampling. The ASTM Guidelines (D5952-96) recommends that equipment should be turned off while collecting samples, if possible. Disposable protective clothing should be worn as well as slip-proof footwear and eye protection, especially when working in wet, potentially contaminated areas and in areas recently treated with biocides, disinfectants, detergents or other chemicals. Half-mask respirators of at least 95% efficiency should be worn when working near potentially contaminated equipment that may generate aerosols.

1.12.2 Sample transport and storage

Samples should be allowed to reach ambient temperature and be protected from extreme temperatures and direct sunlight during transport and storage. In general, temperatures below 3°C and above 30°C should be avoided. The necks of sampling containers should be sealed with tape and the containers wrapped in absorbent paper and

placed in individual plastic bags if not delivered by hand to the laboratory. Samples should preferably reach the laboratory within 24 hours of collection and should be kept at room temperature until testing, which should be commenced within 24 hours of receipt. The longest period of time allowed between sample collection and culture of the concentrate is 14 days (Draft International Guidelines ISO/DIS 11731–1996).

The viable count of bacteria in a water sample can change during transport and storage because bacteria can become attached to the walls of the sample containers, may become non-culturable or die. For legionellae these losses are most apparent when stored between 0°C and 6°C. Thus the ISO method recommends that sample concentrates can be stored at temperatures between 6-18°C in the dark for no longer than14 days. However, some authors recommend refrigeration of the samples at 2-8°C before and after processing (Stout 1998).

1.12.3 Sample concentration

Environmental water samples may be inoculated directly onto agar media if the number of organisms is likely to exceed 10^5 per litre. However, as the number of legionellae in any given sample is usually not known, samples are usually concentrated by either membrane filtration or centrifugation, prior to culture. Both membrane filtration and centrifugation are widely accepted and used for this purpose, but there is no consensus among workers regarding the efficiency and accuracy of either of these methods (Boulanger *et al.* 1995, Brindle *et al.* 1987.

A number of studies were carried out in the late 1980s to early 1990s, comparing the different aspects of filtration and centrifugation. Smith and coworkers published a comprehensive comparison of different filter membranes in 1993. This group evalutated eight different filter types with pore sizes ranging from 0.2-0.8μm and found a polycarbonate filter with a pore size of 0.2μm most appropriate for sterile seeded samples. They reported a recovery rate of 66% after sonication and 59% after vortex resuspension. When using the same filter type with a pore size of 0.4μm, the recovery rate dropped to 49% after sonication and 35% after vortex. These recovery rates were significantly better than those reported with the other filter types they tested. Unfortunately this group did not study the efficiency of these filter types on non-sterile, environmental samples. Reinthaler *et al.* (1992) also preferred polycarbonate or cellulose nitrate filters with a pore size of 0.2μm. Other groups reported on the efficiency of black filters placed directly on agar (Szewzyk *et al.* 1991), sequential filtration through 3.0μm, followed by 1.0μm fibreglass cartridge filters (Payment *et al.* 1989) and nucleopore filters

examined directly by microscopy (Hobbie *et al.* 1977). Some workers reported an increase in recovery from environmental, but not seeded samples, when using flat black filters instead of white cast cellulose nitrate filters (Boulanger *et al.* 1995)

The Centres for Disease Control and Prevention (CDC) (Atlanta GA) recommends filtration of water samples through 0.2μm polycarbonate filters. The use of nylon or polycarbonate filters with pore sizes of 0.22μm or 0.45μm is recommended in the International Standard (ISO/DIS 11731) method. According to the Australian Standard (AS 3896-1991) filtration through 0.22μm and 0.45μm filters is appropriate; this method does not recommend a specific filter type. In South Africa, 0.45μm, type HA Millipore filters, optimised for microbiological analyses of potable water, are used.

For membrane filtration to be effective, several factors have to be taken into account when choosing the type of filter and the pore size to use. Smith *et al.* (1993) reported several defects in some of the filters they tested, including brittleness, wrinkles, non-wetting areas, and inhibitory compounds present on the surface of some filters. Furthermore, blocked pores, abnormalities and pore structure, the method of resuspension after filtration and lot-to-lot differences, even in the same filter type, may complicate sample concentration by filtration. Disadvantages of filtration as a means of concentration also include the slow filtration rate of dirty environmental samples and the presence of biocides in samples that may affect the filtration rate (Brindle *et al.* 1987).

Centrifugation is often used as an alternative for filtration but differences in opinion regarding the optimal centrifugation speed and time have been reported (Voss *et al.* 1984, Brindle *et al.* 1993, Boulanger *et al.* 1995). Some workers prefer centrifugation at 6000 *g* for 10 minutes while others consider 3000 *g* for 30 minutes more appropriate. Brindle *et al.* (1993) reported a good correlation between centrifugation at 6000 *g* for 10 minutes and filtration through 0.45μm pore size, nitro-cellulose filters, with centrifugation having the added advantage of saving on time and effort. The Australian Standard recommends centrifugation at 6000 *g* for 10 minutes whereas the International Standard regards either of the two methods of centrifugation as appropriate. Brindle and coworkers (1993) reported a loss of 57% of organisms after centrifugation at 6100 g for 10 minutes, with an even higher loss at lower centrifugation speeds whereas other authors reported losses of 80-90% with both centrifugation and filtration. Even from very large quantities of water they could only recover 14-55% by filtration, depending on the volume filtered.

Brindle *et al.* (1987) compared membrane filtration and centrifugation for Legionella recovery from seeded samples and found both methods equally successful. For filtration to be effective, several factors have to be taken into account when choosing the

type of filter and the pore size to use. It was also found that direct plating of filter membranes did not enhance recovery but rather experienced that their bacterial counts were inaccurate due to clustering of the colonies on the wet membranes.

1.12.4 Sample resuspension

The method of resuspension of the organisms after concentration may influence the accuracy of recovery from environmental samples. Organisms can be resuspended after filtration by either producing a vortex for a minimum of two minutes, or by placing the concentrate in an ultrasound tank until the membrane appears clean. There have been contradicting reports regarding the efficiency of both these resuspension methods; most authors believe that a combination of methods is necessary to optimise the recovery from different sample types. The general feeling is that each laboratory should find the method that works best for its samples and environment.

The ISO method recommends placing the filter in sterile diluent and shaking vigorously for not less than two minutes, or alternatively to place the container in and ultrasoud tank (sonicator) for 2-10 minutes, depending on the type of tank used. The Australian method recommends resuspension in water from the original sample followed by shaking the concentrate vigorously for 20-30 seconds, Sonication has been used successfully to release intra-amoebal legionellae from seeded samples (Boulanger *et al.* 1995), a method that may further increase the sensitivity of culture methods.

A scraping method to remove bacteria from membranes after filtration has been used in the past. Bacteria were aseptically scraped from filters using commercially available cell scrapers. This method is not routinely used any more.

1.12.5 Sample pretreatment

The presence of non-legionellae in environmental samples may decrease the recovery of legionellae due to overgrowth or inhibition by non-legionellae (Boulanger *et al.* 1995, Lye *et al.* 1997). Legionellae have thermophilic characteristics and are relatively stable in acidic conditions. To improve their isolation from highly contaminated samples, Bopp *et al.* (1981) and Groothuis *et al.* (1983) developed the pretreatment methods (acid treatment and heat treatment respectively) that are still widely used today. However, the efficacy of pretreatment varies among different sample types and institutions and if not carefully controlled, these pre-treatment procedures may inhibit or injure legionellae. Most authors believe that a combination of methods is necessary to optimise the

recovery from different sample types. The general feeling is that each laboratory should find the method that works best for their environment and sample types they process.

It is well known that laboratory-adapted legionellae react differently to pretreatment than environmental strains. Environmental strains from different sources or even from different areas in the same source may react differently to these selective procedures. A study by Roberts and coworkers (1987) indicated that environmental legionellae are more resistant to pretreatment than laboratory-adapted strains.

Acid treatment involves subjecting sample concentrates to a buffer at a pH of 2.2.for a period ranging from 3-15 minutes as preferred by different workers. As legionellae are not entirely acid fast, the length of exposure to this buffer must be carefully controlled. Stout (1998) recommended acid treatment of potable waters for 3 minutes and 15 minutes for non-potable water. Heat treatment relies on the thermophilic characteristics of legionellae and involves subjecting sample concentrates to a temperature of 50°C for 30 minutes.

1.12.6 Culture media

The primary isolation medium for Legionella species is buffered charcoal yeast extract (BCYE) agar supplemented with alpha-ketoglutarate, iron and cysteine. Legionellae do not grow in the absence of iron or cysteine. The presence of certain organisms may inhibit legionellae growth, but the selectivity of agar media can be improved by the addition of antimicrobial agents such as amphotericin B, colistin, cefamandole, polymyxin B, vancomycin and trimethoprim, antifungal agents like anisomycin and cycloheximide and inhibitors like glycine as summarised in Table 1.10. Glycine is an excellent inhibitor of environmental flora, but not effective against respiratory flora (Stout 1998).

The addition of 2.5-5% CO_2 to the atmosphere is essential for the culture of some Legionella species although concentrations above 5% will increase the pH of the medium, thereby inhibiting growth. An extracellular, water–soluble compound is produced when some legionellae are grown on BCYE agar, resulting in fluorescence when exposed to long-wave (365 nm) ultraviolet light. All the known strains of *L pneumophila* provide fluorescence yellow-green while *L bozemanii, L dumoffii, L gormani, L anisa, L cherri, L steigerwaltii, L gratiana, L tusconensis* and *L parisiensis* will produce blue-white and *L rubrilucens* and *L erythra,* red fluorescence. The colour of the fluorescence can assist in differentiation of different species when present in one sample (Figure 1.1). Dyes can be added to solid culture media to aid identification further. It is important to note that certain antibiotics, particularly cafamandole, may inhibit some Legionella species (Stout

1998). *L micdadei* is sensitive to cefamandole but can be recovered on PAV medium. In a study involving 28 species, 11 species failed to grow on a cefamandole-containing medium and 8 species grew only marginally (Lee 1993). The inclusion of a non-selective plate is therefore recommended, even in highly contaminated samples (Stout 1998).

Table 1.10 Supplements in Legionella selective media

Medium	Supplements									
	Aniso[1]	Cefam[1]	Cefal[1]	Colis[1]	Cyclo[1]	Glyc[2]	Pol-B[3]	B-p[1]	B-b[1]	Vanco[1]
BMPAα	80	4	0	0	0	0	80	0	0	0
CCVC	0	0	4	16	80	0	0	0	0	0.5
WY	0	0	0	0	0	3	100	0	0	5
MWY	80	0	0	0	0	3	50	10	10	1
GVPC	0	0	0	0	80	3	80	0	0	1
GVP	0	0	0	0	80	0	80	0	0	1
DGVP	0	0	0	0	0	3	50	10	10	1
PAV	80	0	0	0	0	0	100	0	0	5
GPAV	80	0	0	0	0	3	100	0	0	5

[1]µg/ml; [2]mg/ml; [3]IU/ml; aniso=anisomycin; Cefam=cefamandole; Cefal=cefalothin; Colis=colistin; Glyc=glycine; Pol-B=polymyxin B; B-p=bromocresol purple; B-b=bromothymol blue; Vanco=vancomycin

1.12.7 Standardised culture methods

There is no one generally accepted culture method for legionellae. However, a number of standards and guidelines have been developed in this regard and are widely accepted by different institutions. For example, in Australia the Australian Standard (AS 3896-1991) has been developed, in Europe the International Standard (ISO/DIS 11731-1996) and in the USA, the ASTM Standard Guide for Investigating Water Systems for Legionellae (D 595201996). A method published by Stout in 1998 is also often used in the USA. In South Africa, an adaptation of the Most Probable Number method is also used in addition to the European method.

The Draft International Standard (ISO/DIS 11731), accepted by most European countries as a standard method in 1996, is used to demonstrate the presence of confirmed Legionella species in the majority of environmental sample types, ranging from water to swabs, biofilm and sediment. Water samples are concentrated by centrifugation or membrane filtration and biofilm and scale samples are diluted prior to testing. To reduce the growth of unwanted, non-legionellae organisms, a portion of the sample is subjected to heat treatment and another to acid treatment before culture. Treated and untreated portions are then inoculated onto plates of agar medium and incubated aerobically at 37°C for up to 10 days. After incubation morphologically characteristic colonies (reported as *presumptive* legionellae) are tested for cysteine dependence. For

species identification direct immunofluorescence, latex agglutination or biochemical tests can be done. Results are reported as an estimated number of *confirmed* legionellae per millilitre of sample. Agar media used for this method are bufferened charcoal yeast extract (BCYEα) as a non-selective and GVPC (glycine/vancomycin/polymyxin/cycloheximide) agar as a selective medium.

Stout *et al.* (1998) published a method widely used in the USA. This method recommends slightly different procedures for potable and non-potable waters, biofilms and swab samples. In general, swabs are pretreated with acid for 3 minutes prior to inoculation onto BCYEα and DGVP agar plates. Samples from hot-water tanks are inoculated directly (without concentration or pretreatment) whereas samples collected from faucets and showerheads are concentrated and acid treated before inoculation. Sample concentrates are resuspended in a small volume of the original sample. Cooling water samples are not concentrated and are inoculated directly onto BCYEα. After an initial incubation period the concentrates are acid treated for 15 minutes if overgrowth by non-legionellae occurs and inoculated onto BCYEα, DGVP and CCVC agar. Confirmation is done as stipulated in the ISO method.

The Australian Standard (AS 3896-1991) is applicable to water samples only. Samples are concentrated by centrifugation or membrane filtration and inoculated onto a number of selective and non-selective agar media, either as untreated or as heat-treated portions. Acid treatment is recommended for highly contaminated samples only. Culture plates are incubated aerobically at 37°C for up to 10 days. Legionellae are confirmed by cysteine dependence and species identification by latex agglutination, direct or indirect immunofluorescence or any other suitable method. Results are reported as an estimated number of *confirmed* Legionella organisms in a millilitre of sample. Agar media used are BCYEα as a non-selective and MWY (modified Wadowski-Yee) and BMPAα as selective media.

The Most Probable Number (MPN) is a quantitative method, adapted for enumeration of legionellae in water samples by workers at the Centre for Scientific and Industrial Research (CSIR) (Grabow *et al.* 1991). This method does not make provision for the testing of biofilm, sediment or scale. After concentration by membrane filtration appropriate serial dilutions are made and inoculated in triplicate onto BCYEα agar. No selective media or sample pretreatment steps are included in the method. The plates are incubated aerobically at 37°C for three days where after a representative smear from each agar plate is stained by direct immunofluorescence (DFA). Plates are recorded as positive when they contain morphologically typical Legionella colonies and yield a positive

DFA test. MPN statistical tables are used to calculate the number of Legionella organisms in the original sample and are usually reported as *presumptive* legionellae.

1.12.8 Colony morphology

Colonies of legionellae are smooth with an entire edge and exhibit a characteristic ground-glass appearance (Figure 1.5). The type of agar media used for isolation will influence the colour of the colonies, ranging from white to grey and blue, brown, pink or even green or deep red. When viewed under a plate microscope, some colonies may display pale blue or pink edges. Typical Legionella colones on BCYEα agar are illustrated in Figure 1.1.

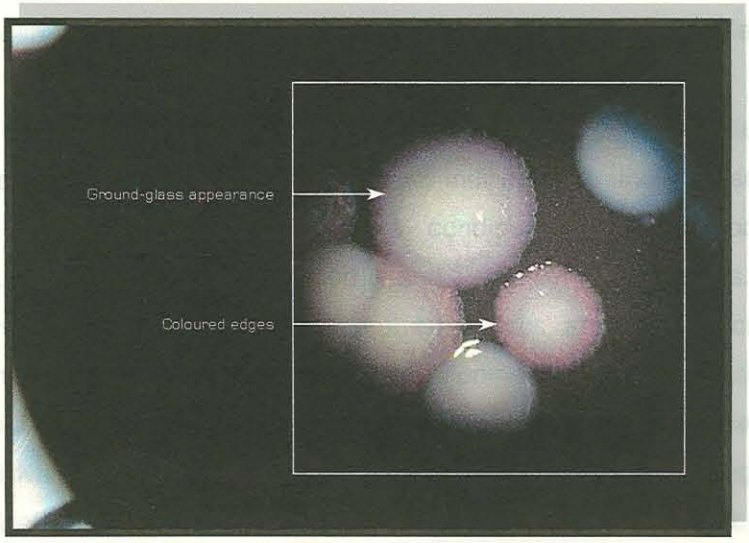


Figure 1.1: Colony morphology on BCYE agar

1.12.9 Cysteine dependence

The first step in Legionella identification is testing morphologically characteristic colonies for cysteine dependence. This is achieved by inoculation onto BCYEα agar (containing cysteine) and blood- or nutrient agar as a negative control. The plates are incubated plates aerobically at 37°C until growth is visible on the BCYEα plate. Colonies growing only on BCYEα are regarded as cysteine dependent and reported as presumptive legionellae.

1.12.10 Direct immunofluorescence

Direct immunofluorescence (DFA) is most commonly used for confirmation of Legionella species from environmental samples. Various polyclonal and monoclonal reagents are available internationally; the reagent available in South Africa is specific for *L pneumophila* SGs 1-6 and *L micdadei.* The test is simple to perform, but interpretation requires a fair amount of experience, especially in highly contaminated samples. Antigen from the sample is fixed to a microscope slide using heat or acetone 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 ultraviolet light.

A number of factors can influence the reactivity of Legionella antigens in surface antigen-dependent tests such as direct and indirect immunofluorescence. These factors include the culture media and culture conditions, the method used for antigen preparation, the fixation method and strain differences. Although direct immunofluorescence (DFA) is very useful to detect antigens in clinical samples when cultures cannot be obtained, its value for environmental samples is controversial although it is used routinely in South Africa (Tobiansky et al 1986). Cross-reactions that may lead to false positive results have been documented to occur between Legionella species and *B fragilis, P alcaligenes, P fluorescens* and *B pertussis* in the DFA test (Grimont 1986, Roig *et al.*1991). Although indirect immunofluorescence (IFA) is routinely used for clinical samples, its value for the identification of Legionella species from environmental samples is not clear.

A typical positive DFA test is illustrated in Figure 1.2.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

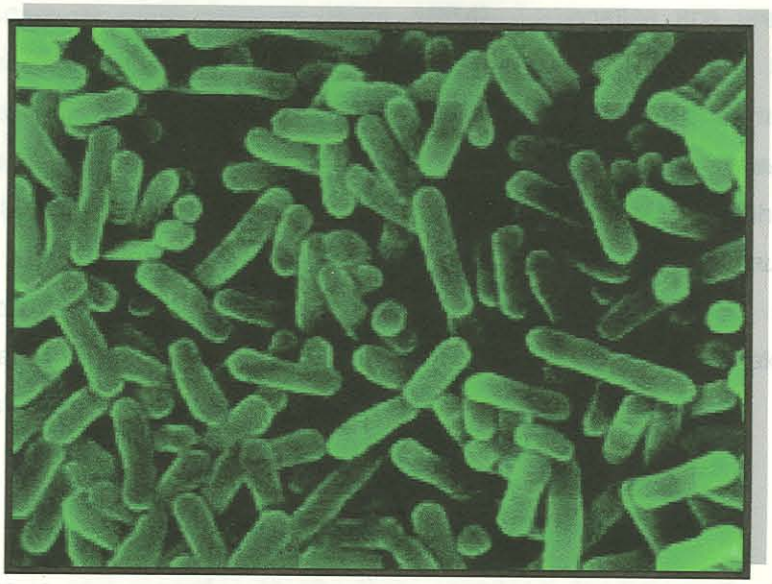


Figure 1.2: Positive DFA

1.12.11 Agglutination tests

A commercially available latex agglutination test is often used in addition to the DFA described above. The three reagents supplied with the test kit commonly used in South Africa are specific for *L pneumophila* SG 1, *L pneumophila* SGs 2-14 and Legionella species respectively. The Legionella species include *L longbeacheae* SGs 1-2, *L bozemanii* SGs 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*. The test is recommended for confirmation of morphologically characteristic colonies on agar media.

Several new test kits have recently been developed. Oxoid has brought out a 'dry spot' test range and several agglutination tests are on the market (Hart et al 2000).

1.12.12 Biochemical tests

Legionella species are not easily distinguished by routine biochemical tests. With the exception of starch, the organisms do not ferment or oxidise carbohydrates (Yu 1990). Most strains of *L pneumophila* and *L feeleii* are hippurate hydrolysis positive but the reaction is variable in *L bozemanii, L micdadei, L dumoffii* and some of the other legionella-like organisms (Hébert 1981). Catalase, peroxidase and superopxide dismutase (SOD) is not present in all legionellae (Pine et al 1986). However, according to Yu (1990),

all legionellae contain catalase but the reaction is weak in comparison with other catalase-positive organisms. *L pneumophila* contains only peroxidase, *L bozemanii* only catalase and *L dumoffii* both catalase and peroxidase. The other legionellae are all catalase positive or weak positive (Winn 1984). Legionellae are urease negative and gelatinase positive, nitrate reduction negative and starch positive (Winn 1984). The oxidase reaction is negative in *L pneumophila* but variable in other legionellae whereas beta-lactamase is produced in *L micdadei, L feeleii* and *L maceachernii* and in *L pneumophila* (Thorpe et al 1981, Yu 1990). Extracellular protease, phosphatase, lipase, ribonuclease and deoxyribonuclease have been detected in *L pneumophila*, whereas elastase, collagenase, lecithinase, hyaloronidase, chondroitase, neuramidase and coagulase reactions were absent and alpha-amylase activity was weak in *L pneumophila* during early studies (Thorpe et al 1981).

1.12.13 Molecular methods

Culture is still regarded as the 'gold standard' for Legionella detection, but has several disadvantages. To culture legionellae require several days of incubation, heterotrophic flora from a sample may overgrow the culture media and make plate counts difficult or even impossible, or the organisms may be in a viable but nonculturable (VBNC) state. However, recent developments in the molecular field opened doors for new detection assays of waterborne pathogens such as Legionella; these methods include DNA probe hybridisation (Grimont *et al.* 1985), restriction enzyme digestion (Saunders *et al.* 1990) and the polymerase chain reaction (Bej *et al.* 1991, Mahbubani *et al.* 1990). Although the sensitivity of most of these techniques is insufficient for direct detection of legionellae in environmental samples, PCR has proven to be a sensitive and rapid alternative to culture (Bej *et al.* 1991, Mahbubani *et al.* 1990, Ng *et al.* 1997, Palmer *et al.* 1993).

Many PCR assays have been described, but relatively few of them have been extensively studied on clinical as well as environmental samples and none are routinely used. Polymerase chain reaction (PCR)-based detection methods are normally highly sensitive and specific. In theory, PCR should be able to amplify the number of targets 10^6-fold, in other words it should be able to detect 1-10 bacteria/litre (Tompkins and Loutit 1993). These workers reported a sensitivity of 1-500 bacteria per sample and specificity of 100% following PCR following amplification of Legionella species and other aquatic bacteria. Waters tested from two outbreaks using their PCR method showed a sensitivity

of 57-60% and specificity of 93-100%. Despite this, the sensitivity of PCR is not yet as high as that of culture.

The sensitivity of PCR depends on a number of factors including effective cell lysis, target DNA availability, competition from non-target DNA and the absence of inhibitory substances that might interfere with polymerase enzyme activity (Maiwald *et al.* 1995, Palmer *et al.* 1993). PCR reactions are especially prone to inhibition from organic chemicals such as humic acids that may be present in environmental samples. In order to overcome this inhibition, workers suggest DNA purification and sample dilution.

According to Tompkins and Loutit (1993) amplification of legionellae before probing with specific chromosomal DNA fragments should overcome the problems of 'non-viable' and 'viable but non-culturable' cells. Primers derived from the 5S rRNA gene detect all Legionella species whereas those developed using the *mip*-gene detect only *L pneumophila* strains (Tompkins and Loutit 1993). Most of their studies were done using seeded samples. Whether these methods will work with environmental samples is not clear. According to Bej *et al.* (1993) all the *L pneumophila* serogroups have been identified using the *mip*-gene for PCR. She recommended combining PCR with endonuclease analysis of a portion of the amplified *mip* gene as a potential tool for identification of the various *L pneumophila* serogroups.

Ligase chain reaction (LCR) is another promising molecular method reported by Tompkins and Loutit in 1993. The difference between PCR and LCR is that LCR oligonucleotides are designed to completely cover the target so that one set of oligonucleotides hybridises with the left and one set with the right half. The ligase enzyme then seals the nick between the two halves, thereby creating a full-length complementary sequence. This is followed by the usual amplification. The key to LCR use is that the ligase enzyme will not seal gaps that are not perfect matches, so even if mismatched nucleotides may hibridise to the target they will not be held together to permit subsequent priming for amplification. The advantage of LCR is that very minor mutations (including single base substitutions or deletions) can be detected by using nucleotides that are complementary to the mutation.

Other molecular tools for the epidemiologic characterisation of Legionella strains involve the use of soluble protein patterns, DNA restriction endonuclease profiles, multilocus enzyme analysis or orthogonal-field-alteration gel electrophoresis (Ehret *et al.* 1993). These authors also compared sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE), restriction enzyme cleaviage of genomic bacterial DNA followed by conventional agarose gel electrophoresis (REA) and analysis of the patterns of DNA fragments separated by pulsed-field gel electrophoresis (PFGE). Their results

indicated that these molecular methods were more efficient than the use of monoclonal antibodies for analysis.

Fluorescent in situ hybridization tests are useful for the detection of legionellae in respiratory tract samples. The method makes use of oligonucleotide probes targeting rRNA and offers a rapid and specific alternative to DFA, culture and urine antigen testing in clinical laboratories but has not been extensively tested in environmental samples. However, Grimm *et al.* (2000 conference posters) reported that fluorescent in situ hybridisation is valuable to identify legionellae in their natural environment. They demonstrated that this method is also valuable for identification of the potential host organisms, Hartmanella and Naeglieria species, from the environment.

1.12.14 Typing methods

Legionella identification is becoming more complex as more species and serogroups are being recognised. The diversity of types and subtypes and the ubiquitous nature of the organisms in water and other moist environments make it difficult to identify epidemic strains in particular. In addition, it is not uncommon to detect more than one species or strain from a single plate of primary culture media. Identification of legionellae below the subspecies level is especially necessary in outbreak situations in order to verify associations between patients and sources of infection.

To simplify this process a number of molecular tools have become available recently in addition to microbiological and immunological methods. These methods include arbitrarily primed PCR (AP-PCR), ribotyping, pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), serologic techniques, plasmid analysis, electrophoretic alloenzyme typing (ET), restriction fragment length polymorphism analysis, RNA or DNA probing of DNA digests and other methods such as pulsed-field electrophoresis of DNA digests. Serologic methods to subtype legionellae include the use of polyvalent [polyclonal] and monoclonal antibodies. A number of monoclonal antibody panels have been developed and are commonly used in the USA, UK and Canada (Barbaree 1993). The majority of these methods have been developed specifically to subtype *L pneumophila* SG 1 (Benson *et al.* 2000 [p29], Lück [030], Lundholm [032], Yu 1990)

- ⇨ Plasmid analysis is of limited use as not all legionellae contain plasmids (Barbaree 1993 book).
- ⇨ Electrophoretic alloenzyme typing (ET), first published by Selander *et al.* in 1985, has been used with some success to subdivide epidemic strains of legionellae but the method is very labour intensive and is not widely used.

- ⇨ Ribotyping involves extracting DNA, nicking it with an endonuclease and probing it with labeled 16S and 23S rRNAs from *E coli* (Grimont *et al.* 1989). This method has been used at the CDC to subtype *L pneumophila* strains implicated in outbreaks (Barbaree 1993).
- ⇨ Restriction endunuclease analysis of total genomic DNA has the advantage of discriminating among strains of *L pneumophila* lacking plasmids, but electrophoretic patterns of the total genomic DNA digests are often difficult to interpret (Bej *et al.* 1993). For this reason an alternative method that uses radiolabeled probe fingerprint analysis following restriction endonuclease digestion of total genomic DNA has been described.
- ⇨ Monoclonal antibodies have been used to screen phenotypic differences between strains of *L pneumophila* (Struelens *et al.* 1993). Several panels of monoclonal antibodies have been developed (Ehret *et al.* 1986, Joly *et al.* 1986).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 2

EVALUATION OF DETECTION METHODS FOR LEGIONELLA USING SEEDED SAMPLES

2.1 SUMMARY

South African laboratories are currently using various methods in a non-standardized approach to detect *Legionella* species in environmental samples. In an attempt to provide guidelines for the development of a standard method, a number of currently available detection methods were evaluated, using seeded samples of sterile and non-sterile tap water, cooling water and make-up water.

The samples were seeded with a type strain of *L pneumophila* serogroup 1 (American Type Culture Collection 33152). The effect of sample concentration by centrifugation and membrane filtration followed by either vortex or sonication for resuspension of organisms was studied. Three currently available culture methods were evaluated, the International standard method (ISO/DIS 11731), the Australian standard method (AS 3896 - 1991) and a locally-developed adaptation of the most probable number method (MPN). In addition, the direct immunofluorescence test and a commercially available latex agglutination test kit were included in the evaluations. The usefulness of treatment with acid or heat prior to culture was also compared.

Our results indicated that concentration by membrane filtration using nitro-cellulose filters with a pore size of 0.45 μm, followed by sonication for 10 minutes, would be the most appropriate concentration and resuspension method for the samples. In the absence of sample pretreatment with acid or heat, organism recovery from sterile seeded samples on BCYE ranged from 85.9 – 98.7%. However, in the non-sterile samples, these figures dropped to 8.1 – 38.5%. Sample pretreatment resulted in a further loss of at least 50% of organisms in all the samples, regardless of the pretreatment method or culture medium used. In general, the ISO and AS methods were more appropriate than the MPN method for organism recovery from sterile seeded samples. However, for the non-sterile samples, the MPN method yielded better recovery.

2.2 INTRODUCTION

Large numbers of legionellae in water distribution systems present a potentially serious health risk to workers and the public. Since the first isolation of legionellae in 1976, numerous legionellosis outbreaks have been documented and there has been a steady increase in the incidence of sporadic cases (Lye et al. 1997). For example, the two most recently reported outbreaks, one at a flower show in the Netherlands (Den Boer et al. 2000) and the other in an aquarium in Australia (Tallis et al. 2000), resulted in about 246 confirmed Legionnaires' disease cases. This clearly illustrates the importance of the disease and highlights the need for appropriate detection methods.

Despite new developments in the detection of *Legionella* in environmental sources, it remains problematic. Legionellae were initially isolated by the inoculation of guinea pigs, but with the development of suitable media these expensive and time-consuming techniques were replaced by culturing. Additional methods like radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), agglutination tests and nucleic acid probes have since been developed and tested in attempts to simplify *Legionella* identification. More recently, a number of polymerase chain reaction-based (PCR) assays have been documented (Mahbubani et al. 1990, Bej et al. 1991, Lye et al. 1997). Although some of these methods were proven to be very successful, culturing remains the method of choice for detection of *Legionella* species from the environment. To improve the recovery of *Legionella* by culturing, the use of certain treatment steps to minimize contamination by non-legionellae, have been introduced (Bopp et al. 1981, Groothuis and Veenendal 1983). However, despite these developments, no one method has thus far proven to be ideal for all samples in all given circumstances and environments.

Standard culture methods for *Legionella* detection have been formulated in the USA, Britain and Australia, but such standards have not been set for South Africa. Local laboratories have been testing water samples using a variety of culture methods, using a non-standardized approach. Some of these methods are time-consuming, require special reagents and culture media and a high degree of technical skill in their application. The apparent preference of *Legionella* for biofilm conditions and the potential role of protozoa in their multiplication and distribution are not considered in these conventional methodologies. This resulted in contradictory results regarding water quality in South Africa and a lack of confidence in local water testing, specifically for the presence of legionellae.

With this in mind, a research project was launched in 1996 to address some of the controversial issues regarding *Legionella* detection in South Africa. The first stage of the project dealt with the evaluation of a number of isolation and identification methods, using water samples seeded with a type strain of *L pneumophila* (ATCC 33152). These results are reported here. Three currently available identification methods were evaluated: the Draft International Standard (ISO) method (ISO/DIS 11731, 1996), the Australian standard (AS) method (AS3896-1991) and a locally-developed Most Probable Number (MPN) method (Grabow et al. 1991). In addition, the direct immunofluorescence (DFA) and a commercially available latex agglutination (LA) test kit were evaluated. The ISO and AS methods require the confirmation of *Legionella* in environmental samples to species level. They are the same in principle, but make use of different culture media and sample pretreatment steps. After concentration, water samples are treated with heat and/or acid to reduce the number of non-legionellae present. Appropriate dilutions of the sample concentrates are then inoculated onto selective and non-selective agar media and incubated. Thereafter, confirmation tests are carried out. Buffered charcoal yeast extract agar containing alpha-ketoglutarate (αBCYE) is used as a non-selective medium for both methods. The ISO method makes use of αBCYE supplemented with cycloheximide, glycine, polymyxin B and vancomycin (GVPC) as a selective medium. The AS method recommends αBCYE supplemented with anisomycin, cefamandole and polymyxin B (BMPA); and αBCYE supplemented with anisomycin, glycine, polymyxin B, vancomycin, bromocresol purple and bromothymol blue (MWY) as selective media. The MPN method involves sample concentration followed by dilution and inoculation in triplicate onto αBCYE agar plates. No selective media or pretreatment steps are used. After incubation, smears of growth are examined microscopically using the DFA for confirmation. MPN statistical tables are used to calculate the number of *Legionella* organisms in the original sample.

The results highlight the differential efficiencies of concentration, pretreatment and culturing methods when applied to different types of samples. Following the equivalent comparison of the above methods on a variety of specified samples, one was able to conclude with some specific and useful recommendations in *Legionella* diagnostics.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.3.1 Seeding of samples

A type strain of *L pneumophila* SG 1 was obtained from the American type culture collection (ATCC 33152) and maintained under normal laboratory conditions. Freeze-dried cultures were reconstituted in 0.5ml sterile distilled water and aliquots of 0.1ml were stored at –20°C until required. Strain viability and purity was tested by inoculation onto αBCYE agar and incubation, aerobically, at 37°C for 3-5 days. Plate cultures were stored at 4-6°C.

Fresh sub-cultures were prepared on αBCYE medium before each experiment.

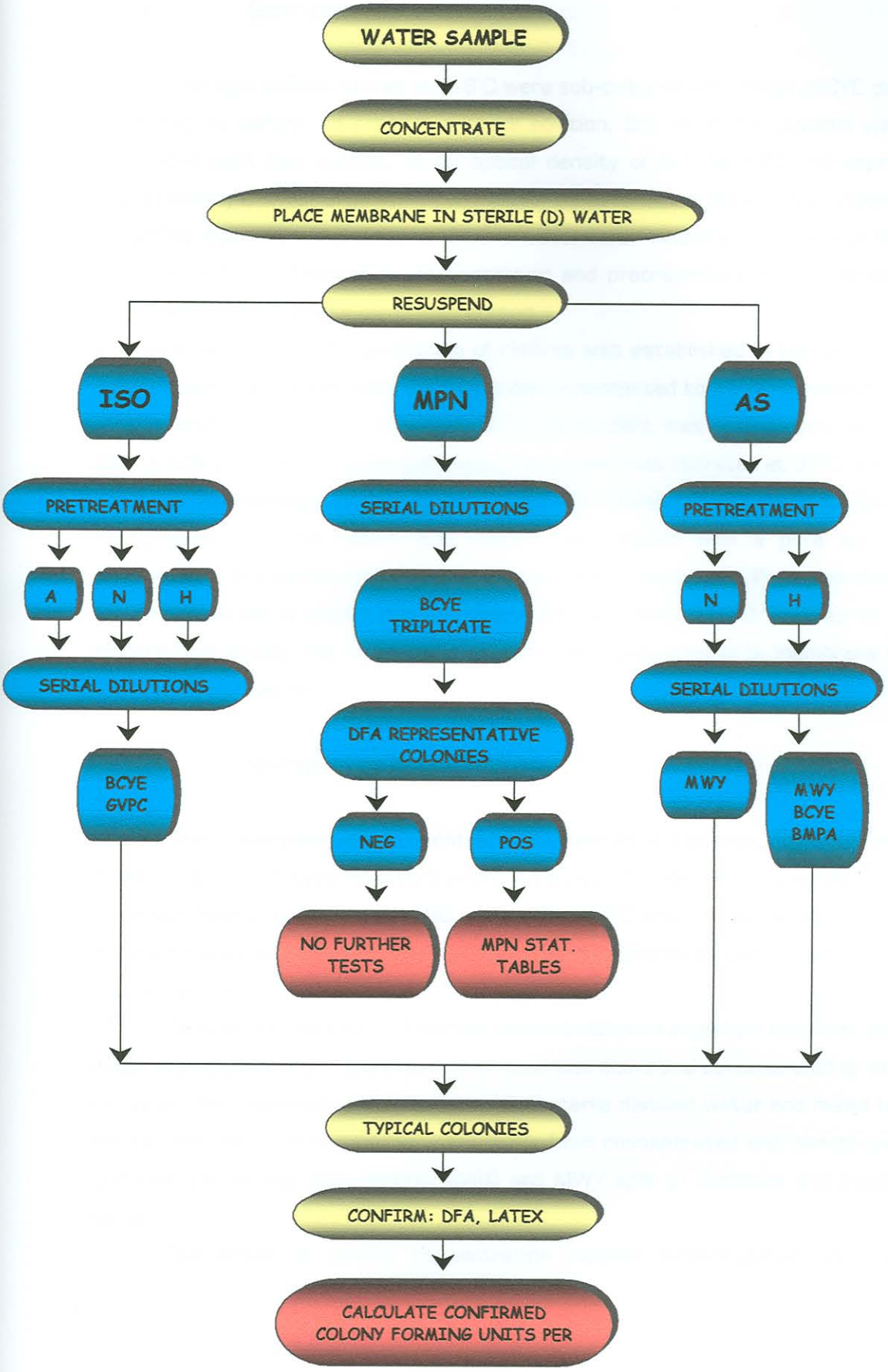
Stock solutions for seeding of samples were prepared by inoculating sterile distilled water with this culture, to an optical density of 0.1 (wavelength 620 nm), representing $8x10^7$ organisms per milliliter, as determined previously by colony counts on αBCYE agar using standard methodology. The final seeding was done by inoculating 5 ml of this stock into 500 ml of sample. Sterile and non-sterile samples of tap water, cooling water and make-up water were seeded for evaluation. Evaluations were carried out immediately after seeding.

2.3.2 Samples evaluated

The seeded samples evaluated are listed in Table 2.1 and consisted of sterile and non-sterile tap-, cooling- and makeup water as well as biofilm seeded with *L pneumophila.*

Table 2.1 Seeded samples evaluated

NUMBER	SAMPLE IDENTIFICATION
1	Sterile seeded tap water
2	Sterile seeded cooling water
3	Sterile seeded makeup water
4	Non-sterile seeded tap water
5	Non-sterile seeded cooling water
6	Non-sterile seeded makeup water
7	Seeded biofilm
8	Seeded biofilm
9	Water colledted with seeded biofilm
10	Water collected with seeded biofilm


WATER SAMPLE
CONCENTRATE
PLACE MEMBRANE IN STERILE (D) WATER
RESUSPEND
ISO
MPN
AS
PRETREATMENT
SERIAL DILUTIONS
PRETREATMENT
A
N
H
BCYE TRIPLICATE
N
H
SERIAL DILUTIONS
SERIAL DILUTIONS
BCYE GVPC
DFA REPRESENTATIVE COLONIES
MWY
MWY BCYE BMPA
NEG
POS
NO FURTHER TESTS
MPN STAT. TABLES
TYPICAL COLONIES
CONFIRM: DFA, LATEX
CALCULATE CONFIRMED COLONY FORMING UNITS PER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.3.3 Sample inoculation

The type strains stored at 4-6°C were sub-cultured onto fresh αBCYE plates and incubated as before. To prepare a stock solution, 5ml of sterile distilled water was inoculated with this culture, to an optical density of 0.1 (at 620 nm) representing approximately 8 X 10^7 organisms per milliliter as determined previously by colony counts on αBCYE agar. For final seeding, 500ml water was inoculated with 5ml of the stock solution. This was followed by concentration and pretreatment as stipulated in each method evaluated, unless otherwise stated.

A reactor for the generation of biofilms was established in the laboratory. The system consisted of a modified Pedersen device connected to a 5-litre reservoir through which water, obtained from an industrial cooling system, was continuously circulated, to form a biofilm on glass microscope slides. The system was operated at 37°C. Using phase contrast microscopy, biofilm formation on the slides was monitored daily. After establishment of the biofilm, the system was seeded with a pure culture of *L pneumophila.* The biofilm slides and water circulated through the Pedersen device were screened, placed in sterile distilled water and sonicated for 10 minutes to dislodge biofilms from slides. The water samples were then concentrated by membrane filtration followed by sonication.

2.3.4 Sample concentration

Water samples were concentrated by membrane filtration, using a 3-piece PVC manifold fitted with type HA filters with a pore size of 0.45μm (Millipore SA). Filter cups were sterilised by autoclaving for 30 minutes at 121°C prior to use. After concentration, the membranes were aseptically removed, placed into sterile containers with 10ml sterile distilled water.

To evaluate the effect of sample concentration on organism recovery, sterile tap water was seeded with *L pneumophila* as described above and concentrated by membrane filtration. The membrane was placed in 10ml sterile distilled water and mixed by vortex for two minutes. Serial dilutions were made from concentrated and non-concentrated portions, plated out onto αBCYE, GVPC and MWY agar in duplicate and incubated as usual.

The effect of sample concentration method (centrifugation *vs.* membrane

filtration) on organism recovery was evaluated by seeding sterile tap water with *L pneumophila*, concentrating one portion by membrane filtration as above and centrifuging the other portion at 6000g for 10 minutes. The sediment was resuspended in sterile distilled water, serial dilutions were made and inoculated onto αBCYE agar in duplicate.

2.3.5 Sample resuspension

To evaluate the effect of sample concentration and resuspension by vortex on organism recovery, sterile tap water was seeded with *L pneumophila* and concentrated as described above. The membrane was placed in 10ml sterile distilled water and mixed by vortex for 2 min. Serial tenfold dilutions of concentrated and non-concentrated portions were made in sterile distilled water, plated onto αBCYE agar in duplicate and incubated as usual. Sample concentration by membrane filtration and centrifugation was compared by centrifuging one portion of the sample at 6000g for ten minutes. The sediment was resuspended in sterile distilled water where after serial dilutions were made and inoculated onto αBCYE agar in duplicate.

2.3.6 Sample pretreatment

Acid buffer was prepared by adding 3.9ml of a 0.2mol/l hydrochloric acid solution to 25ml of a 0.2mol/l solution of potassium chloride. The pH was adjusted to between 2.0 and 2.2. The solution was stored at room temperature in the dark, for no longer than two weeks. Acid treatment was done following the method of Bopp *et al.* (1981). One milliliter of sample concentrate was centrifuged at 3000g for 30 minutes, 0.5ml of the supernatant was removed and the sediment resuspended in the remaining 0.5 ml of supernatant by vortex. To this, 0.5ml acid buffer as added, gently mixed by inverting and left to stand at room temperature for 5 minutes. Serial dilutions were made and culture media inoculated immediately afterwards.

For heat treatment the method of Groothuis and Veenendal (1983) was followed. One milliliter of sample concentrate was heated in a water bath at 50°C for 30 minutes. Serial dilutions and agar inoculations were done immediately after incubation.

The effect of sample pretreatment by acid and heat was studied on seeded sterile and non-sterile tap-, cooling- and makeup water samples. Seeding, sample concentration and pretreatment steps were carried out as described. Resuspension of bacteria after membrane filtration was done by sonication for 10 minutes.

2.3.7 Evaluation of culture media

Culture media were prepared as indicated in the ISO and AS methods. Serial tenfold dilutions were made in sterile distilled water. The agar plates were inoculated with 0.1ml of each dilution and incubated aerobically at 37°C. For evaluation of the ISO method, sample concentrates were divided into three portions, namely untreated, acid- and heat-treated and dilutions were made as above. Each of the portions was inoculated onto αBCYE and GVPC agar. For evaluation of the AS, serial dilutions made from the untreated portion were inoculated onto MWY agar and those from the heat-treated portion were inoculated onto MWY, BMPA and αBCYE agar. The MPN was evaluated by inoculating serial tenfold dilutions of the untreated portion onto αBCYE agar in triplicate (Grabow *et al.* 1991).

2.3.8 Comparison of culture methods

For comparison of the three methods (ISO, AS and MPN), agar media were inoculated as follows: For the ISO method, sample concentrates were divided into 3 portions: no pretreatment, acid pretreatment and heat pretreatment. Tenfold serial dilutions of each of these portions were inoculated onto αBCYE and GVPC agar. Only cysteine dependent colonies were confirmed by DFA and/or latex agglutination and reported. For the AS method, tenfold dilutions of each portion were inoculated onto MWY and BMPA agar.

2.3.9 Cysteine dependence

Morphologically characteristic colonies on agar media were tested for cysteine dependence by inoculating αBCYE and nutrient agar plates and incubating these until growth was observed on the αBCYE agar. Colonies growing on αBCYE but not on nutrient agar were regarded as cysteine dependent (*ie.* cysteine was required for growth), reported as presumptive legionellae and confirmed by direct immunofluorescence and/or latex agglutination.

2.3.10 Direct immunofluorescence (DFA)

Direct immunofluorescence (DFA) was done as follows: For the MPN method, representative smears were made from each of the three αBCYE plates of each dilution. For the ISO and AS methods, only CD+, single colonies were confirmed by DFA. Suspensions were made in sterile distilled water, of which 5 µl was placed onto a 12-well glass microscope slide, air-dried and heat fixed. An equal volume (5 µl) of DFA reagent (*L pneumophila* serogroups 1-6 and *L micdadei* polyvalent conjugate A, Zeus Scientific, Raritan, USA) was added and the slides incubated at 37°C in a moist chamber for 30 min. After incubation, the slides were rinsed twice for 10 min in phosphate buffered saline (PBS) at pH 7.6, air-dried and mounted in IFA mounting fluid (Zeus Scientific, Raritan, USA). The slides were read on an Olympus Model BH2 standard fluorescence microscope, equipped with an HBO-100 mercury-incident light source. Observations were made under a dark field using 10x ocular, 100x objective, oil immersion lenses. Only strongly fluorescent, typical short rod-shaped organisms were reported as DFA positive.

2.3.11 Latex agglutination

A latex agglultination test kit (LA) (Oxoid DR800M) was evaluated according to the manufacturers' instructions. Reagents supplied with the kit are specific for *L pneumophila* SG 1, *L pneumophila* SG 2-14, and Legionella species (including *L longbeacheae* SG 1-2, *L bozemannii* SG 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*).

2.3.12 Reporting and interpretation

Colony counts were performed on all agar media, for all of the dilutions. For the ISO and AS methods, counts of confirmed (*ie.* CD and DFA and/or latex positive) Legionella colonies as well as non-legionellae were recorded wherever possible. For the MPN method, the DFA result for each dilution was recorded (*ie.* DFA positive plates were not confirmed by latex agglutination). Overgrown plates were recorded as yielding a colony count of >300. For final calculations, colony counts between 30 and 300 were used. Where <30 colonies were observed in all dilutions, the number of colonies in the highest dilution was recorded.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2.3.13 Calculations

Cfu/ml was calculated as follows:

$$\text{Cfu/ml} = \frac{\text{CxDx10x10}}{\text{V}}$$

Where C = colonies counted
D = dilution
V = volume of concentrate

2.4 RESULTS AND DISCUSSION

2.4.1 Effect of sample concentration on organism recovery

For optimal recovery of legionellae from the environment, water samples have to be concentrated before culturing. Although both membrane filtration and centrifugation are widely accepted and used for this purpose, there is no consensus among workers regarding the efficiency and accuracy of either of these methods (Voss *et al.* 1984, Brindle *et al.* 1987, Boulanger and Edelstein 1995). For filtration to be effective, several factors have to be taken into account when choosing the type of filter and the pore size to use. The resuspension method used after filtration, the presence of biocides in the samples, the type of samples analysed and certain physical characteristics of the filters, like the brittleness, composition and pore structure (Brindle *et al.* 1987) may also influence the accuracy of organism recovery through filtration. The Centers for Disease Control (Atlanta GA) recommends the use of polycarbonate filters with a pore size of 0.2μm but it was found to be difficult to concentrate cooling- and makeup water samples through membranes with such a small pore size. The efficiency of different filter types was not investigated and Millipore Type HA filters with a pore size of 0.45μm that are commercially available and used by most laboratories in South Africa were used throughout the study.

The method of resuspension of the organisms after sample concentration may influence the accuracy of organism recovery from environmental samples. Organisms can be resuspended after filtration by either producing a vortex for a minimum of two minutes, or by placing the concentrate in an ultrasound tank for a maximum of ten minutes. However, to ensure optimal recovery by sonication, it is recommended in the ISO method that the time of immersion should be adjusted for different sample types and ultrasound tanks. South African laboratories generally accept sonication for 10 minutes as appropriate.

Different combinations of concentration and resuspension methods were compared. Our results (Tables 2.2 and 2.3) indicated a recovery rate of 14.4% in the filtered portion and 35.4% in the centrifuged portion after resuspension by vortex. However, when membrane filtration was followed by sonication, the organism recovery rate increased to 85.6%. These results correlated well with previously published reports of recovery rates of between 39% and 93% from environmental samples, using different types of membrane filters (Wolford et al. 1988). It was concluded that concentration by membrane filtration using nitro-cellulose filters with a pore size of 0.45μm, followed by sonication for 10 minutes, would be the most appropriate method for the samples.

Table 2.2 Effect of sample concentration on organism recovery

MEDIUM	UNCONCENTRATED (CFU)	CONCENTRATED (CFU)	RECOVERY (%)
BCYE	253	138	54.5
MWY	790	392	49.6
GVPC	640	384	60.0

Table 2.3 Effect of concentration and resuspension method on organism recovery

CONCENTRATION	RESUSPENSION	AVERAGE CFU/PLATE	RECOVERY (%)
Unconcentrated		195	
Centrifugation	Vortex	69	35.4
Filtration	Vortex	30	15.4
Filtration	Sonication	166	85.1

2.4.2 Comparison of culture media

The data summarized in Table 2.4 represent a comparison of the results obtained from sterile and non-sterile seeded samples, cultured on each of the four media (αBCYE, GVPC, BMPA and MWY) in the absence of pretreatment, after acid treatment and after heat treatment. In the absence of sample pretreatment, the recovery of confirmed *L pneumophila* from the sterile samples was high on the non-selective αBCYE plates (85.9, 98.7 and 89.7% for tap water, cooling water and makeup water respectively). The use of selective media resulted in a considerable decrease in organism recovery, depending on the culture medium used (Table 2.4). Acid treatment resulted in a further loss of organisms from the sterile samples, especially after culture on selective media. The number of organisms recovered after heat treatment was negligible in all the sterile samples that were evaluated, regardless of the culture medium used. These results were not surprising, given the fact that laboratory-adapted type strains of *Legionella* are known to be more sensitive to adverse conditions such as sample pretreatment than environmental strains (Roberts *et al.* 1987). In view of the sensitivity of the laboratory-adapted strains of *Legionella* used to seed the samples in this investigation, these results may not necessarily be directly applicable to environmental samples. However, this aspect needs to be investigated further. The recovery rate of confirmed legionellae from the non-sterile seeded samples was considerably lower than that of the sterile samples (Table 2.4). In the absence of sample pretreatment, culture on αBCYE yielded confirmed *L pneumophila* in only 8.1% of tap water samples, 23.1% of the makeup and 38.5% of the cooling water samples. Pretreatment resulted in a further loss of approximately 50% of organisms in all the samples, regardless of the pretreatment method or culture medium used. The confirmation of single colonies was complicated to some extent by the presence of non-legionellae on all the culture media, even after sample pretreatment with acid or heat.

Table 2.4. Recovery (%) of *L pneumophila* from seeded samples: comparison of culture media and methods.

Sample	Treatment	MPN	Culture media			
			Non-selective	Selective		
			BCYE(ISO, AS)	GVPC (ISO)	BMPA (AS)	MWY (AS)
Sterile TW	N	59.0	85.9	4.9	48.7	-
	A		76.9	1.4	44.9	20.0
	H		<1	<1	<1	<1
Sterile CW	N	26.9	98.7	6.7	18.2	42.3
	A		8.5	<1	2.3	<1
	H		<1	<1	<1	-
Sterile MW	N	3.6	89.7	3.1	6.8	57.8
	A		35.9	1.5	28.2	-
	H		<1	<1	<1	<1
Non-sterile TW	N	59.0	8.1	<1	4.5	10.1
	A		<1	<1	<1	<1
	H		<1	<1	<1	<1
Non-sterile CW	N	59.0	38.5	<1	2.6	1.5
	A		<1	<1	<1	<1
	H		12.8	<1	<1	2.6
Non-sterile MW	N	99.5	23.1	<1	14.4	20.5
	A		12.8	<1	<1	<1
	H		9.9	2.2	4.6	1.2

- Not done; A: acid treatment; H: heat treatment; N: no treatment; TW: tap water; CW: cooling water; MW: makeup water

2.4.3 Comparison of culture methods

When these results are interpreted in accordance with the ISO and AS specifications and compared with the MPN method, the following observations were made: Whereas the ISO and AS both provide a means of confirming legionellae to species level, this is not possible using the MPN. In general, the ISO and AS were more useful than the MPN for organism recovery from the sterile seeded samples (eg. 99.5% vs. 26.9% with the sterile cooling water samples). This may have been due to the increased specificity of the ISO and AS methods which made it possible to perform colony counts of confirmed legionellae, a step that is excluded from the MPN method. However, for the non-sterile seeded samples, the MPN method consistently yielded a higher recovery of Legionella. In explanation, it is likely that the legionellae could have been masked by non-legionellae on the non-selective agar plates used in the ISO and AS which complicated the recovery of single colonies for confirmation. Because the confirmation of single colonies as legionellae is not required by the MPN method, it was more sensitive than the other two methods for recovery of legionellae from the

non-sterile seeded samples. However, the DFA is known to detect non-legionellae, decreasing the specificity of the method considerably. In all the samples (sterile and non-sterile), the sample pretreatment steps required by the ISO and AS decreased the recovery of organisms significantly.

2.5 CONCLUSION

It was found that the methods used for sample concentration and resuspension may influence the outcome of culture experiments significantly. For the conditions and the sample types tested, the use of membrane filtration followed by sonication was preferred. The use of heat and acid treatment had a detrimental effect on the recovery of legionellae from seeded environmental samples. Therefore the application of the MPN method, which excludes these pretreatment methods, resulted in higher recovery rates and accounted for more accurate Legionella numbers in the samples evaluated. These findings provided a basis for further study, in which a survey of the prevalence of legionellae in specific environmental and industrial and water samples, not seeded with laboratory-adapted legionellae is investigated.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 3

EVALUATION OF DETECTION METHODS USING INDUSTRIAL SAMPLES

3.1 SUMMARY

Laboratories responsible for *Legionella* diagnostics around the world use a number of different culturing methods of non-equivalent sensitivities and specificities, to detect *Legionella* species in environmental samples. Specific countries usually standardize and use one approved method. For example, laboratories in Australia use the Australian Standard and those in Europe, the International Standard method. However, no standard culturing methods have been established in South Africa to date. As a result, there is uncertainty about the true prevalence and most common species of legionellae present in the South African environment. In an attempt to provide guidelines for the development of a standard method specific for South Africa, the International standard method (ISO), Australian standard method (AS) and a most probable number method (MPN) were evaluated and compared. In addition, the effect of sample re-incubation with autochthonous amoebae on culture outcome was studied. Samples were collected from four environments, representing industrial water, mine water and biofilm. The samples were concentrated by membrane filtration and divided into three portions and cultured without pretreatment, after acid treatment and after heat treatment, on four culture media namely αBCYE, BMPA, MWY and GVPC agar. A selective approach, incorporating heat treatment, but not acid treatment, combined with culture on αBCYE and GVPC or MWY, was most appropriate for legionellae detection in the samples evaluated. Legionellae were cultured from 82% of the environmental samples we evaluated. In 54% of the samples tested, legionellae were present in numbers equal to or exceeding 10^2 colony-forming units per milliliter (cfu/ml). *Legionella pneumophila* serogroups 1-14 were the most prevalent species and were present as single, or a combination of two or more serogroups in a number of samples tested. Re-incubation of sample concentrates with autochthonous amoebae improved the culturability of legionellae in 50% of cultures on αBCYE and 25% on GVPC.

3.2 INTRODUCTION

Since the first isolation of *Legionella pneumophila* in 1976, diagnostic methods and global surveillance have improved considerably, resulting in an increased awareness of the health risks involved in *Legionella* infections. Numerous outbreaks have been documented and there has been a steady increase in the incidence of sporadic cases reported. For example, at least three major outbreaks have been reported during the year 2000 alone, together resulting in more than 200 cases of Legionnaires' disease (Den Boer *et al.* 2000, Reuters 2000, Tallis *et al.* 2000).

The detection of *Legionella* species originally relied on the inoculation of susceptible guinea pig hosts. Although quite selective, these methods were expensive and time consuming and were soon replaced by culturing and identification on a variety of agar media, with subsequent addition of selective supplements and pretreatment methods to reduce heterotrophic growth. Despite recent advances in molecular technology and the development of diagnostic applications of the polymerase chain reaction (PCR), culturing is still considered the 'gold standard' for *Legionella* detection from the environment. However, several factors complicate the interpretation of culturing results. Culturing legionellae remains time consuming and labor intensive, the organisms may be present in a viable but non-culturable form or their growth on agar media may be inhibited by the presence of other organisms, especially in environmental samples.

Laboratories around the world use a variety of methods of non-equivalent sensitivities and specificities for culturing legionellae from environmental samples. This problem has been addressed in various countries through standardized protocols, for example in Australia (AS 3896-1991) and Europe (ISO/DIS 11731-1996). However, in Southern Africa, no such standards are available and local laboratories use a number of different detection methods. This has resulted in contradictory results regarding the quality of water in industrial distribution systems, especially with regard to *Legionella* contamination, which in turn led to a lack of confidence in local water testing results (personal communication, F Goede, Sasol, South Africa). This study was undertaken to provide Southern African laboratories and industries with guidelines for the development of a culturing method appropriate for our environment, and with information on the prevalence of legionellae in our industrial waters. In a previous study, a variety of sample types seeded with a reference strain of *L pneumophila* serogroup 1 (ATCC 33152) were used to compare different detection methods (Bartie *et al.* 2001). In this study, these

methods were applied to industrial samples. Two of the internationally accepted culturing methods and a locally developed most probable number method were evaluated.

The International Standard method (ISO/DIS 11731-1996), accepted in Europe as a standard and used by some laboratories in South Africa, demonstrates the presence of confirmed Legionella species in a variety of environmental sample types, ranging from water to biofilm and sediment. The test involves sample concentration followed by acid and heat treatment prior to culturing on selective (GVPC) and non-selective (αBCYE) agar. Cysteine dependent colonies are confirmed as legionellae by a variety of methods including direct or indirect immunofluorescence and latex agglutination. The Australian Standard method (AS 3896-1991) differs from the ISO method in that acid treatment is only recommended for highly contaminated samples, in the selective media used (BMPA and MWY agar) and in the sample dilutions recommended.

The quantitative most probable number (MPN) method has been adapted for enumeration of Legionella in water samples (Grabow *et al.* 1991). It involves similar sample concentration methods as above, followed by inoculation of serial dilutions of the concentrate in triplicate, using αBCYE agar. No selective media or sample pretreatment methods are incorporated into this method. After an incubation period of 3-7 days, representative smears are stained by direct immunofluorescence for confirmation. Plates are recorded as positive when they contain morphologically typical Legionella colonies and yield a positive DFA test. MPN statistical tables are then used to calculate the number of Legionella-like organisms in the original sample. No further confirmatory tests are carried out.

Our results indicated a high prevalence of *L pneumophila* in the industrial water samples we studied. Sample pretreatment with heat improved our culturing results considerably whereas acid treatment did not. In addition the viability of non-culturable legionellae was demonstrated by re-incubation in the presence of autochthonous amoebae. From these results, guidelines for a standard culturing method for detection of *Legionella* in non-potable industrial samples in South Africa are proposed.

3.3 MATERIALS AND METHODS

3.3.1 Samples evaluated

Twenty-eight industrial samples were evaluated. These consisted of 13 cooling waters from three sources, three biofilms from two sources and 12 waters from a gold mine, four from underground sites and eight from sites of the surface operations (Table 3.1). These 12 samples were chosen to represent areas that were previously tested and were positive by direct immuofluorescence but could not be cultured. (Bartie and Klugman 1997). The samples were concentrated immediately upon arrival at the laboratory. Two samples of wall scrapings were collected in sterile containers with a small volume of water from the same source.

Staff members at the various sources collected the samples in sterile, one-litre containers. The samples were placed in a cooled container and transported to the laboratory on the same day, where they were kept at 6-10°C until concentration. Concentrates that could not be processed immediately were stored in the dark, at room temperature, for no longer than 2 days.

Table 3.1 Industrial samples evaluated

SOURCE	DESCRIPTION	#	SAMPLE IDENTIFICATION
A	Manufacturer of plastics	1	Cooling water
		2	Cooling water
		3	Cooling water
		4	Cooling water
		5	Cooling water
B	Coal burning power station	6	Cooling water
		7	Cooling water
		8	Cooling water
		9	Cooling water
		10	Wall scraping from cooling tower sump
		11	Wall scraping from cooling tower sump
C	Petrochemical company	12	Cooling water
		13	Cooling water
		14	Cooling water
		15	Makeup water
		16	Biofilm
D	Gold mine – underground sites	17	Shaft 1, level 72
		18	Shaft 2, level 68
		19	Shaft 1, change house
		20	Shaft 3, level 14
		21	Shaft 4, change house
		22	Shaft 4, level 34
E	Gold mine – surface sites	23	Acclimatisation centre – inside
		24	Acclimatisation centre – outside
		25	Hostel 1, shower 3 east side, south tap 3
		26	Hostel 1, shower 3 south side, east tap 3
		27	Hostel 1, shower 7 north side, south tap 1
		28	Hostel 1, shower 15 south side, north tap 1

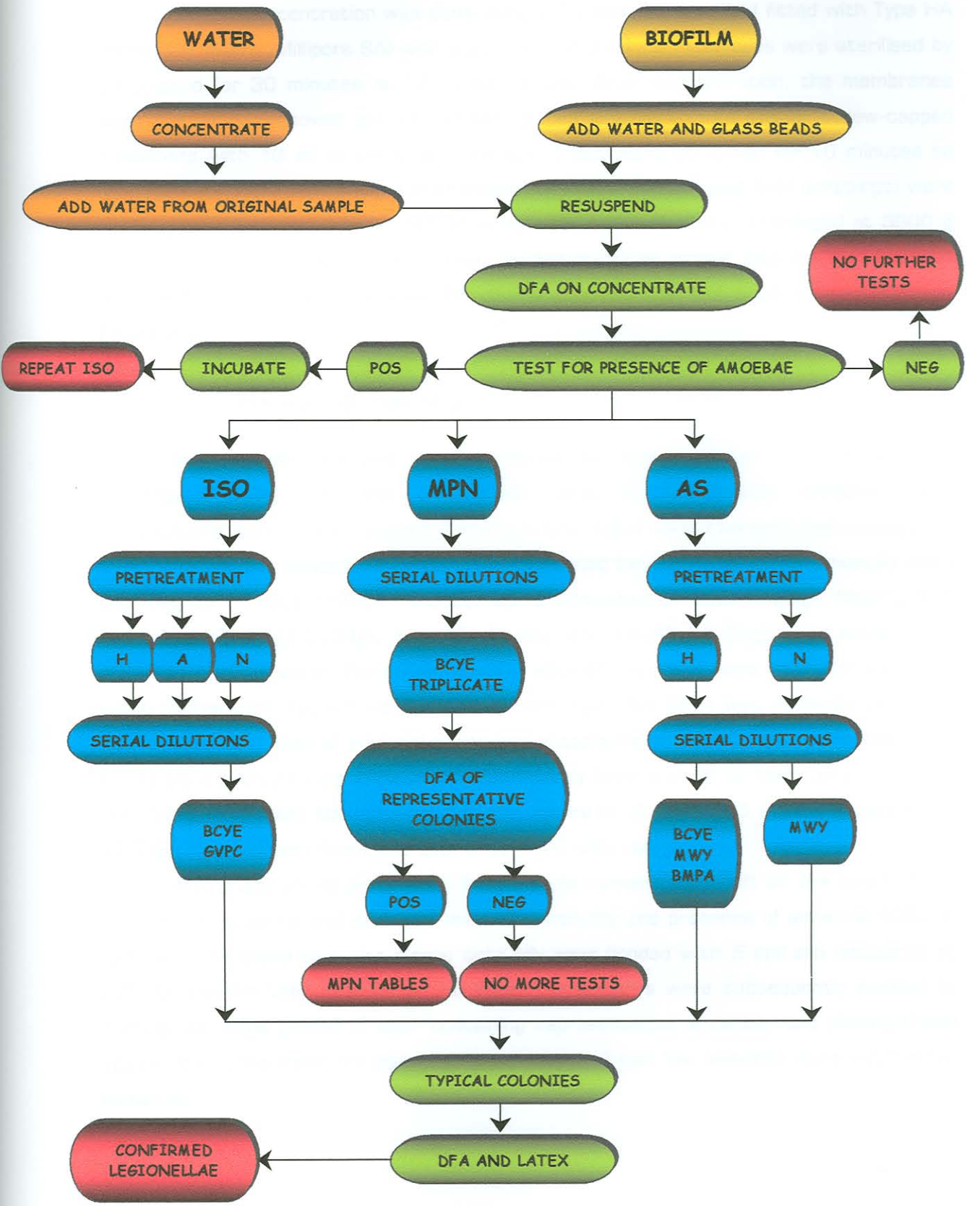


FIGURE 3.1: General procedure for evaluation of environmental samples

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.3.2 Sample concentration and re-suspension

Sample concentration was done using a 3-piece PVC manifold fitted with Type HA membrane filters (Millipore SA) with a pore size of 0.45μm. Filter cups were sterilised by autoclavind for 30 minutes at 121°C before use. After concentration, the membranes were aseptically removed, cut into smaller pieces and placed into sterile, screw-capped containers with 10 ml of the original sample. These were sonicated for 10 minutes to dislodge bacterial cells from the membranes. The sediment samples (wall scrapings) were made up to 50 ml with sterile distilled water and the biofilm was centrifuged at 3000 g for 30 minutes and the sediment resuspended in 10 ml sterile distilled water. These represented the prepared samples. The general procedure for this study is represented in Figure 3.1.

3.3.3 Sample screening

The prepared samples were screened for the presence of *L pneumophila* serogroups (SGs) 1-6 and *L micdadei* using a commercially available direct immunofluorescence (DFA) reagent (Zeus Scientific, USA) using standard methodology.

The sample concentrates were also screened for the presence of amoebae using the method of Page (1976). Amoebal saline consisted of NaCl (1.20g), $MgSO_4.7h_2O$ (0.04g), $CaCl_2.2H_2O$ (0.04g), Na_2HPO_4 (1.42g) and KH_2PO_4 (1.36g), each made up to 100ml in distilled water. For saline, 10ml of each of the above were combined and made up to 1 litre with distilled water. Non-nutrient agar (NN agar) was prepared by adding 15g of agar to 1 litre of amoebal saline and autoclaving at 121°C for 20 minutes. Agar plates were poured to a thickness of approximately 5mm and left at room temperature to set. The plates were flooded with a broth culture of *E coli* (ATCC 8739), incubated at 37°C until growth was fluent and stored at 4-6°C until use.

Wet preparations were made from sample concentrates, left on the bench for 5-10 minutes to settle and examined microscopically for the presence of amoebae. 100μl of sample concentrate was also placed onto NN agar flooded with *E coli* and incubated at 37°C to confirm the presence of amoebae. The cultures were subsequently purified by cutting out small pieces of agar containing representative amoebae, and placing these upside down onto fresh NN agar plates containing *E coli.* The amoebae were not further identified.

3.3.4 Sample pretreatment

To reduce the number of non-legionellae present, portions of the sample concentrates were treated with acid and heat. For heat treatment, 1 ml of the sample concentrate was incubated for 30 minutes in a water bath at 50°C. Serial dilutions were made immediately after incubation.

Acid treatment was carried out using the method of Bopp *et al.* (1981). Acid buffer was prepared by adding 3.9ml of a 0.2mol/l hydrochloric acid solution to 25ml of a 0.2mol/l solution of potassium chloride. The pH was adjusted to between 2.0 and 2.2. The solution was stored at room temperature, in the dark, for no longer than two weeks. For acid treatment, 1ml of the sample concentrate was centrifuged at 3000g for 30 minutes, 0.5ml of the supernatant was removed and the sediment resuspended in the remaining 0.5mlof supernatant by vortex. To this, 0.5ml of acid buffer was added, gently mixed by inverting and left to stand at room temperature for 5 minutes. Serial dilutions were made and culture media inoculated immediately afterwards.

3.3.5 Agar inoculation

Culture media were prepared as indicated in the ISO and AS methods. A series of tenfold dilutions were made in sterile distilled water. The agar plates were inoculated with 0.1 ml of each dilution and incubated aerobically at 37°C for 10 days in a humid atmosphere. The untreated portion of each sample was inoculated onto three αBCYE plates (as indicated in the MPN method) and one of each GVPC, BMPA and MWY plates to represent the selective media indicated in the ISO and AS methods. The acid- and heat-treated portions were inoculated onto one of each of the different agar media.

To compare the three methods, agar inoculations were done as stipulated in each of the methods. The sample concentrates were divided into three portions: no pretreatment, acid pretreatment and heat pretreatment. For the ISO method, tenfold dilutions of each of the portions were inoculated onto αBCYE and GVPC agar. Agar inoculation for the AS method was done as follows: the 10^{-1} dilution of the untreated portion was inoculated onto MWY agar, the undiluted, heat-treated portion onto BMPA agar and the 10^{-1} dilution of the heat-treated portion onto αBCYE and MWY agar (AS 3896-1991). In addition, the AS method was adapted to include serial dilutions as indicated in the ISO method (designated the ASM method). Both acid- and heat-treated portions of all these dilutions were inoculated onto BMPA and MWY agar. Cysteine dependent colonies were confirmed using direct immunofluorescence and/or latex

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

agglutination. For the MPN method, each dilution of the untreated portion was inoculated onto αBCYE agar in triplicate. Cysteine dependence was not confirmed. The incubation period for all three methods was 7-10 days.

3.3.6 Cysteine dependence

Morphologically characteristic single colonies were tested for cysteine dependence (CD) by inoculation onto αBCYE and nutrient agar and incubating until growth was observed on the αBCYE plate. Colonies that grew on both media were considered CD negative and were reported as non-legionellae.

3.3.7 Direct immunofluorescence (DFA)

For the ISO and AS methods, CD positive colonies were confirmed as Legionella species by direct immunofluorescence (DFA) and/or latex agglutination. For the MPN method, a representative smear from each agar plate of each dilution was tested by DFA as follows: 5 µl of concentrate was placed onto a glass slide in triplicate, air-dried and heat fixed. To this 5µl DFA reagent was added and the slides incubated at 37°C for 30 minutes in a moist chamber. After incubation, the slides were rinsed twice for 10 minutes in phosphate buffered saline (PBS) at pH 7.6, air dried and mounted. The slides were read on an Olympus Model BH2 standard fluorescence microscope. Observations were made under a dark field using 10x ocular, 100x objective, oil-immersion lenses. Only strongly fluorescent, typical rod-shaped organisms were reported as DFA positive.

3.3.8 Latex agglutination

A commercially available latex agglutination test kit (Oxoid, DR800M) was used for final confirmation. Cysteine dependent colonies were stained by DFA as described above. DFA positive colonies were reported as such and CD positive, DFA negative colonies were confirmed by latex agglutination. Reagents supplied in the kit allow confirmation of organisms as either *L pneumophila* SG 1, *L pneumophila* SGs 2-14, or *Legionella* species (including *L longbeacheae* SGs 1-2, *L bozemannii* SGs 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*).

3.3.9 Recording and interpretation of culturing results

Culturing results were recorded as follows: For the ISO, AS and ASM, the highest dilution yielding colonies confirmed as Legionella species by either DFA or agglutination or both, were reported. For the MPN, DFA results of representative smears for each dilution were recorded and no additional confirmation tests were carried out.

3.3.10 Sample re-incubation with autochthonous amoebae

Samples that were negative by culturing, or where the presence of legionellae could not be confirmed during the first culturing experiment, but contained amoebae, were re-incubated as described by Sanden and colleagues (1992): The concentrates were stored at 4-6°C until the results from the first experiment were available. After repeating the sonication step to remove aggregates that may have formed on the filter membranes during storage, the liquid was poured off into sterile, screw-capped, glass containers and incubated at 37°C for 10 days. The process of pretreatment and serial dilutions was then repeated as for the first experiment. In this case, only αBCYE and GVPC agar plates were inoculated to represent non-selective and selective media respectively.

3.4 RESULTS

3.4.1 Screening for the presence of Legionella and amoebae

Twenty-eight industrial water samples were evaluated for the presence of legionellae in a comparison of the ISO, AS, ASM and MPN methods. Of these, 26 (92.9%) were positive for *L pneumophila* SGs 1-6 or *L micdadei* by direct immunofluorescence (DFA) screening. Of the DFA positive samples, 18 (69.2%) were confirmed to contain legionellae with at least one of the culturing methods. Amoebae were present in 14 (87.5%) of the industrial water and biofilm samples screened, and n 3 (25.0%) of the mine water samples (Figure 3.2).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



Figure 3.2 Amoebae on NN agar

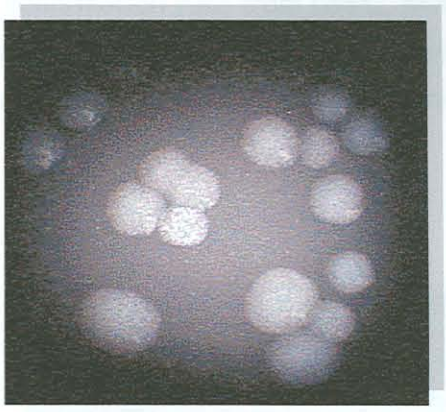


Figure 3.3 Ground-glass appearance

3.4.2 Evaluation of culture media

As expected, αBCYE agar was the most sensitive for legionellae of the culture media evaluated. However, confirmation in the absence of sample pretreatment was complicated by overgrowth of the plates and difficulties in distinguishing legionellae from non-legionellae (Table 3.2). In only 17.9% of the samples could legionellae be confirmed from this medium in the absence of sample pretreatment. A further 53.5% of αBCYE plates yielded presumptive legionellae after representative smears from the plates were stained by DFA. However, these organisms could not be confirmed by cysteine dependence or latex agglutination. The distinction between legionellae and non-legionellae on αBCYE was simplified to some extent by microscopic examination of colony morphology for the ground-glass appearance typical of Legionella species (Figure 3.3). Although most Legionella colonies had the typical sticky consistency (Figure 3.4) and some fluoresced under long-wave fluorescent light (Figure 3.5), this finding was not consistent and could therefore not be used for identification. In a number of cases the only means of visualizing presumptive legionellae on this medium was by staining a representative smear of growth by DFA.

In general, the use of selective agar media (BMPA, GVPC and MWY) in the absence of sample pretreatment improved the confirmation rate of legionellae considerably (Table 3.2) by decreasing the number of non-legionellae present, thereby simplifying the differentiation between legionellae and non-legionellae. However, the numbers recovered on these media were mostly one or two orders of magnitude lower than on αBCYE agar.

Although there was no significant difference in confirmation rate from GVPC, MWY and BMPA (32.2%, 28.6% and 25.0% respectively), the differentiation of legionellae from non-legionellae based on colony morphology was more difficult on BMPA agar than on the other two media.

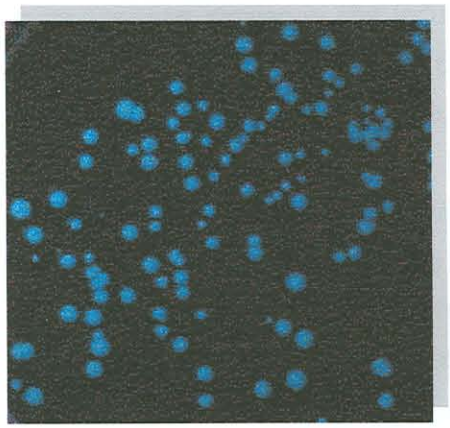


Figure 3.4 Autofluorescence on BCYE



Figure 3.5 Sticky consistency of colonies

3.4.3 Evaluation of sample pretreatment methods

The effect of sample pretreatment with acid and heat was investigated. In order to determine the effect of sample pretreatment on the recovery rates, all the samples were subjected to both treatment methods.

Compared to no treatment or acid treatment of samples, heat treatment was (Table 3.2). Generally, the presumptive positive numbers were comparatively lower, but the percentage confirmation of these presumptive isolates was consistently high (average = 75.0%). In comparison, agar plates were also easier to read as a result of the lower number of viable and culturable organisms in the samples after heat treatment. Compared to no treatment, acid treatment usually allowed less growth on all media, but with similar or higher confirmation rates of the cultured organisms (Table 3.2).
shown to yield the highest number of confirmed positive samples except on MWY agar

3.4.4 Comparison of detection methods

Two well established culturing methods, the ISO and AS methods and the modification of the AS method (ASM), were compared with the MPN method. The comparison of these four methods with regard to their relative ability to detect legionellae from the samples and industries evaluated indicated considerable differences in the results obtained (Table 3.3). In general, the sensitivity of the ISO, ASM and MPN methods was similar in the industrial samples and biofilms (average 80.0%, 80.0% and 81.0% respectively). Similarly, the samples from workings on the mine surface yielded fairly comparable results (88.0%, 63.0% and 75.0% respectively).

However, this was not the case with the underground mine water samples. Whereas all these samples yielded confirmed legionellae when using the ISO method, only 25.0% were positive when using the MPN method and no legionellae were detected when using the AS and the ASM methods (Table 3.3). On average, the ISO method was by far the most appropriate for isolation of legionellae from the mine water samples (88.0%).

In general, the ISO method was the most appropriate for detection of legionellae from the samples evaluated during this study (83.0%) when compared to the MPN, ASM and AS methods (69.0%, 61.0% and 34.0% respectively) (Table 3.3).

Table 3.2
Comparison of culture media and pretreatment methods for Legionella detection

CULTURE MEDIUM	RESULT	PRETREATMENT METHOD		
		NONE	ACID	HEAT
BCYE	Total positive	71.4	64.3	53.6
	Confirmed	17.9	25.0	32.2
	Pure	0	9.2	0
BMPA	Total positive	67.9	35.7	35.7
	Confirmed	25.0	17.9	25.0
	Pure	0	0	0
GVPC	Total positive	64.3	57.1	53.6
	Confirmed	32.2	35.7	42.9
	Pure	0	0	22.3
MWY	Total positive	53.6	57.1	39.3
	Confirmed	28.6	46.4	35.7
	Pure	0	0	23.6

Total positive = percentage positive samples (presumptive and confirmed); Confirmed = percentage of total positive samples that were confirmed by either DFA or latex agglutination; Pure = percentage of confirmed positive samples that yielded pure cultures.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 3.3
Comparison of *Legionella* prevalence by different detection methods

Source	N	Percentage of samples positive for Legionella			
		MPN	ISO	AS	ASM
Industrial water & biofilm					
Plastics manufacturer	5	100	100	60	100
Power station	6	83	100	50	100
Petrochemical company	5	60	40	20	40
Average (IW&B)	n=16	81	80	43	80
Mine water					
Underground	4	25	100	0	0
Surface	8	75	75	38	63
Average (MW)	n=12	50	88	19	32
AVERAGE (ALL)	n=28	69	83	34	61

MPN: most probable number method; ISO: international standard method; AS: Australian standard method; ASM: Australian standard method with modifications; PCR: polymerase chain reaction.

3.4.5 Legionella prevalence

The prevalence of legionellae in samples from a manufacturer of plastics, a power station, a petrochemical company and underground and surface operations of a gold mine was studied, using the four methods described above. A comparison of the four methods with regard to their relative ability to detect legionellae in the samples evaluated is shown in Table 3.3. Legionellae were detected from an average of 89.3% of the samples tested (range 35.7 - 82.1%) depending on the detection method used (not shown); in 21.4% of the samples with all the methods and in 14.3% of the samples with only one method. No single method appeared to be superior in all the samples evaluated. The numbers of legionellae detected exceeded 10^2 cfu/ml in 54% of the samples (organism numbers ranged from 10^{1-4} cfu/ml). The majority of positive samples contained *L pneumophila* SG 1 and SGs 2-14, often in combination. Only two samples, both from the petrochemical company, contained Legionella species other than *L pneumophila* as confirmed by latex agglutination (Figure 3.6). Both these samples yielded pure cultures of this organism after pretreatment: one after acid treatment and the other after heat treatment.

All the samples from the plastics manufacturer and the power station contained confirmed *L pneumophila* SGs 1-14, in numbers $\geq 10^3$ cfu/ml and often in pure culture after heat treatment (not shown). The prevalence was slightly lower but still high in the petrochemical company (80.0% in the absence of sample pretreatment and selective media) but this figure dropped considerably with the use of a combination of sample pretreatment and selective media. Using latex agglutination for confirmation, the

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

organisms present in these samples were confirmed as *L pneumophila* SG 1 and *L* species.

In order to compare the prevalence of legionellae in different sample types, the samples were divided into two categories namely industrial water and biofilm (n=16) and mine water (n=12) (Table 3.3). Our results indicated the prevalence of legionellae in an average of 43.0-81.0% of industrial water and biofilm and 0-88.0% of mine water, depending on the detection method used (Table 3.3).

3.4.6 Effect of re-incubation on culture outcome

The effect of re-incubation of Legionella presumptive but unconfirmed samples that contained amoebae in their concentrates was investigated (n=8). The results are summarized in Table 3.4. Amoebae were present in 87.5% of the industrial water and biofilm samples and 25.0% of the mine water samples on initial screening (not shown). In the untreated and acid treated portions, 50.0% of the previously unconfirmed samples yielded confirmed legionellae after 10 days incubation of sample concentrates and subsequent culturing on αBCYE agar. The use of selective culture media and heat treatment decreased the yield of confirmed legionellae after re-incubation considerably.

Table 3.4
Effect of re-incubation of culture unconfirmed, amoebae positive samples

		% Positive after re-incubation	
Portion	N	BCYE	GVPC
Untreated	8	50	25
Acid treated	8	50	13
Heat treated	8	25	13

3.5 DISCUSSION

To date, very little has been published on *Legionella* in Southern Africa. As a result, there has been a general lack of awareness regarding the health risks of *Legionella* contamination of water distribution systems until fairly recently. After the initial introduction of diagnostic laboratory tests in 1979, a number of sporadic legionellosis cases were identified in the early 1980s. By 1982, 10% of over 2000 pneumonia patients have been diagnosed with *Legionella* infections (Mauff *et al.* 1984), a figure that was confirmed in hospitalized pneumonia patients in 1994 (Maartens *et al.* 1994).

Ratshikhopha (1990) reported a high prevalence of antibodies in healthy blood donors and Bartie and Klugman (1997) found high levels of antibodies and seroconversion in gold miners. Using a locally developed most probable number (MPN) method, Grabow and colleagues (1991) reported the presence of *L pneumophila* in 77% of over 500 cooling towers. Despite these figures, only one outbreak occurred to date (Strebel *et al.* 1988) and 31 sporadic cases have been reported since 1990, when legionellosis became a notifiable disease in South Africa.

Culturing is generally accepted as the 'gold standard' for *Legionella* detection from the environment, but the lack of standardization of culturing methods, especially for environmental legionellae, complicates the interpretation of results. Despite numerous international studies there is still no consensus about the most appropriate culturing methods and agar media to use. The general feeling is that no single method or agar medium is appropriate for the culturing of legionellae from all environmental samples. South African laboratories currently use a variety of non-standardized culturing methods; this project was thus undertaken in an attempt to provide guidelines for the development of a standard method to be used by all the local laboratories.

Direct immunofluorescence (DFA) was used in the past to screen environmental samples for the presence of legionellae prior to culturing. Although useful for the detection of non-viable and viable but non-culturable organisms, this method had several disadvantages. Cross-reactions with non-legionellae gave rise to false positive results, the reading of slides was time consuming and labor intensive and the interpretation of the test was often subjective. Furthermore, the limited specificity of available DFA reagents often resulted in false negative results. Although this screening step is not used routinely any more, it was included in this study to provide an indication of the number of samples expected to yield legionellae and the possible presence of viable but non-culturable legionellae. Despite the limited specificity of the DFA reagent available in South Africa (specific for detecting *L pneumophila* SGs 1-6 and *L micdadei*), over 90% of the samples evaluated were positive during this initial screening step. For this reason, the inclusion of sample screening by DFA may be useful, especially during outbreak situations when quick test results are critical.

The recovery of legionellae from environmental samples is often hampered by overgrowth of culture media by faster growing non-legionellae that may inhibit legionellae or may mask the presence of colonies on agar media (Lye *et al.* 1997). Because legionellae have thermophilic characteristics and are relatively stable in acidic conditions, sample concentrates can be pretreated with heat or acid prior to culturing to inhibit background

flora. If not carefully controlled however, these pretreatment methods may also inhibit or injure legionellae. Even in controlled conditions, workers differ in their opinions regarding the use of these selective procedures. Some prefer the combination of acid treatment with selective agar while others consider acid treatment too drastic for legionellae (Palmer *et al.* 1993, Reinthaler *et al.* 1993, Harley *et al.* 1997). De Luca *et al.* (1999) and others suggested the combination of heat treatment with selective agar media. However, not all environmental samples and Legionella species and strains will react similarly to these selective procedures, a factor that has to be taken into account when attempting to standardize culture methods. In general, sample pretreatment improved the confirmation rate of legionellae from the samples evaluated in this study, regardless of the treatment method and culture medium used. The best overall confirmation rate was obtained when combining culturing on GVPC agar with heat treatment but this finding was not consistent in all the samples tested.

The primary isolation medium for legionellae is buffered charcoal yeast extract (BCYE) agar supplemented with alpha-ketoglutarate, iron and cysteine (αBCYE). The selectivity of αBCYE agar can be increased through the addition of antimicrobial and antifungal agents and other inhibitors like glycine (Yu 1990). Dyes are sometimes added to aid Legionella identification. The major disadvantage of all these media is their black color resulting from the addition of activated charcoal but although transparent culture media have been developed (Armon and Payment 1990), these media have not been sufficiently validated and are therefore not used routinely.

Four agar media (αBCYE as a non-selective medium, BMPA and MWY agar as suggested in the AS method and GVPC agar as suggested in the ISO method) were evaluated for their relative ability to support the culturing of environmental legionellae. MWY and GVPC agar both contain glycine, polymyxin B and vancomycin as antibacterial agents. To reduce fungal growth, anisomycin is added to MWY agar and cycloheximide to GVPC agar. MWY agar also contains bromocresol purple and bromothymol blue to aid in the distinction between legionellae and non-legionellae. BMPA agar contains cefamandole and polymyxin B as antibacterial agents and anisomycin as an antifungal agent.

In general, the addition of selective supplements to αBCYE agar improved Legionella detection from all the samples tested, even in the absence of sample pretreatment with acid or heat. Although BMPA agar has been reported to inhibit legionellae that do not form cephalosporinase (mainly *L micdadei, L bozemanii* and certain subtypes of *L pneumophila* SG 1) (Edelstein 1982, Reinthaler *et al.* 1993), both *L micdadei* and *L pneumophila* SG 1 were isolated from this medium in the samples

evaluated. However, the plates were often overgrown with non-legionellae. For this reason, the use of BMPA as a selective medium in South Africa is not recommended.

The confirmation rate improved considerably when using GVPC and MWY agar despite a slight decrease in the total number of legionellae detected. The dyes incorporated into the MWY agar did not simplify Legionella detection in this study. These results correlated well with those of other workers who reported both GVPC and MWY agar useful selective media for Legionella detection from environmental samples (Reinthaler *et al.* 1993, Ta *et al.* 1995). Following the recent withdrawal of cycloheximide from world markets (personal communication JM Kusnetsov, National Public Health Institute, Helsinki, Finland) GVPC agar will not be commercially available until a replacing supplement has been validated. αBCYE is therefore recommended as a non-selective and MWY as a selective medium for *Legionella* detection from environmental samples in South Africa.

Two internationally accepted culture methods, the ISO and AS methods, were compared with a locally developed MPN method. In addition, a modification of the AS method, designated the ASM method, was evaluated. The AS method as described in the Australian Standard AS 3896-1991 was not useful for detecting *Legionella* in the samples evaluated, but appropriate sample dilutions improved the results considerably (the ASM approach). Similarly, acid treatment did not improve the yield of legionellae in the samples evaluated, therefore only heat treatment is recommended. Quantitative culturing to determine the risk of infection (as indicated in the MPN method) is controversial for a number of reasons. Estimations of *Legionella* concentrations in industrial waters are generally regarded as imprecise and of low sensitivity. However, some of the samples evaluated were very contaminated, therefore the MPN approach may be useful for screening purposes, provided it is combined with culturing on selective media and/or heat treatment. This approach may be particularly useful in situations where culturing results are required urgently, for instance during outbreaks. For these reasons, a combination of the three methods was found most useful for the samples evaluated in this study.

The results from this study indicated a high prevalence of legionellae in South African industrial waters. The reasons for the low prevalence in the underground mine waters are unknown but these results correlate well with previously reported data (Bartie and Klugman 1997). It would appear that the workings on the surface of the mine (for example change houses at the different shafts and showers in hostels) are more likely

sources of legionella contamination and possible infection for mine workers at this particular mine.

Protozoa, including amoebae, play a key role in the persistence of legionellae in the environment (Brieland *et al.* 1997, Rohr *et al.* 1998). In low-nutrient environments, legionellae are able to enter a viable but non-culturable (VBNC) state (Steinert *et al.* 1997). Bacteria in this state are still virulent and able to cause human infection. Resuscitation of legionellae in the VBNC state by incubation in the presence of autochthonous amoebae has been described (Steinert *et al.* 1997, Sanden *et al.* 1992). Legionellae were detected and confirmed in 50% of the samples that were negative by culturing or where the presence of legionellae could not be confirmed during the first experiment. One such sample contained a pure growth of legionellae on BCYE and GVPC agar in the untreated portion after incubation and another, in the acid treated portion. This confirmed previous findings by Sanden and colleagues (1992) who reported that incubation of environmental samples with autochthonous amoebae improved the sensitivity of culture methods for legionellae considerably. This experiment also confirmed that non-culturable legionellae may remain viable and may in fact increase in numbers, to culturable levels, during periods of nutrient starvation when they are found intracellularly within amoebae and protozoa.

3.6 CONCLUSIONS

Legionella species, particularly *L. pneumophila* SGs 1-14 are prevalent in South African industrial water distribution systems. Culturing on αBCYE as a non-selective and MWY as a selective agar medium, using non-treated and heat-treated concentrates, is recommended. The MPN approach may be useful in some situations when combined with heat treatment and culturing on selective media. Screening for, and re-incubation of sample concentrates with autochthonous amoebae may improve the sensitivity of culturing further and may allow detection of legionella-like amoebal pathogens (LLAPs), a group of bacteria not previously studied in South Africa.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 4

CONCLUDING REMARKS

4.1 INTRODUCTION

Recent industrial development, population growth and urbanisation have increased the demand for water, forcing industries to recycle and re-utilise available water for cooling and industrial use. The formation of biofilms in industrial water distribution systems making use of recycled water creates favourable conditions for harmful microorganisms like legionellae to grow and multiply, thereby increasing the health risks to workers and the general public.

The current worldwide interest in emerging and re-emerging pathogens and the resulting movement towards a better understanding of Legionella species, the ongoing outbreaks reported in other countries and the high incidence of legionellosis cases among workers, travellers and immunocompromised individuals worldwide, has prompted the National Centre for Occupational Health (NCOH) to initiate the first Legionella Seminar and Workshop in South Africa, which was held in collaboration with the Centre for Scientific and Industrial Research (CSIR) during September 1995. This event was very well received, highlighted the unique problems regarding Legionella detection in South Africa for the first time and resulted in the formation of the South African Legionella Action Group (LAG), a non-profit organisation representing industry, academic institutions and the medical profession. Apart from the standardisation of detection methods for legionellae from environmental samples, research and the recommendations of appropriate prevention and treatment methods for contaminated water systems, the main objectives of the LAG are to disseminate information and increase the awareness of this organism and its health effects among medical personnel and workers in the water treatment industry.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.2 EMERGING PATHOGENS

By nature, infectious diseases have the potential to spread internationally. With the development of vaccines and the discovery of antimicrobial drugs during the previous century, many infectious diseases could be cured and prevented. Epidemics were reported less often than before and the battle against infectious diseases was believed to be over. But the microbial world is complex, dynamic and constantly evolving. Microorganisms proliferate, mutate and adapt to new environments and hosts and have the potential to develop drug resistance.

As a result, the world currently finds itself in a situation where epidemics are again spreading around the globe unhindered and faster than before due to the increase in global travel and trade. Once an infectious disease invades a new country or continent, it can prove very difficult, if not impossible, to control. The magnitude of the problem is clearly illustrated by the appearance of several new pathogens (for example HIV and other retroviruses, hantaviruses and the Ebolavirus and bacteria like Legionella) and the re-emergence of old pathogens like cholera, plague, dengue, hemorrhagic fever and yellow fever. Furthermore, microorganism mutations are resulting in drug and multidrug-resistant strains of pathogens like *Mycobacterium tuberculosis*. New or newly recognised diseases are being reported at the rate of approximately one per year (Heymann 2001). In recent years, considerable attention has been paid to the global threat of these emerging and re-emerging diseases.

South Africa has the highest prevalence of HIV/AIDS in the world, has a mining population in which tuberculosis and infections with non-tuberculous mycobacteria and pneumonia are very high. The country recently experienced a cholera outbreak affecting thousands of people and is well known as a high risk for malaria. These diseases are leaving the country with an increased number of immunocompromised people at risk of opportunistic infections and thus in need of treatment, often requiring hospitalisation and specialised medical care. This decreases our healthy workforce considerably and creates an enormous economic burden.

Another serious and costly problem is the development of resistance to inexpensive but effective antimicrobial drugs. The bacterial infections that contribute most to human disease are also those in which emerging resistance is of most concern: diarrhoeal diseases such as dysentry, respiratory tract infections including pneumonia and multidrug-resistant tuberculosis, as well as a host of nosocomial

infections that are very difficult and costly to treat. Antimicrobial resistance is a global problem that requires a global solution.

In June 1995 the Pan American Health Organisation (PAHO) published a plan of action for addressing the problems regarding emerging diseases. Given the current situation regarding Legionella and legionellosis in South Africa, this plan of action can be a useful guide for further development in this field. The PAHO plan of action had four main objectives

- Strengthening surveillance networks for infectious diseases;
- The establishment of infrastructures for early warning and rapid response to infectious diseases;
- The promotion of applied research in the areas of rapid diagnosis, epidemiology and prevention and
- Strengthening the capacity for prevention and control of disease.

4.3 SURVEILLANCE

The development of strong national, regional and global surveillance systems is central to the prevention and control of emerging and re-emerging infectious diseases. Traditionally, one of the main factors undermining the effectiveness of infectious disease surveillance has been the reluctance of countries to report outbreaks due to fear of the negative impact it would have on travel, trade and tourism. This traditional reluctance is now starting to change. In line with the growth of electronic media, more than half of the world's first news about infectious disease events has recently come from informal sources, including press reports and the Internet. Transparency about outbreaks and prompt reporting has therefore become increasingly important; unverified rumours of an outbreak or unusual disease can have a very negative impact on trade, travel and tourism. The World Health Organisation (WHO) expressed its concern about the situation in a resolution of the World Health Assembly in 1995, and advised member states to strengthen their surveillance for infectious diseases. This has resulted in several international initiatives to restore and improve the surveillance and control of emerging, re-emerging and communicable diseases.

The main purpose of disease surveillance is to promptly and efficiently detect, investigate and monitor emerging and re-emerging pathogens and the diseases they cause and notifiable medical conditions. Furthermore, surveillance is necessary to contain known risks, to monitor disease trends and progress made toward disease

control and to estimate the magnitude of particular health problems. In addition, surveillance networks make the detection of infectious disease outbreaks and evaluation of interventions and preventive programs possible. Without proper surveillance, addressing specific research needs in would not be possible.

Disease surveillance in a country should ideally focus on certain priority diseases. For each disease or group of diseases the ability to carry out the core and support functions of surveillance and response should be reviewed. Although all surveillance systems involve the same core functions of case detection, confirmation, reporting, analysis, investigation, response, feedback and monitoring, and common support functions (training, supervision, communications and other resources), specialised surveillance systems are important, especially when surveillance methods are complex and the systems have specific information needs. For a surveillance system to be adequate in its function as an early warning system, the reporting, confirmation, decision-making and response should be rapid. On the other hand, data also have to be considered carefully in order to adjust or target the control programme. These activities are made possible by a number of support functions that lead to better performance of the core activities. Standardisation of case definitions, case management and procedures for investigation is essential. Training should be provided in the areas of surveillance, epidemiology and laboratory methodology and proper supervision should be provided for these activities. Furthermore, communications systems should be in place and appropriate human, equipment and financial resources should be provided.

The disease surveillance system in South Africa is based on government law (Health Act, Act No. 63 of 1977) and other regulations that specify the reporting of specific infectious disease to Provincial Health Departments, who then report to the National Department of Health. Given the high prevalence of HIV/AIDS and tuberculosis in South Africa, surveillance systems are in place for these diseases, and rightly so, but in the process surveillance of other notifiable diseases, for example legionellosis, is grossly neglected. This is clearly illustrated by the small number of Legionnaires' disease cases notified in the country.

Disease surveillance in South Africa comprises four types. The notifiable disease-reporting system involves the reporting of confirmed cases of certain notifiable conditions, of which legionellosis is one, to the local authority who then reports it to the Department of Health. This type of surveillance is extremely important, especially in the case of legionellosis, where underreporting is undeniable and probably

substantial. Although there is no easy solution to this problem, public awareness campaigns may assist in the process. In order to increase the public awareness, health professionals should become involved in the notification process. A multidisciplinary outbreak response team has been established at national level, consisting of representatives from Disease Control and Prevention, Health Promotion and Communication, Health Information Evaluation and Research. Outbreak control in South Africa is the responsibility of the various provinces.

Laboratories are essential to disease surveillance and most epidemiological surveillance systems require a laboratory component for confirmation. These serve both for the routine confirmation of clinical syndromes and for rapid confirmation of the causative agent in outbreaks. In some cases surveillance is purely laboratory-based, therefore the laboratory's capacity for surveillance should be adequate. To assist in laboratory-based surveillance, the NCOH has taken the initiative to develop a legionellosis database. South African laboratories involved in legionellosis diagnosis will be asked to participate in the surveillance program, which may assist in identifying unreported cases. The database will then be used to generate information that will be used for formulating preventative and control measures for legionellosis in South Africa and will provide South African public health practitioners with important data on which to plan intervention activities. Hospital discharge information as well as mortality data can be used to monitor disease trends and disease burden in the particular area served by the hospital. A population-based surveillance system can assist by collecting and analysing medical data in well defined populations, to provide baseline data and assist in the evaluation of disease trends and the success of intervention programs (http://www.sadap.org.za/edl/notify/htm).

In terms of Section 45 of the Health Act of 1977 (Act No. 63 of 1977), legionellosis has been added to the list of notifiable diseases in 1990. Medical practitioners are therefore responsible for reporting all confirmed legionellosis cases to the Department of Health. Despite this responsibility, there appears to be gross underreporting of cases; in fact, only 32 cases have been reported to date (Table 4.1), clearly illustrating the general lack of awareness among medical practitioners and the general public regarding the health risks of Legionella contamination of water distribution systems. As a result, the prevalence and distribution of Legionella species and the incidence of legionellosis, particularly among workers, is not known.

It has become clear that the surveillance of legionellosis in South Africa is not up to standard. For this reason, the NCOH has embarked on a program in collaboration

with the Gauteng Provincial Government, to provide assistance in the reporting of legionellosis cases and to keep a database of all the cases reported. As a National Centre, support will also be given to other provinces in an attempt to improve the surveillance of legionellosis. To this effect, an information brochure was developed, specifically for the medical profession, which includes a section on disease surveillance

4.4 PREVALENCE

The CDC estimates that between 10,000 and 20,000 cases of Legionnaires' disease occur each year in the United States. Of these only 1500-1800 are reported to public health authorities. There has been a general increasing trend in the number of cases reported per year, probably because of improvements in physicians' ability to diagnose the disease associated with the introduction of new diagnostic assays. Of the Legionnaires' disease cases reported to the CDC, 23% are nosocomial with a mortality rate of 49%; the mortality of community-acquired cases is 20%. In a national survey of 192 hospitals, 60% had onsite testing facilities but only 21% routinely tested for Legionnaires' disease.

In 1998, a total of 32 outbreaks were reported in Europe. 14 were caused by hot or cold water stystems in 9 hospitals, 2 were associated with communities and 3 with travel. Contaminated cooling towers were responsible for 5 outbreaks. During that year, 1442 cases of Legionnaires' disease were reported in Europe. *L pneumopnila* SG1 accounted for 60% of the cases, nearly half these were diagnosed by the recently developed urinary antigen test. Only 5.3% of cases were caused by species other than *L pneumophila.*

A study by Yu (1998) reported the following data from hospital surveys (Table 4.1):

Table 4.1 Prevalence of Legionella in hospitals

Location	# hospitals tested	% with Legionella
UK (1987)	40	70
Quebec (1992)	84	68
Western PA (1987)	15	60
UK (1997)	69	55
Nova Scotia (1994)	39	23
UK (1983)	17	12
San Antonio (1999)	15	73

The European Working Group on Legionella Infections (EWGLI) estimates that 12,000 European tourists (ten times the number of reported cases) have contracted Legionnaires' disease since 1995, of whom 10% are expected to have died. Most cases were linked to hotels in southern Europe. As a result, the Netherlands Ministry of public health, welfare and sport, plans to publish the names of all European hotels that have not succeeded in bringing legionellosis outbreaks under control within four weeks, on the Internet. (Source: IRC International Water and Sanitation Centre and the Water Supply and Sanitation Collaborative Council (WSSCC), The Netherlands, www.irc.nl).

Numerous Legionnaires' disease outbreaks have occurred in Spain during the last few years. (Sopena et al. 1999): hospitalised patients with CAP were studied and they could only achieve definitive diagnosis in 58%. *S pneumoniae* (23.9%), *C pneumoniae* (13.5%), *L pneumophila* (12.5%), *H influenza* (2.3%), *P aeroginosa* (1.5%), *M pneumoniae* (1.3%), *C burnetti* (1%). A surprising finding was the increased occurrence of Legionella infections in patients under the age of 60 and without underlying disease. *S pneumoniae* (29%) and *L pneumophila* (26.3%) was mostly implicated in the requirement to admit patients to intensive care units. The authors concluded that *C pneumoniae* and Legionella species should be taken into cosideration when ordering laboratory tests and giving empirical treatment. In San Antonio, Texas, 92% of hospitals were tested positive for legionellae (Kool *et al.* 1999). This study reported no link between the number of legionellae present in the water distribution system and the number of nosocomial cases in the hospital, but the proportion of water-system sites that tested positive.

Very little is known about the prevalence of legionellosis in South Africa. After the initial introduction of diagnostic laboratory tests in 1979, cases were identified in Durban, Port Elizabeth and Johannesburg during the early 1980s. The first fatal case was diagnosed at autopsy and reported in 1980 by Goldstein and coworkers from the NCOH. Sera from 2200 pneumonia patients have been tested for antibodies to *L pneumophila* by 1982; 10% of these patients were confirmed as having Legionnaires' disease. This figure was confirmed in hospitalised community-acquired pneumonia patients in a study published by Maartens *et al.* in 1994. Despite the high prevalence of antibodies in healthy blood donors (Ratshikhopha *et al.* 1990) and a prevalence rate of 36% in workers in the mining industry (Bartie and Klugman 1994) only one outbreak

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

has so far been reported in the country, affecting 12 patients in a Johannesburg hospital.

Table 4.2 Notified legionellosis cases in South Africa

YEAR	CASES	DEATHS	TOTAL
1990	2	0	2
1991	14	0	14
1992	2	0	2
1993	4	1	5
1994	4	0	4
1995	-	-	-
1996	-	-	-
1997	4	0	4
1998	-	-	-
1999	-	-	-
2000	-	-	-
2001	1	0	1

-: data not available. Source: Department of Health, Statistics.

4.5 RESOURCE DEVELOPMENT

For effective response to Legionnaires' disease outbreaks in South Africa, the improvement of laboratory services and the development of training programs are essential and should be developed. Appropriately trained personnel will be a critical component of this infrastructure.

4.5.1 Improvement of laboratory services

There is a need for a survey of suitable laboratory facilities in South Africa and their capabilities in terms of Legionella detection from the environment and the diagnosis of legionellosis. All laboratories involved in Legionella testing should be listed and assessed for implementation of quality control programs.

At present, there are two laboratories known to be involved in the routine testing of water samples for Legionella, and one laboratory actively involved in Legionella research. The detection methods for Legionella have not yet been standardised in South Africa and the laboratories involved in routine testing are using a number of different methods. As a result, contradictory results are obtained. In fact, the results from a recent inter-laboratory study showed extensive discrepancies in results, even

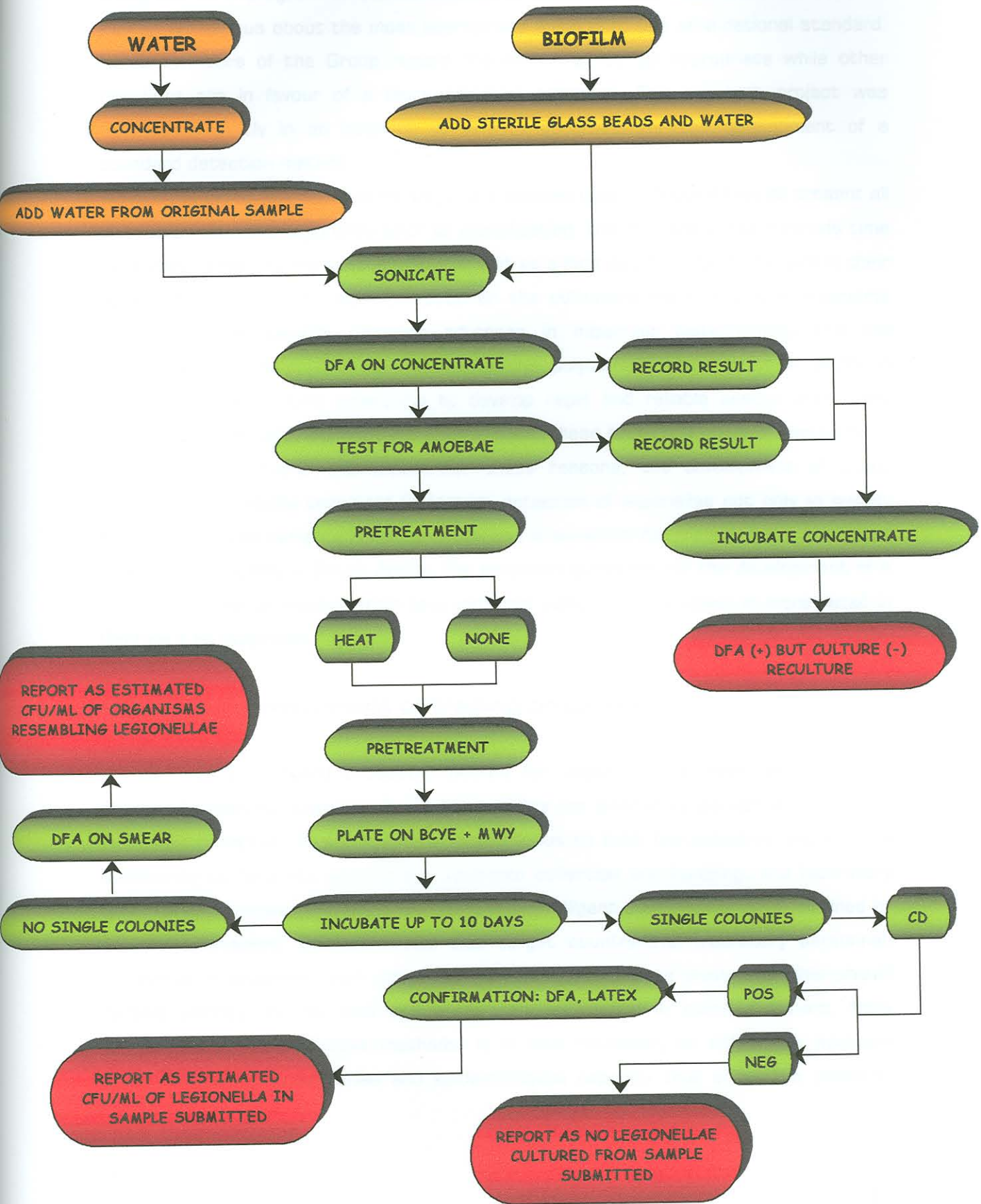


FIGURE 4.1: General procedure for proposed method

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

when using seeded samples. For these reasons, the Legionella Action Group has embarked on a program of standardisation of detection methods. However, there is still no consensus about the most appropriate method to use as a national standard. Some members of the Group regard the MPN method as appropriate while other members are in favour of a more selective approach. This research project was undertaken partly in an attempt to provide guidelines for the development of a standard detection method.

The detection methods for Legionella species used in South Africa at present all depend on culturing legionellae prior to identification. Not only are these methods time consuming, they require special media as well as a high degree of technical skill in their application and only provide information on the culturable fraction of the organisms present in the sample. Although advances in molecular biotechnology and the development of diagnostic applications of the polymerase chain reaction (PCR) in particular, have enabled scientists to develop rapid and reliable assays with many advantages over conventional culturing methods, these tests are not yet standardised sufficiently for routine application. For these reasons, the development of quick, standardised, reliable tests for laboratory detection of legionellae not only in water, but also in biofilm samples is long overdue and is essential for proper risk assessment studies, particularly in South Africa. The proposed guidelines for the development of a standard detection method from environmental samples is discussed in more detail in Section 4.6: Guidelines.

4.5.2 Development of training programs

Once a standard detection method for Legionella has been developed and accepted, practical training should be provided for laboratory personnel involved in Legionella detection. These activities should focus on both the industrial and medical community to facilitate appropriate specimen collection and handling, the laboratory resources for optimum specimen handling, the intelligent utilisation of data obtained by program managers. Training should also target country-level laboratory personnel. Collaborative programs with other countries that have been involved in this should receive priority. In this way specialists can be trained in state-of-the-art, field-applicable and cost-effective methods. It is also necessary to define the minimum requirements for laboratories and epidemiological capacity that should be available,

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

develop guidelines and standard procedures, and assist provincial governments in implementing these guidelines.

The Legionella Action Group has been involved in training activities since 1995 when the first Legionella Seminar and Workshop was held. Since then, training seminars were held in Johannesburg, Cape Town and Pietermaritzburg during February 1998 and in Pretoria during February 2000. A workshop providing information on clinical aspects of Legionnaires' disease, laboratory diagnosis and outbreak control and a one-day training course for Legionella prevention were presented during 2001. In addition, the NCOH published an information brochure for medical practitioners, titled "Legionellosis: a general guide" as part of an occupational disease series. Given the increased interest in Legionella and legionellosis among occupational health practitioners, a training module is planned for the Diploma in Occupational Health of the University of the Witwatersrand.

4.6 GUIDELINES FOR PREVENTION AND CONTROL

Continuing outbreaks of Legionnaires' disease have raised doubts about the ability of industry to effectively manage water systems. Good water system management involves operating procedures being laid down in a manual, management responsibilities being well defined, operations staff being well trained and all aspects of the system operation being recorded. Water systems service companies cannot be effective if the building occupier does not operate the system as instructed.

No official guidelines exist in South Africa for the prevention and control of legionellae present in water distribution systems. In general, treatment procedures currently available are expensive and only successful in the temporary reduction of bacterial numbers in these systems. Although certain biocides have been proven effective against legionellae in laboratory conditions, their efficiency in field conditions has not been studied to date. The exact role of protozoa, especially in biofilm conditions in the protection of legionellae against biocide treatment is not clear and needs to be studied. The CSIR in collaboration with the Water Research Commission recently embarked on a program of developing guidelines and a code of practice for the prevention and control and a health risk assessment for legionellae in water distribution systems. These guidelines were discussed at a workshop during September 2001 and are in the final development stage.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

The genus Legionella is classified as a Category 2 biological hazard (Regulations for Hazardous Biological Agents, Occupational Health and Safety Act No. 85 of 1993). Organisms in this category can cause human disease, may be a hazard to employees and may spread to the community. Although there is usually effective prophylaxis or treatment available for organisms in this category, the onus is on the employer to follow the necessary containment measures outlined in the Regulations.

4.7 SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

4.7.1 Seeded samples

The first stage of the project dealt with the optimisation and comparison of conventional detection methods for legionellae. The experiments were carried out using seeded samples, enabling us to control and manipulate the experimental conditions.

Numerous difficulties were experienced in the culture of legionellae from the seeded samples. In general, there appeared to be a lack of consistency in the quality of culture media obtained in a ready-made form. Batch-to-batch differences were experienced in all the media evaluated. BCYEα agar was often totally overgrown with non-legionellae, to such an extent that no single colonies could be distinguished even in the higher dilutions. Several batches of BMPA agar could not be used at all, due to contamination. These problems were discussed with the suppliers and were subsequently rectified. The isolation and identification of Legionella species were found to be extremely time consuming and labour intensive. Difficulties were also experienced in obtaining media and reagents and the cost was very high. Specific findings of importance were:

- The sample concentration method may influence the outcome of Legionella culture from environmental samples;
- The method used for re-suspension of organisms after membrane filtration may result in a considerable loss of organisms. In this regard, sonication appears to be superior to vortex;
- The pretreatment steps indicated in the ISO and AS methods further decrease the number of organisms recovered in sterile as well as non-sterile seeded samples;
- The different types of supplements in the various selective media influence the efficiency of distinction between legionellae and non-legionellae on culture media;

- Quality control of culture media is extremely important. This aspect should be taken up with the relevant suppliers;
- GVPC and MWY agar are similar in selectivity with GVPC being slightly more selective;
- The latex agglutination test currently available in South Africa is suitable for confirmation of legionellae from agar media. Its applicability to colony suspensions from MPN culture plates needs to be confirmed;
- The direct immunofluorescence test is easy to perform but is only specific form *L pneumophila* SGs 1-6 and *L micdadei*, which may decrease its sensitivity for environmental samples;
- The reproducibility of culture experiments are generally low;
- In this study the MPN method was superior to the ISO and AS methods for isolation of legionellae from non-sterile, seeded samples.

4.7.2 Environmental samples

Having established optimal conditions for common treatments and for the different detection methods individually, with modifications where applicable, the different methods were compared with respect to their applicability on industrial water systems, with particular reference to cooling waters. This study has confirmed that legionellae are highly prevalent in South African cooling water systems. The evaluation of different culture methods emphasised the importance of proper training of laboratory personnel involved in the evaluation of environmental samples for the presence of legionellae. From this work, a standard method for use by South African laboratories in the isolation and identification of legionellae from industrial samples is proposed. Specific findings of importance are:

- For complex samples, selective media are more appropriate than a non-selective isolation approach;
- Appropriate sample dilution reduces inhibition by non-legionellae and simplifies the isolation from these complex samples;
- Heat treatment is preferred over acid treatment where complex samples with a high microbial load is concerned;
- The prevalence of legionellae in the industrial cooling water samples tested was found to be high to very high;
- The majority of samples contained legionellae in numbers $\geq 10^3$;

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

- *L pneumophila* SGs 1-14 were the most prevalent species and were present as single, or a combination of two or more serogroups in a number of samples tested;
- The presence of free-living amoebae play a supportive role in the replication and survival of legionellae and may be a useful additional way of isolating legionellae.

4.7.3 Most probable number (MPN) method

Most probable number tests are usually based on liquid media and a statistical analysis of numbers of replicates at dilutions (usually 10-fold) that are positive. The numbers provide only a probable number of organisms that may be present. The MPN method used for Legionella detection was recently proposed as a standard method for Legionella detection in South Africa. The matter was discussed at length with various experts in the field of Legionella detection from environmental samples (personal communication: Legionella e-group). The general feeling is that the MPN method as done in South Africa is a basic colony count method at various dilutions. According to members of the group the MPN method has serious weaknesses making it unsuitable for use as a standard method for Legionella detection.

It has been suggested that the storage of reagents at 4-8°C after autoclaving may cause 'cold shock' of some cells, particularly those that are already damaged in some way. There is general consensus about the storage and use of reagents at room temperature. The absence of antibiotics in the culture medium and the lack of treatment with heat and/or acid to remove competitor species will cause serious overgrowth, leaving legionellae invisible. Furthermore, some competitor species (for example Pseudomonas species) secrete anti-bacterial compounds that significantly impair Legionella growth.

Although there is no consensus about the best pretreatment method to use, workers agree that some form of pretreatment has to be incorporated into culture methods, especially from non-potable waters. It is important to be able to subtype legionellae from environmental samples in order to find the species or serogroups responsible for infections in outbreak situations. The MPN method does not make provision for subtyping in addition to culture. The present method only identifies *L pneumophila* SGs 1-6 and *L micdadei*. Although these two species are important they may not be the only ones present in some samples.

There is general consensus about filtration through membranes with a pore size of 0.45 but in some cases centrifugation is better. Some workers regard sonication for

10 minutes too long. In some circles a maximum of 4 minutes is recommended. He says the British standard recommends either 2 minutes sonication or 2 minutes vortex.

It has been suggested that one of the problems of using an selective media is inhibition of the serogroup-specific antigen by the antibiotics. The antigen may not be expressed if antibiotics are present. For this reason, all suspected legionellae are subcultured onto BCYEα without antibiotics before attempting confirmation by DFA or latex. It was found that expression of the antigen is not affected by the use of the supplements in MWY agar, therefore some workers recommend using only MWY agar for primary isolation. Presumptive colonies are then tested by DFA and subcultured for confirmation.

The use of sterile tap water for resuspension of the filter in is not acceptable due to the possible presence of chlorination by-products that may inhibit or kill any organisms that may have been damaged during sample collection. Some workers recommend the use of 1/40 Ringers or Page's saline and others prefer resuspension in a portion of the original sample. Finally, an incubation period of 3-5 days is too short for legionellae. An incubation period of at least 7-10 days is generally accepted.

4.8 GUIDELINES FOR THE DEVELOPMENT OF A STANDARD DETECTION METHOD

Following the results obtained from this study, guidelines are proposed for the development of a standard detection method for Legionella in South Africa. The proposed guidelines were derived from a combination of methods evaluated during this research project and are intended for discussion and development. The proposed method is applicable to environmental waters, biofilm, sediment, scale and slime samples and should provide an estimated number of confirmed legionellae in a given sample. A flow diagram of the proposed method is presented in Figure 4.1.

4.8.1 Definition

Legionella is a genus of gram negative bacteria that stain weakly with normal gram stain and do notgrow in the absence of L-cysteine and iron. The organisms may take up to 10 days or in some cases even longer to grow. The colony morphology may differ on different culture media, and even on the same media during different stages of growth.

Colonies usually have a ground-glass appearance when viewed under a stereo microscope, but this feature may not always be present and should therefore not be used as a sole identification characteristic. The colonies from some species fluoresce under long-wave ultraviolet light and the colonies may vary in colour according to species, age of the culture and the culture media used.

4.8.2 Principle

Bacteria in water samples are concentrated by filtration followed by resuspension by sonication. Biofilm, sediment, slime and scale samples are suspended by sonication. A portion of the sample is subjected to treatment with heat. Both treated and untreated portions are inoculated onto non-selective and selective culture media, containing L-cysteine, iron and various antimicrobial agents. These are incubated aerobically. Presumptive identification is done by direct immunofluorescence of representative growth on agar medi. Morphologically characteristic single colonies are tested for cysteine dependence, followed by confirmation by DFA and/or latex agglutination. Results are reported as an estimated number of colony forming units of water sample or weight of solid samples submitted.

4.8.3 Quality control of culture media

Quality control of culture media is extremely important. The following should be taken into account when preparing these media (ISO/DIS 1996): BCYE and its additives are heat sensitive and prolonged heating or heating to high temperatures may affect the nutritional qualities of the medium. Batch-to-batch variation of ingredients (particularly α-ketoglutarate) can severely affect the performance of the medium, therefore the quality of each newly prepared batch of media should be checked for its ability to promote the growth of *L pneumophila* within three days of incubation. It is not recommended to use previously isolated Legionella species that have been maintained by passage on agar slopes for assessing growth media, as legionellae become easily adapted to growth under laboratory conditions and will grow on media that will not necessarily support primary isolation of environmental ('wild') strains;

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.8.4 Concentration and resuspension

Water samples should preferably be concentrated by membrane filtration through filters with a pore size of 0.2-0.45μm. As different filter types were not evaluated during this study, type HA filters, generally used for concentration of water samples for microbiological analyses, is recommended. For highly contaminated samples, the sample may be divided into portions and more than one filter membrane used. To assist resuspension of organisms, membranes may be cut aseptically into smaller pieces and placed in a sterile container with 10 ml of water from the original sample. Resuspension is done by sonication for approximately 10 minutes, depending on the type of sonicator used. Biofilm samples may be concentrated further if necessary, but centrifugation at 3000g for 30 minutes. The sediment is resuspended in 10 ml of supernatant. Sediment and scale samples are not concentrated.

4.8.5 Sample pretreatment

For heat treatment a portion of the sample concentrate is placed in a water bath at 50°C for 30 minutes before making serial dilutions. Acid treatment is not recommended to replace heat treatment, but may be added as en extra step if a high level of contamination is suspected. Acid buffer consists of 3.9ml of a 0.2mol/ml hydrochloric acid solution added to 25ml of a 0.2mol/ml solution of potassium chloride. Adjust the pH to 2.0-2.2 and store in a dark container at room temperature for no longer than 2 weeks. Acid treatment is done as follows: Centrifuge a portion of sample concentrate at 3000g for 30 minutes, remove half the supernatant, and replace it with acid buffer. Leave on the bench for 5 minutes and make serial dilutions immediately afterwards.

4.8.6 Sample dilution

Serial tenfold dilutions of both untreated and treated portions are made in sterile distilled water.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.8.7 Direct immunofluorescence (DFA)

Using a portion of the sample concentrate the DFA is performed by placing a drop of concentrate onto a glass microscope slide and allowing to air dry before fixing with heat or acetone. Commercially available DFA reagent is added and the slide incubated in a moist chamber at 37°C for 30 minutes. After incubation, the slides are rinsed twice for ten minutes each time in phosphate buffered saline (pH 7.6) using a magnetic stirrer, air dried and mounted with buffered glycerol.

4.8.8 Testing for the presence of amoebae

Amoebal saline consists of NaCl (1.20g), $MgSO_4.7H_2O$ (0.04g), $CaCl_2.2H_2O$ (0.04g), Na_2HPO_4 (1.42g) and KH_2PO_4 (1.36g), each made up to 100ml in distilled water. For saline, 10ml of each of the above are combined and made up to 1000ml using distilled water. Non-nutrient agar (NN agar) is prepared by adding 15g agar to 1000ml of amoebal saline and autoclaving at 121°C for 20 minutes. Agar plates are poured to a thickness of approximately 5mm and left at room temperature to set. The plates are flooded with a pure broth culture of a type strain of *E coli*, incubated overnight at 37°C and stored stored at 4-6°C until use. To test for the presence of amoebae, place 100µl of sample concentrate in the centre of the plate and incubate (inverted) at 37°C until a clear zone is observed, indicating the presence of amoebae. Check the plates microscopically for the presence of trophozoites and/or cysts and record the results.

4.8.9 Agar inoculation

For each dilution, inoculate one each of BCYE and MWY agar with 100µl of sample concentrate and spread over the entire surface of the plate using a glass rod.

4.8.10 Incubation

Cover inoculated plates with plastic to avoid drying out and incubate aerobically at 35-37°C for up to 10 days, checking regularly for the presence of legionellae during the incubation period.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

4.8.11 Presumptive identification

If single colonies are present, test all the characteristic colonies for cysteine dependence. In cases where no single colonies can be distinguished in any of the dilutions, stain a representative smear from each dilution by DFA. If positive, report as presumptive legionellae.

4.8.12 Cysteine dependence

Inoculate single colonies onto BCYE and nutrient- or blood agar and incubate as usual. Colonies that grow on the BCYE but not on the other media are considered cysteine dependent.

4.8.13 Confirmation

Cysteine dependent colonies are confirmed by DFA and/or latex agglutination.

4.8.14 Expression of results

Results are expressed as an estimated number of cfu/ml of original water sample of weight of original solid sample, taking the dilution factor into account. The highest dilutions yielding confirmed legionellae is reported.

4.9 THE WAY FORWARD

4.9.1 Surveillance and notification

South Africa has a well developed disease surveillance network. The prevalence of disease like tuberculosis, HIV/AIDS, malaria and cholera is well known. Unfortunately, the surveillance of other respiratory infections, including Legionnaires' disease and legionellosis has been neglected in the past, with the result that their prevalence is not known. The NCOH has embarked on a program of assistance in the reporting of legionellosis cases to the Gauteng Provincial Gavernment. This program also includes a

continued effort to increase the awareness of this disease, not only in medical professionals, but also in workers, management and the general public.

Only one Legionnaires' disease outbreak has been reported in South Africa to date. However, there is always a possibility of outbreaks occurring in the country; in order to recognise such outbreaks and be able to respond timeously and efficiently to these threats, cases have to be reported to the proper officials. This program is designed to assist in this process.

4.9.3 Standardisation of detection methods

There is no standard method for the detection of legionellae from environmental samples in South Africa. This project served to point out some of the problems regarding legionella detection and propose a standard method for development and discussion. This method will be subjected to extensive interlaboratory testing before being standardised and used routinely.

4.9.4 Research

- The presence of legionellae in dental unit water lines

  The NCOH is currently involved in a research project, studying the presence of Legionella organisms in dental unit water lines at the Dental School, University of the Witwatersrand. This project will be expanded during 2002 to study the prevalence of antibodies in students and personnel at the Dental School. Preliminary results have shown a high proportion of dental unit water lines to contain legionellae. The findings from this study will be used to initiate appropriate prevention and control measures and will be of great value to increase the awareness of the risk of legionellosis in these systems.

- Legionella infections in HIV positive mine workers

  Pneumonia is one of the most frequent complications of HIV/AIDS patients. Apparently *Pneumocystis carinii* is the leading cause. The prevalence of *L pneumophila, C pneumoniae, C trachomatis* and *M pneumoniae* in this population is unknown. The reason for this may be the currently used diagnostic methods that make use of the measurement of antibody levels which is of limited value in HIV/AIDS patients due to their compromised immune response.

- Legionella infections in miners with tuberculosis

  Similarly, the high number of mine workers with tuberculosis increases the immunocompromised population in this sector. In order to provide the best primary and occupational health care, these patients should be tested for the presence of antibodies to respiratory pathogens like *C pneumoniae, L pneumophila* and other pathogens. To this effect, a research project is planned by the NCOH for the near future.

- Legionella-like amoebal pathogens (LLAPs)

  Intracellular replication within protozoa plays a major role in the ecology and pathogenesis of Legionella species. The recent emphasis on the presence of pathogenic free-living amoebae in water distribution systems, legionella-like amoebal pathogens and their potentially serious health risks in both the occupational and public health sectors have received much attention in scientific literature. A research project in collaboration with the Water Research Centre at the Tennessee Technological University is in the planning stage. A visit to the Centre has provided excellent information and training into the methodologies to be developed for this work, which will be a first in South Africa.

- Molecular methods

  Polymerase chain reaction (PCR)-based methods have been used fairly successful in detecting legionellae in clinical specimens, but problems remain in environmental samples. Although an effective PCR methods for Legionella has been developed in a recent South African study, the results obtained from using this method for Legionella detection from the samples evaluated in this study did not correlate with those obtained by culture. This was especially true for the samples from the underground areas of the mine (results not shown). Further research is therefore needed on this area of Legionella detection. Molecular typing methods should be developed for use in outbreak situations where it is essential to link isolates from patients and environments.

- Immunomagnetic separation techniques

  Immunomagnetic separation has been shown to have potential as a simple way of extracting bacteria from environmental samples prior to detection by other methods, by workers at the University of Pretoria. Research into this aspect of Legionella detection may prove useful.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

REFERENCES

Abu Kwaik Y., Gao L.Y., Harb O.S. and Stone B.J. (1997). Transcriptional regulation of the macrophage-induced gene (gspA) of *Legionella pneumophila* and phenotypic characterisation of a null mutant. *Mol. Microbiol.* 24 (3): 629-642.

Adams S.L. (1999). Legionella: A new millenium bug. *Clin. Lab. Sci.* 12 (5): 309-315.

Adeleke A.A. (1996). Legionella-like amoebal pathogens – phylogenetic status and possible role in respiratory disease. *Emer. Infect. Dis.* 2 (3): http://www.cdc.gov/ncidod/eid/vol2no3/adeleke.htm

Adeleke A.A., Fields B.S., Benson R.F., Daneshvar M.I., Pruckler J.M., Ratcliff R.M., Harrison T.G., Weyant R.S., Birtles R.J., Raoult D. and Halablab M.A. (2001). *Legionella drozanskii* sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov., three unusual new Legionella species. *Int. J. Sys. Evol. Microbiol.* 51 (3): 1151-1160.

Alary M. and Joly J.R. (1992). Factors contributing to the contamination of hospital water distribution systems by legionellae. *J. Infect. Dis.* 165: 565-569.

Allen K.W., Prempeh H. and Osman M.S. (1999). Legionella pneumonia from a novel industrial aerosol. *Commun. Dis. Public Health* 2 (4): 294-296.

Amano K. and Williams J.C. (1983). Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysosome hydrolysis correlates with a high degree of peptide cross-linking. *J. Bacteriol.* 153 (1): 520-526.

Amemura-Maekawa J., Kura F. and Watanabe H. (1996). Cloning and nucleotide sequences of iron and copper-zinc superoxide dismutase genes of *Legionella pneumophila* and their distribution among Legionella species. *Jpn. J. Med. Sci. Biol.* 49 (4): 167-186 (abstract only).

Amemura-Maekawa J., Mishima-Abe S., Kura F., Takanashi T. and Watanabe H. (1999). Identification of a novel periplasmic catalase-peroxidase KatA of *Legionella pneumophila*. *FEMS Microbiol. Lett.* 176 (2): 339-344.

Andrews H.L., Vogel J.P. and Isberg R.R. (1998). Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. *Infect. Immun.* 66 (3): 950-958.

Aragon V., Kurtz S., Flieger A., Neumeister B. and Cianciotto N.P. (2000). Secreted enzymatic activities of wild-type and pilD-deficient *Legionella pneumophila*. *Inf. Imm.* 68 (4): 1855-1863.

Armon R. and Payment P. (1990). A transparent medium for isolation of *Legionella pneumophila* from environmental water sources. *J. Microbiol. Methods* 11: 65-71.

Armon R., Potasman I. and Green M. (1990). Biochemical fingerprints of Legionella species by the BIOLOG system: presumptive identification of clinical and environmental isolates. *Lett. Appl. Microbiol.* 11: 290-292.

ASTM Guidelines D5952 (1996). Standard guide for inspecting water systems for legionellae and investigating possible outbreaks of legionellosis (Legionnaires' disease and Pontiac fever).

Australian Standard: Waters – Examination for legionellae. Standards Australia (AS 3896-1991).

Aye T., Wachmuth K.., Feeley J.C., Gibson R.J. and Johnson S.R. (1981). Plasmid profiles of Legionella species. *Curr. Microbiol.* 6: 389-394.

Bandyopadhyay P. and Steinman H.M. (2000). Catalase-peroxidases *of Legionella pneumophila*: cloning of the katA gene and studies of KatA function. *J. Bacteriol.* 182 (23): 6679-6686.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Bangsborg J.M., Collins M.T. and Høiby N. (1986). Antigenic homogeneity among *Legionella pneumophila* serogroups 1 to 6 evaluated by crossed immunoelectrophoresis. *Int. J. Syst. Bacteriol.* 36 (3): 399-404.

Barbaree J.M. (1993). Selecting a subtyping technique for use in investigations of legionellosis epidemics. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Barbaree J.M., Fields B.S., Feeley J.C., Gorman G.W. and Martin W.T. (1986). Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila. Appl. Environ. Microbiol.* 51 (2): 422-424.

Barbaree J.M., Gorman G.W., Martin W.T., Fields B.S. and Morrill W.E. (1987). Protocol for sampling environmental sites for legionellae. *Appl. Environ. Microbiol.* 53 (7): 1454-1458.

Barker J., Brown M.R.W., Collier P.J., Farrell I. and Gilbert P. (1992). Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Appl. Environ. Micrbiol.* 58 (8): 2420-2425.

Barker J., Brown M.R.W., Gilbert P., Collier P.J. and Farrell I.D. (1993). The physiological status of *Legionella pneumophila* and its susceptibility to chemical inactivation. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Barker J. and Brown M.R. (1995). Speculations on the influence of infecting phenotype on virulence and antibiotic susceptibility of *Legionella pneumophila. J. Antimicrob. Chemother.* 36 (1): 7-21.

Barker J. and Clement P. (1994). Reducing the risk of legionellosis: the role of amoebae. *Occup. Health. Rev.* Jan/Feb. 20-23.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Barker J., Farrell I.D. and Hutchinson J.G. (1986). Factors affecting growth of *Legionella pneumophila* in liquid media. *J. Med. Microbiol.* 22 (2): 97-100.

Barker J., Lambert P.A. and Brown M.R. (1993). Influence of intra-amoebic and other growth conditions on the surface properties of *Legionella pneumophila. Infect. Immun.* 61 (8): 3503-3510.

Bartlett J. (2000). Treatment of community-acquired pneumonia. *Chemotherapy* 46: Suppl. 1: 24-31.

Bartie C. and Klugman K.P. (1997). Exposures to *Legionella pneumophila* and *Chlamydia pneumoniae* in South Afrcan mine workers. *Int. J. Occup. Env. Health* 3 (2): 120-127.

Bej A.K., Mahbubani M.H., Miller R., DiCesare J.L., Haff L. and Atlas R.M. (1990). Multiplex PCR amplification and immobilised capture probes for detection of bacterial pathogens and indicators in water. *Mol. Cell. Probes* 4: 353-365.

Bej A.K., Mahbubani M.H. and Atlas R.M. (1991). Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. *Appl. Environ. Microbiol.* 57 (2): 597-600.

Bej A.K., Mahbubani M.H., DiCesare J.L. and Atlas R.M. (1991). Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Appl. Environ. Microbiol.* 57 (12): 3529-3534.

Bej A.K., Mahbubani M.H. and Atlas R.M. (1993). Detection and molecular serogrouping of Legionella pneumophila by polymerase chain reaction amplification and restriction enzyme ananalysis. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Bej A.K., Mahbubani M.H., DiCesare J.L. and Atlas R.M. (1991). Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Appl. Environ. Microbiol.* 57: 3529-3534.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Benson R.F. and Fields B.C. (1998). Classification of the genus Legionella. *Semin. Respir. Infect.* 13 (2): 90-99.

Benson R.F., Fields B.S., Besser R. and Benin A. (2000). Application of amplified fragment length polymorphism to subtyping of *Legionella pneumophila* SG1. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Bentham R.H. (2000). Routine sampling and the control of Legionella species in cooling water systems. *Curr. Microbiol.* 41 (4): 271-275.

Bohach G.A. and Snyder I.S. (1983). Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. *Appl. Environ. Microbiol.* 46 (2): 528-531.

Boissinot M., Ramsay D., Barthe C. and Joly J.R. (1987). Antigenic variability of the outer membrane antigens of *Legionella pneumophila* SGs 1-8. *Can. J. Microbiol.* 33 (7): 607-613.

Bollin G.E., Plouffe J.F., Para M.F. and Hackman B. (1985). Aerosols containing *Legionella pneumophila* generated by showerhead and hot-water faucets. *Appl. Env. Microbiol.* 50 (5): 1128-1131.

Bopp C.A., Sumner J.W., Morris G.K. and Wells J.G. (1981). Isolation of Legionella species from environmental water samples by low-pH treatment and use of a selective medium. *J. Clin. Microbiol.* 13 (4): 714-719.

Boulanger C.A. and Edelstein P.H. (1995). Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. *Appl. Environ. Microbiol.* 61: 1805-1809.

Brand B.C., Sadosky A.B. and Shuman H.A. (1994). The *Legionella pneumophila* icm locus: a set of genes required for intracellular multiplication in human macrophages. *Mol. Microbiol.* 14 (4): 797-808.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Brenner D.J., Feeley J.C. and Weaver R.E. (1984). Family VII. Legionellaceae, p279. In NR Krieg and JG Wolf (eds). Bergey's Manual of systematic bacteriology. Vol 1. Williams and Wilkins, Baltimore.

Brenner D.J., Steigerwalt A.G. and McDade J.E. (1979). Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, family nova. *Ann. Intern. Med.* 90 (4): 656-658.

Brenner D.J. (1986). Classification of Legionellaceae. *Isr. J. Med. Sci.* 22 (9): 620-633.

Brenner D.J. (1987). Classification of the legionellae. *Semin. Respir. Infect.* 2 (4): 190-205.

Brenner D.J., Steigerwalt A.G., Epple P., Bibb W.F., McKinney R.M., Starnes R.W., Colville J.M., Selander R.K., Edelstein P.H. and Moss C.W. (1988). *Legionella pneumophila* SG Lansing 3 isolated form a patient with fatal pneumonia, and descriptions of *L pneumophila* subsp. *pneumophila* subsp. nov., *L pneumophila* subsp. *fraseri* subsp. nov. and *L pneumophila* subsp. *pascullei* subsp. nov. *J. Clin. Microbiol.* 26 (9): 1695-1703.

Breiman R.F. (1993). Modes of transmission in epidemic and nonepidemic Legionella infections: directions for further study. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC.

Breiman R.F., Fields B.S., Sanden G.N., Volmer L., Meier A. and Spika J.S. (1990). Association of shower use with Legionnaires' disease: possible role of amoebae. *J. Am. Med. Assoc.* 2924 (3):

Brindle R.J., Stannett P.J. and Cunliffe R.N. (1987). *Legionella pneumophila*: comparison of isolation from water specimens by centrifugation and filtration. *Epidem. Inf.* 99: 241-247.

Broadbent C.R. (1987). Practical measures to control Legionnaires' disease hazards. *Australian Refrigeration, Air Conditioning and Heating*, July: 22-30.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Brown C.M., Nuorti P.J., Breiman R.F., Hathcock A.L., Fields B.S., Lipman H.B., Llewellyn G.C., Hofman J. and Detron M. (1999). A community outbreak of Legionnaires' disease linked to hospital cooling towers: an epidemiological method to calculate dose of exposure. *Int. J. Epidemiol.* 28: 353-359.

Brown A., Vickers R.M., Elder E.M., Lema M. and Garrity G.M. (1982). Plasmid and surface antigen markers of endemic and epidemic *Legionella pneumophila* strains. *J. Clin. Microbiol.* 16 (2): 230-235.

Brundett G. (1989). Water Services and Maintenance Systems. *Occup Health* June: 159-161.

Butler C.A. and Hoffman P.S. (1990). Characterisation of a major 31-kilodalton peptidoglycan-bound protein of *Legionella pneumophila. J. Bacteriol.* 172 (5): 2401-2407.

Butler C.A., Street E.D., Hatch T.P. and Hoffman P.S. (1985). Disulfide-bonded outer membrane proteins in the genus Legionella. *Infect. Immun.* 48 (1): 14-18.

Byrd T.F. and Horwitz M.A. (2000). Aberrantly low transferrin receptor expression on human monocytes is associated with non-permissiveness for *Legionella pneumophila* growth. *J. Infect. Dis.* 181 (4): 1394-1400.

Byrd J.J., Xu H.S. and Colwell R.R. (1991). Viable but non-culturable bacteria in drinking water. *Appl. Env. Microbiol.* 57 (3): 875-878.

Chandler F.W., Cole R.M., Hicklin M.D., Blackmon J.A. and Callaway C.S. (1979). Ultrastructure of the Legionnaires' disease bacterium. *Ann. Intern. Med.* 90 (4): 642-647.

Chandler F.W., Roth I.L., Callaway C.S., Bump J.L., Thomason B.M. and Weaver R.E. (1980). Flagella on Legionnaires' disease bacteria. *Ann. Intern. Med.* 93: 711-714.

Cianciotto N.P., Bangsborg J.M., Eisenstein B.I. and Engleberg N.C. (1990). Identification of mip-like genes in the genus Legionella. *Infect. Immun.* 58: 2912-2919.

Cianciotto N.P. and Fields B.S. (1992). *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages.

Cianciotto (2001). Pathogenicity of *Legionella pneumophila*. *Int. J. Med. Microbiol.* 291 (5): 331-343.

Ciecielski C.A., Blaser M.J. and Wang W.L. (1986). Serogroup specificity of *Legionella pneumophila* is related to lipopolysaccharide characteristics. *Infect. Immun.* 51 (2): 397-404.

Cirillo S.L., Bermudez L.E., El-Etr S.H., Duhamel G.E. and Cirillo J.D. (2001). *Legionella pneumophila* entry gene rtxA is involved in virulence. *Infect. Immun.* 69 (1): 508-517.

Coers J., Kagan J.C., Matthews M., Nagai H., Zuckman D.M. and Roy C.R. (2000). Identification of icm protein complexes that play distinct roles in the biogenesis of an organelle permissive for *Legionella pneumophila* intracellular growth. *Mol. Microbiol.* 38 (4): 719-736.

Colbourne J.S. and Dennis P.J. (1985). Distribution and persistence of Legionella in water systems. *Microbiol. Sc.* 2 (2): 40-43.

Colbourne J.S. and Dennis P.J. (1989). The ecology and survival of *Legionella pneumophila*. *J.IWEM.* 3:345-350.

Collins M.T., Cho S.N., Høiby N., Espersen F., Baek L. and Reif J.S. (1983). Crossed immunoelectrophoretic analysis of *Legionella pneumophila* SG1 antigens. *Inf. Immun.* 39 (3): 1428-1440.

Craeger J.G., Black J.G. and Davison V.E. (eds). (1990). Microbiology: Principles and Applications. Prentice Hall, Englewood Cliffs, New Jersey 07632.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

De Gheldre Y., Maes N., Presti F.L., Etienne J. and Struelens M. (2001). Rapid identification of clinically relevant legionella spp. by analysis of transfer DNA intergenic spacer length polymoprhism. *J. Clin. Microbiol.* 39 (1): 162-169.

Dennis P.J. (1993). Potable water systems: insights into control. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legonella: current status and emerging perspectives. American Society for Microbiology. Washington, DC.

Dennis P.J., Bartlett C.L.R. and Wright A.E. (1993). Comparison of isolation methods for Legionella species. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Desai R., Welsh C., Summy M., Farone M. and Newsome A.L. (1999). The potential of in situ hybridisation and an immunogold assay to identify Legionella associations with other microorganisms. *J. Microbiol. Methods* 37 (2): 155-164.

Dietrich C., Heuner K., Brand B.C., Hacker J. and Steinert M. (2001). Flagellum of *Legionella pneumophila* positively affects the early phase of infection in eukaryotic host cells. *Infect. Immun.* 69 (4): 2116-2122.

Diogo A., Veríssímo A., Nobre M.F. and Da Costa M.S. (1999). Usefulness of fatty acid composition for differentiation of Legionella species. *J. Clin. Microbiol.* 37 (7): 2248-2254.

Dominguez J., Gali N., Matas L., Pedroso P., Hernandez A. and Padilla E. (1999). Evaluation of a rapid immunochromatographic assay for the detection of Legionella antigen in urine. *Eur. J. Clin. Microbiol. Infect. Dis.* 18 (12): 896-898.

Draft British Standard for Identification of Legionella from environmental samples (1992). DD 211:1992.

Draft Internation Standard ISO/DIS 11731 (1996). Water quality – detection and enumeration of Legionella.

Dumais-Pope C., O'Connell W. and Cianciotto N.P. (1993). Distribution and regulation of the Legionella mip gene. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds).

Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC.

Edelstein P.H. (1981). Improved semiselective medium for isolation of *L pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* 14 (3): 298-303.

Edelstein P.H. (1982). Comparative study of selective media for isolation of *Legionella pneumophila* from potable water. *J. Clin. Microbiol.* 16 (4): 697-699.

Edelstein P.H., Edelstein M.A., Higa F. and Falkow S. (1999). *Proc. Natl. Acad. Sci. USA* 96 (14): 190-195.

Ehret W., Von Specht B.U. and Ruckdeschel G. (1986). Discrimination between clinical and environmental strains of *Legionella pneumophila* by a monoclonal antibody. *Isr. J. Med. Sci.* 22 (10): 715-723.

Ehret W. and Ruckdeschel G. (1985). Membrane proteins of Legionellaceae. I. Membrane proteins of different strains and serogroups of *Legionella pneumophila*. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* 259 (4): 433-445.

Ehret W., Anding G., Tartakovski I. and Ruckdeschel G. (1993). Molecular epidemiology of outbreak-associated Serogroup 1 isolates. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legonella: current status and emerging perspectives. American Society for Microbiology. Washington, DC.

Ehret W., Von Specht B.U. and Ruckdeschel G. (1986). Discrimination between clinical and environmental strains of *Legionella pneumophila* by a monoclonal antibody. *Isr. J. Med. Sci.* 22 (10): 715-723

Engelhart S., Sennekamp J., Gilges S., Pleischl S. and Exner M. (2000). Workplace-related complaints due to exposure to contaminated humidifier water and the VDI guideline 6022. *Eur. J. Med. Res.* 5 (3): 126.

England A.C., Fraser D.W., Plikaytis B.D., Tsai T.F., Storch G. and Broome C.V. (1981). Sporadic legionellosis in the United States: the first thousand cases. *Am. J. Med.* 70: 707-711.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Engleberg N.C., Howe D.C., Rogers J.E., Arroyo J. and Eisenstein B.I. (1991). Characterisation of a *Legionella pneumophila* gene encoding a lipoprotein antigen. *Mol. Microbiol.* 5 (8): 2021-2029.

Engleberg N.C. (1993). Genetic studies of Legionella pathogenesis. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp 63-68.

Feeley J.C., Gibson R.J., Gorman G.W., Langford N.C., Rasheed J.K., Mackel D.C. and Baine W.B. (1979). Charcoal yeast extract agar: primary isolation medium for *Legionella pneumophila*. *J. Clin. Microbiol.* 10 (4): 437-441.

Feeley J.C., Gorman G.W., Weaver R.E., Mackel D.C. and Smith H.W. (1978). Primary isolation media for Legionnaires' disease bacterium. *J. Clin. Microbiol.* 8 (3): 320-325.

Fields B.S. (1991). The role of amoebae in legionellosis. *Clin. Microbiol. News.* 13 (12): 92-93 (editorial).

Fields B.S. (1996). The molecular ecology of legionellae. *Trends Microbiol.* 4 (7): 286-290.

Fields B.S. (1997). Legionella and Legionnaires' disease, p 666-675. In: Walter M.V., Knudsen G.R., McInerney M.J., Stetzenbach L.D. and Hurst C.J. (eds). Manual of environmental microbiology. American Society for Microbiology, Washington, DC.

Fields B.S., Sanden G.N., Barbaree J.M., Morrill W.E., Wadowski R.M., White E.H. and Feeley J.C. (1989). Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. *Curr. Microbiol.* 18: 131-137.

Finch R. (1988). Minimising the risk of Legionnaires' disease. *British Med. J.* 296: 1343-1344.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Fiore A.E., Butler J.C., Emori T.G. and Gaynes R.P. (1999). A survey of methods used to detect nosocomial legionellosis among participants in the National Nosocomial Infections Surveillance System. *Infect. Control Hosp. Epidemiol.* 20 (6): 412-416.

Flieger A., Gong S., Faigle M., Deeg M., Bartmann P. and Neumeister B. (2000). Novel phospholipase A activity secreted by Legionella species. *J. Bacteriol.* 182 (5): 1321-1327.

Fouchet P., Jayat C., Héchard Y., Ratinaud M.H. and Frelat G. (1993). Recent advances of flow cytometry in fundamental and applied microbiology. *Biol. Cell* 78: 95-109.

Freije M.R. (1996). Legionellae control in health care facilities. A guide for minimising risk. HC Information Resources, Inc., Indianpolis, USA.

Gabay J.E., Blake M., Niles W.D. and Horwitz M.A. (1985). Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. *J. Bacteriol.* 162 (1): 85-91.

Gabay J.E. and Horwitz M.A. (1985). Isolation and characterization of the cytoplasmic and outer membranes of the Legionnaires' disease bacterium (*Legionella pneumophila*). *J. Exp. Med.* 161: 409-422.

Girod J.C., Reichman R.C., Winn W.C., Laucke D.N., Vogt R.L. and Dolin R. (1982). Pneumonic and non-pneumonic forms of legionellosis: the result of a common-source exposure to *Legionella pneumophila*. *Arch. Intern. Med.* 142: 545-547.

Glick T.H., Gregg M.B., Berman B., Mallison G., Rhodes W.W. and Kassanoff I. (1978). Pontiac fever: an epidemic of unknown etiology in a health department: I. Clinical and epidemiological aspects. *Am. J. Epidemiol.* 107 (2): 149-160.

Goldstein B., Cole L.R., Koornhof H.J. and Dubery B. (1980). A fatal case of Legionnaires' disease. *SA Mediese Tydskrif* 58: 24-27.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Gorman G.W., Feeley J.C., Steigerwalt A., Edelstein P.H., Moss C.W. and Brenner D.J. (1985). *Legionella anisa*: a new species of Legionella isolated from potable waters and a cooling tower. *Appl. Environ. Microbiol.* 49 (2): 305-309.

Gosting L.H., Cabrian K., Sturge J.C. and Goldstein L.C. (1984). Identification of a species-specific antigen in *Legionella pneumophila* by a monoclonal antibody. *J. Clin. Microbiol.* 20 (6): 1031-1035.

Grabow N.A., Kfir R. and Grabow W.O.K. (1991). A most probable number method for the enumeration of Legionella bacteria in water. *Wat. Sci. Tech.* 24 (2): 143-147.

Grabow N.A., Pienaar E.J. and Kfir R. (1991). The occurrence of Legionella bacteria in cooling towers in South Africa. *Wat. Sci. Tech.* 24 (2): 149-152.

Graman P.S., Quinlan G.A., Rank J.A. (1997). Nosocomial legionellosis traced to a contaminated ice machine. *Infect. Control Hosp. Epidemiol.* 18 (9): 637-640.

Gregersen P., Grunnet K., Uldum S.A., Andersen B.H. and Madsen H. (1999). Pontiac fever at a sewage treatment plant in the food industry. *Scand. J. Work Environ. Health* 25 (3): 291-295.

Grimm D., Ludwig W., Brand B.C., Schleifer K.H., Michel R., Hacker J. and Steinert M. (2000). Specific detection of Legionella and its amoebic hosts by fluorescence in situ hybridisation with 16S and 18S rRNA oligonucleotide probes. Presented at the 5th International Conference on Legionella. September 26-29, Neu-Ulm, Germany.

Grimont P.A.D., Grimont F., Desplaces N. and Tchen P. (1985). DNA probe specific for *Legionella pneumophila*. *J. Clin. Microbiol.* 2: 431-437.

Grimont F., Lefevre M., Ageron E., and Grimont P.A.D. (1989). rRNA gene restriction patterns of Legionella species: a molecular identification system. *Res. Microbiol.* 140: 615-626.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Groothuis D.G. and Veenendal H.R. (1983). Heat treatment as an aid for the isolation of *Legionella pneumophila* from clinical and environmental samples. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* 255 (1): 39-43.

Hacker J., Ott M., Wintermeyer E., Ludwig B. and Fischer G. (1993). Analysis of virulence factors of *Legionella pneumophila*. *Int. J. Med. Microbiol. Parasitol. Infect. Dis.* 278 (2-3):348-358.

Hales L..M. and Shuman H.A. (1999). The *Legionella pneumophila* rpoS gene is required for growth within *Acanthamoeba castellanii*. *J. Bacteriol.* 181 (16): 4879-4889.

Hales L.M. and Shuman H.A. (1999). *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infect. Immun.* 67 (7): 3662-3666.

Haley C.E., Cohen M.L., Halter J. and Meyer R.D. (1979). Nosocomial Legionnaires' disease: a continuing common-source epidemic at Wadsworth Medical Centre. *Ann. Intern. Med.* 90 (4): 583-586.

Harb O.S. and Abu Kwaik Y. (2000). Interaction of *Legionella pneumophila* with protozoa provides lessons. *ASM News* 66 (10): 609-616.

Hariparsad D., Ramsaroop R., Pather M. and Wesley A. Legionnaires' disease in Durban. A case report. *SA Mediese Tydskrif* 59: 977-979.

Hart I., Serman S., Lee P.A. and Lee J.V. (2000). Evaluation of the Oxoid Dryspot Legionella test range. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Hébert G.A. (1981). Hippurate hydrolysis by *Legionella pneumophila*.. *J. Clin. Microbiol.* 13 (1): 240-242.

Hébert G.A., Callaway C.S. and Ewing E.P. Jr (1984). Comparison of *Legionella pneumophila, L micdadei, L bozemanii* and *L dumoffii* by transmission electron microscopy. *J. Clin. Microbiol.* 19 (2): 116-121.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Helbig J.H., Luck P.C., Steinert M., Jacobs E. and Witt M. (2001). Immunolocalisation of the Mip protein of intracellularly and extracellularly grown *Legionella pneumophila*. *Lett. Appl. Microbiol.* 32 (2): 83-88.

Hellard M.E., Sinclair M.I., Streeton C.L. and Fairly C.K. (1997). Commentary: Drinking water and microbial pathogens – issues and challenges for the year 2000. *J. Pub. Health Med.* 19 (2): 129-131.

Heuner K., Brand B.C. and Hacker J. (1999). The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. *FEMS Microbiol. Lett.* 175 (1): 69-77.

Heuner K., Dietrich C., Steinert M., Gobel U.B. and Hacker J. (2000). Cloning and characterisation of the Legionella pneumophila-specific gene encoding a member of the LysR family of transcriptional regulators. *Mol. Gen. Genet.* 264 (1-2): 204-211.

Heymann D.L. (2001). Strengthening global preparedness for defence against infectious disease threats. Committee on Foreign Relations, United States Senate. Hearing on *The threat of bioterrorism and the spread of infectious diseases*, 5 September.

Higa F. and Edelstein P.H. (2001). Potential virulence role of *Legionella* pneumophila ptsP ortholog. *Infect. Immun.* 69 (8): 4782-4789.

Hindahl M.S. and Iglewski B.H. (1984). Isolation and characterisation of *Legionella pneumophila* outer membrane. *J. Bacteriol.* 159 (1): 107-113.

Hindahl M.S. and Iglewski B.H. (1986). Outer membrane proteins from Legionella pneumophila serogroups and other Legionella species. *Infect. Immun.* 51 (1): 94-101.

Hindahl M.S. and Iglewski B.H. (1987). Cloning and expression of a common Legionella outer membrane antigen in *Escherichia coli*. *Microb. Pathog.* 2 (2): 91-99.

Hobbie J.E., Daley R.J. and Jasper S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33 (5): 1225-1228.

Hoffman P. (1984). Bacterial physiology. In: *Legionella, Proceedings of the 2nd International Symposium* ed. Thornsberry C, Balows A, Feeley JC and Jakubowski W: pp 61-67. Washington DC: American Society for Microbiology.

Hoffman P.S. and Garduno R.A. (1999). Surface-associated heat shock proteins of *Legionella pneumophila* and *Helicobacteri pylori*: roles in pathogenesis and immunity. *Infect. Dis. Obstet. Gynecol.* 7 (1-2): 58-63 (abstract only)

Hoffman P.S., Pine L. and Bell S. (1983). Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: catalytic decomposition of charcoal. *Appl. Env. Microbiol.* 45 (3): 784-791.

Hoffman P.S., Ripley M. and Weeratna R. (1992a). Cloning and nucleotide sequence of a gene (OmpS) encoding the major outer membrane protein of *Legionella pneumophila. J. Bacteriol.* 174 (3): 914-920.

Hoffman P.S., Seyer J.H. and Butler C.A. (1992b). Molecular characterisation of the 28- and 31-kilodalton subunits of the *Legionella pneumophila* major outer membrane protein. *J. Bacteriol.* 174 (3): 908-913.

Hübner I., Obst U., Steinmetz I. and Bitter-Suermann D. (1992). Microbiological-immunological rapid method to detect legionellae in water. *Acta Hydrochim. Hydrobiol.* 20: 205-207.

Hughes M.A. and Steele T.W. (1994). Occurrence and distribution of Legionella species in composted plant materials. *Appl. Environ. Microbiol.* 60 (6): 2003-2005.

Hurley M.C., Balazovich K., Albano M., Engleberg N.C. and Eisenstein B.I. (1993). *Legionella pneumophila* Mip inhibits protein kinase C. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp 69-70.

Hussong D., Colwell R.R., O'Brien M., Weiss E., Pearson A.D., Weiner R.M. and Burge W.D. (1987). Viable *Legionella pneumophila* not detectable by culture on agar media. *Biotechnology* 5: 947-950.

Ikedo M. and Yabuuchi E. (1986). Ecological studies of Legionella species. I. Viable counts of *Legionella pneumophila* in coolint tower water. *Microbiol. Immunol.* 30 (5): 413-423.

Inglis (2000).

Ishimatsu S., Miyamoto H., Hori H., Tanakas I. and Yoshida S. (2001). Sampling and detection of *Legionella pneumophila* aerosols generated from an industrial cooling tower. *Ann. Occup. Hyg.* 45 (6): 421-427.

Jantzen E., Sonesson A., Tangen T. and Eng J. (1993). Hydroxy-fatty acid profiles of Legionella species: diagnostic usefulness assessed by principal component analysis. *J. Clin. Microbiol.* 31 (6): 1413-1419.

Johnson W., Elliot J.A., Helms C.M. and Renner E.D. (1979). A high molecular weight antigen in the Legionnaires' disease bacterium: isolation and partial characterisation. *Ann. Intern. Med.* 90 (4): 638-641.

Joly J.R., Alary M. (1993). Occurrence of nosocomial Legionnaires' disease in hospitals with contaminated potable water supply. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC.

Joly J.R., McKinney R.M., Tobin J.O., Bibb W.F., Watkins I.D. and Ramsay D. (1986). Development of a standardised subgrouping sceme for *Legionella pneumophila* serogroup 1 using monoclonal antibodies. *J. Clin. Microbiol.* 23: 768-771.

Jousimies-Somer H.R., Waarala S. and Väisänen M.L. (1993). Recovery of Legionella species from water samples by four different methods. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp. 200-201.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Kaplan C., Zwi S., Kallenbach J., Singer J., Koornhof H.J., Mauff A.C. and Dubery B. (1980). Legionnaires' disease in Johannesburg. *South African Med. J.* 58: 13-17.

Kazandjian D., Chiew R. and Gilbert G.L. (1997). Rapid diagnosis of *Legionella pneumophila* Serogroup 1 infection with the Binax Enzyme Immunoassay urinary antigen test. *J. Clin. Microbiol.* 35 (4): 954-956.

Keevil C.W. (2000). Biofilms and Legionella. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Keleti G. and Shapiro M.A. (1987). Legionella and the environment. *CRC Crit. Rev. Env. Control* 17 (2): 133-181.

Keller D.W., Hajjeh R., DeMaria A., Fields B.S., Pruckler J.M., Benson R.S., Kludt P.E., Lett S.M., Mermel L.A., Giorgio C. and Breiman R.F. (1996). Community outbreak of Legionnaires' disease: an investigation confirming the potential for cooling towers to transmit Legionella species. *Clin. Infect. Dis.* 22 (2): 257-261.

Kilvington S. and Price J. (1990). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *J. Appl. Bacteriol.* 68 (5): 19-25.

Kirby J.E., Vogel J.P., Andrews H.L. and Isberg R.R. (1998). Evidence of pore-forming ability by *Legionella pneumophila. Mol. Microbiol.* 27 (2): 323-336.

Knirel Y.A., Moll H. and Zähringer U. (1996). Structural study of a highly O-acetylated core of *Legionella pneumophila* serogroup 1 lipopolysaccharide. *Carbohydr. Res.* 293 (2): 223-234.

Kniros C., High A.S. and Rodgers F.G. (1999). Role of the 25kDa major outer membrane protein of *Legionella pneumophila* in attachment to U-937 cells and its potential as a virulence factor for chick embryos. *J. Appl. Microbiol.* 86 (2): 237-244.

Knudson G.B. and Mikesell P. (1980). A plasmid in *Legionella pneumophila*. *Infect. Immun.* 29 (1): 1092-1095.

Kool J.L., Bergmire-Sweat D., Butler J.C., Brown E.W., Peabody D.J., Massi D.S., Carpenter J.C., Pruckler J.M., Benson R.F. and Fields B.S. (1999). Hospital characteristics associated with colonisation of water systems by Legionella and risk of nosocomial Legionnaires' disease: a cohort study of 15 hospitals. *Infect. Control Hosp. Epidemiol.* 20 (12): 798-805.

Kuchta J.M., States S.J., McGlauchlin J.E., Overmeyer J.H., Wadowski R.M., McNamara A.M., Wolford R.S. and Yee R.B. (1985). Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium-passaged strains. *Appl. Env. Microbiol.* 50 (1): 21-26.

Kusnetsov J.M., Martikainen P.J., Jousimies-Somer H.R., Väisänen M.L., Tulkki A.I., Ahonen H.E. and Nevalainen A.I. (1993). Physical, chemical and microbiological water characteristics associated with the occurrence of Legionella in cooling tower systems. *Wat. Res.* 27 (1): 85-90.

Kusnetsov J.M., Jousimies-Somer H.R., Nevalainen A.I. and Martikainen P.J. (1994). Isolation of Legionella from water samples using various culture methods. *J. Appl. Bacteriol.* 76: 155-162.

Kusnetsov J.M., Ottoila E. and Martikainen P.J. (1996). Growth, respiration and survival of Legionella pneumophila at high temperatures. *J. Appl. Bacteriol.* 81: 341-347.

Lambert M.A. and Moss W. (1989). Cellular fatty acid compositions and isoprenoid quinone contents of 23 Legionella species. *J. Clin. Microbiol.* 27 (3): 465-473.

La Scola B., Mezi L., Weiller P.J. and Raoult D. (2001). Isolation of *Legionella anisa* using an amoebic coculture procedure. *J. Clin. Microbiol.* 39 (1): 365-366.

LeChevalier M.W., Cawthon C.D. and Lee R.G. (1988). Inactivation of biofilm bacteria. *Appl. Env. Microbiol.* 54 (10): 2492-2499.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Leoni E., Legnani P.P. (2001). Comparison of selective procedures for isolation and enumeration of Legionella species from hot water systems. *J. Appl. Microbiol.* 90 (1): 27-33.

Lever M.S. (1993). Production and characterisation of a *Legionella pneumophila* specific monoclonal antibody. *FEMS Microbiol. Lett.* 107 (1): 5-9.

Lin Y.E., Vidic R.D., Stout J.E. and Yu V.L. (1998). Legionella in water distribution systems. *J. Am. Wat. Works. Assoc.* 90 (9): 112-121.

Ludwig B., Schmid A., Marre R. and Hacker J. (1991). Cloning, genetic analysis, and nucleotide sequence of a determinant coding for a 19-kDa peptidoglycan-associated protien (Ppl) of *Legionella pneumophila*. *Infect. Immun.* 59 (8): 2515-2521.

Lo Presti F., Riffard S., Meugnier H., Reyrolle M., Lasne Y., Grimont P.A., Grimont F., Vandenesch F., Etienne J., Fleurette J. and Freney J. (1999). *Legionella taurinensis* sp. nov., a new species antigenically similar to *Legionella spiritensis*. *Int. J. Syst. Bacteriol.* 49 Pt 2: 397-403.

Lück P.C. (2000). Standardisation of typing of Legionella. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Lundholm M., Jacobson K., Zettersten U. and Bernander S. (2000). Variations in *Legionella pneumophila* non-1 strains in nosocomial outbreaks (1993-1999) and environment at a Swedish University hospital. PFGE and AFLP analysis. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Maartens G., Lewis S.J., de Goveia C., Bartie C. *et al.* (1994). Atypical bacteria are a common cause of community-acquired pneumonia in hospitalised patients. *South African Med. J.* 84 (10): 678-682.

Mahbubani M.H., Bej A.K., Miller R., Haff L., DiCesare J. and Atlas R.M. (1990). Detection of Legionella with polymerase chain reaction and gene probe methods. *Mol. Cell. Probes* 4: 175-187.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Maher W.E., Plouffe J.F. and Para M.F. (1982). Plasmid profiles of clinical and environmental isolates of *Legionella pneumophila* SG1. *J. Clin. Microbiol.* 18 (6): 1422-1423.

Maiwald M., Schill M., Stockinger C., Helbig J.H., Luck P.C., Witzleb W. and Sonntag H.G. (1995). Detection of Legionella DNA in human and guinea pig urine samples by the polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis.* 14: 25-33.

Makin T. and Hart C.A. (1989). Detection of *Legionella pneumophila* in environmental water samples using a fluorescein conjugated monoclonal antibody. *Epidem. Inf.* 103: 105-112.

Marrie T.J., Haldane D., Bezanson G. and Peppard R. (1992). Each water outlet is a unique ecological niche for *Legionella pneumophila*. *Epid. Infect.* 108: 264-270.

Martin W.T., Fields B.S. and Hutwagoner L.C. (1993). Comparison of culture and polymerase chain reaction to detect legionellae in environmental samples. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp. 175.

Martinelli F., Caruso A., Moschini L., Turano A., Scarcella C. and Speziani F. (2000). A comparison of *Legionella pneumophila* occurrence in hot water tanks and instantaneous devices in domestic, nosocomial and community environments. *Curr. Microbiol.* 41 (5): 374-376.

Mauff A.C. and Koornhof H.J. (1984). Legionellosis in South Africa. In: Thornsberry C., Balows A., Feeley J.C., Jakubowski W. (eds). Legionella, Proceedings of the 2nd International Symposium. American Society of Microbiology, Washington, DC.

McClain M.S., Hurley M.C., Brieland J.K. and Engleberg N.C. (1996). The *Legionella pneumophila* hel locus encodes intracellularly induced homologs of heavy-metal ion transporters of Alcaligenes species. *Infect. Immun.* 64 (5): 1532-1540.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

McDade J.E., Sheppard C.C., Fraser D.W., Tsai T.S., Redus M.A. and Dowdle W.R. (1977). Legionnaires' disease. Isolation of the bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* 297: 1197-1203.

McNealy T., Newsome A., Johnson R. and Berk S. (2000). Impact of amoebae, bacteria and tetrahymenae on *Legionella pneumophila* multiplication and distribution in an aquatic environment. Presented at the 5th International Conference on Legionella, September 26-29, Neu-Ulm, Germany.

Moss C.W., Weaver R.E. and Dees S.B. (1977). Cellular fatty acid composition of isolates from Legionnaires' disease. *J. Clin. Microbiol.* 6: 140-143.

Muder R.R., Yu V.L. and Woo A.H. (1986). Mode of transmission of Legionella pneumophila. A critical review. *Arch. Intern. Med.* 146: 1607-1612.

Muraca P.W., Stout J.E., Yu V.L. and Yee Y.C. (1988). Legionnaires' disease in the work environment: implications for environmental health. *Am. J. Hyg. Assoc.* 49 (11): 584-590.

Neblett T.R., Riddle J.M. and Dumoffi M. (1979). Surface topography and fine structure of the Leigonnaires' disease bacterium: a study of six isolates from hospitalised patients. *Ann. Intern. Med.*90 (4): 648-651.

Ng D.L.K., Koh B.B., Tay L. and Heng B.H. (1997). Comparison of the polymerase chain reaction and conventional culture for the detection of legionellae in cooling tower waters in Singapore. *Lett. Appl. Microbiol.* 24: 214-216.

Nielsen K., Bangsborg J.M. and Høiby N. (2000). Susceptibility of Legionella species to five antibiotics and development of resistance by exposure to erythromycin, ciprofloxacin and rifampin. *Diagn. Microbiol. Infect. Dis.* (36 (1): 43-48.

Nolte F.S., Conlin C.A., Soisin A.J.M. and Redmond S.R. (1984). Plasmids as epidemiological markers in nosocomial Legionnaires' disease. *J. Infect. Dis.* 149 (2): 251-256.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

OSHA Technical Manual. Section III: Chapter 7.

Osterholm M.T., Chin T.D., Osborne D.O., Dull H.B., Dean A.G., Fraser D.W., Hayes P.S. and Hall W.N. (1983). A 1957 outbreak of Legionnaires' disease associated with a meat packing plant. *Am. J. Epidemiol.* 117 (1): 60-66.

Ott M., Bender L., Chirinos E., Ehret W. and Hacker J. (1991). Phenotype versus genotype of the 19kDa peptidoglycan associated protein of Legionella (PplA), among Legionellae and other gram-negative bacteria. *Microb. Pathog.* 11 (5): 357-365.

Otten S., Iyer S., Johnson W. and Montgomery R. (1986). Serospecific antigens of *Legionella pneumophila. J. Bacteriol.* 167 (3): 893-904.

Palmer C.J., Tsai Y.L., Paszko-Kolva C., Mayer C. and Sangermano L.R. (1993). Detection of Legionella species in sewage and ocean water by the polymerase chain reaction, direct fluorescent-antibody and plate culture methods. *Appl. Environ. Microbiol.* 59: 3618-3624.

Paszko-Kolva C., Hacker P.A., Stewart M.H. and Wolfe R.L. (1993). Inhibitory effect of heterotrophic bacteria on cultivation of *Legionella dumoffii*. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp. 203-205.

Paszko-Kolva C., Yamamoto H., Shahamat M., Sawyer T.K., Morris G. and Colwell R.R. (1991). Isolation of amoebae and Pseudomonas and Legionella from eyewash stations. *Appl. Environ. Microbiol.* 57 (1): 163-167.

Paszko-Kolva C., Yamamoto H., Shahamat M. and Colwell R.R. (1993). Polymerase chain reaction, gene probe and direct fluorescent antibody staining of *Legionella pneumophila* SG1 in drinking water and environmental samples. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp. 181-183.

Pearlman E., Engleberg N.C. and Eisenstein B.I. (1985). Identification of protein antigens of *Legionella pneumophila* serogroup 1. *Inf. Immun.* 47 (1): 74-79.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Petersen L.R. and Catchpole M. (2001). Surveillance for infectious diseases in the European Union. A small European centre may have an important coordinating role (editorial). *British Med. J.* 323: 818-819.

Petitjean F., Dournon E., Strosberg A.D. and Hoebeke J. (1990). Isolation, purification and partial analysis of the lipopolysaccharide antigenic determinant recognised by a monoclonal antibody to *Legionella pneumophila* SG1. *Res. Microbiol.* 141 (9): 1077-1094.

Pine L., Franzus M.J. and Malcolm G.B. (1986). Guanine is a growth factor for Legionella species. *J. Clin. Microbiol.* 23 (1): 163-169.

Pine L., Hoffman P.S., Malcolm G.B., Benson R.F. and Franzus M.J. (1986). Role of keto-acids and reduced-oxygen-scavenging enzymes in the growth of Legionella species. *J. Clin. Microbiol.* 23 (1): 33-42.

Pine L., Hoffman P.S., Malcolm G.B., Benson R.F. and Keen M.G. (1984). Determination of catalase, peroxidase, and superoxide dismutase within the genus Legionella. *J. Clin. Microbiol.* 20 (3): 421-429.

Randall T.W., Naidoo P., Newton K.A., Botha P.W.A., Koornhof H.J. and Dubery B. (1980). Legionnaires' disease in Port Elizabeth. *South African Med. J.* 58 (17):17-23.

Reeves M.W., Pine L., Hutner S.H., George J.R. and Harrell W.K. (1981). Metal requirements of *Legionella pneumophila*. *J. Clin. Microbiol.* 13 (4): 688-695.

Reinthaler F.F., Sattler J., Schaffler-Dulling K., Weinmayr B. and Marth E. (1993). Comparative study of procedures for isolation and cultivation of *Legionella pneumophila* from tap water in hospitals. *J. Clin. Microbiol.* 31 (5): 1213-1216.

Rietschel E.T., Brade H., Brade L., Kaca W., Kawahara K., Lindner B., Luderitz T., Tomita T., Schade U. and Seydel U. (1985). Newer aspects of the chemical structure and biological activity of bacterial endotoxins. *Prog. Clin. Biol. Res.* 189: 31-51.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Riffard S., Douglas S., Brooks T., Springthorpe S., Fillion L.G. and Sattar S.A. (2001). Occurrence of Legionella in groundwater: an ecological study. *Water Sci. Tech.* 43 (12): 99-102.

Ristroph J.D., Hedlund K.W. and Gowda S. (1981). Chemically defined medium for *Legionella pneumophila* growth. *J. Clin. Microbiol.* 13 (1): 115-119.

Roberts K.P., August C.M. and Nelson J.D. (1987). Relative sensitivities of environmental legionellae to selective isolation procedures. *Appl. Environ. Microbiol.* 53 (12): 2704-2707.

Robinson P.N., Heidrich B., Tiecke F., Fehrenbach F.J. and Rolfs A. (1996). Species-specific detection of Legionella using polymerase chain reaction and reverse dot-blotting. *FEMS Microbiol. Lett.* 140: 111-119.

Roig J., Aguilar X., Ruiz J., Domingo C., Mesalles E., Manterola J. and Morera J. (1991). Comparative study of *Legionella pneumophila* and other nosocomially acquired pneumonias. Chest 99: 344-350.

Rowbotham T.J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* 33: 1179-1183.

Rowbotham T.J. (1983). Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. *J. Clin. Pathol.* 36: 978-986.

Ruef C. (1998). Nosocomial Legionnaires' disease – strategies for prevention. *J. Microbiol. Methods* 33: 81-91.

Sadoski A.B., Wiater L.A. and Shuman H.A. (1993). Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect. Immun.* 61 (12): 5361-5373.

Saint C.P. and Ho L. (1999). A PCR test for the identification and discrimination of Legionella longbeacheae serogroups 1 and 2. *J. Microbiol. Methods* 37 (3): 245-253.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Sampson J.S., Plikaytis B.B. and Wilkinson H.W. (1986). Immunologic response of patients with legionellosis against major protein-containing antigens of *Legionella pneumophila* SG1 as shown by immunoblot analysis. *J. Clin. Microbiol.* 23 (1): 92-99.

Selander R.K., McKinney R.M., Whittam T.S., Bibb W.F., Brenner D.J., Nolte F.S. and Pattison P.E. (1985). Genetic structure of populations of *Legionella pneumophila*. *J. Bacteriol.* 163: 1021-1037.

Segal G. and Shuman H.A. (1998). Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components of IncQ plasmid RSF1010. *Mol. Microbiol.* 30 (1): 197-208.

Shelton B.G., Kerbel W., Witherell L. and Millar J.D. (2000). Review of Legionnaires' disease. *Am. Ind. Hyg. Assoc. J.* (61): 738-742.

Smith L., Carroll K. and Mottice S. (1993). Comparison of membrane filters for recovery of legionellae from water samples. *Appl. Env. Microbiol.* 59 (1): 344-346.

Sonesson A., Jantzen E., Bryn K., Larsson L. and Eng J. (1989). Chemical composition of a lipopolysaccharide from *Legionella pneumophila*. *Arch. Microbiol.* 153 (1): 72-78.

Sonesson A., Jantzen E., Bryn K., Tangen T., Eng J. and Zähringer U. (1994). Composition of 2,3-dihydroxy fatty acid-containing lipopolysaccharides from *Legionella israelensis, Legionella maceachernii* and *Legionella micdadei*. *Microbiology* 140 (6): 1261-1271.

Sonesson A., Jantzen E., Tangen T. and Zähringer U. (1994). Lipopolysaccharides of *Legionella erythra* and *Legionella oakridgensis*. *Can. J. Microbiol.* 40 (8): 666-671.

Sonesson A., Jantzen E., Tangen T. and Zähringer U. (1994). Chemical characterisation of lipopolysaccharides from *Legionella feeleii, Legionella hackeliae* and *Legionella jordanis*. *Microbiology* 140 (10): 2663-2671.

Sonesson A., Jantzen E., Tangen T. and Zähringer U. (1994). Chemical composition of lipopolysaccharides from *Legionella bozemanii* and *Legionella longbeacheae*. *Arch. Microbiol.* 162 (4): 215-221.

Soni R., Seale J.P. and Young I.H. (1999). Fulminant psittacosis requiring mechanical ventilation and demonstrating serological cross-reactivity between Legionella longbeacheae and Chlamydia psittaci. *Respirology* 4 (2): 203-205 (abstract).

Sopena N., Sabria M., Pedro-Botet M.L., Manterola J.M., Dominguez J., Modol J.M., Tudela P., Ausina V. and Foz M. (1999). Prospective study of community-acquired pneumonia of bacterial etiology in adults. *Eur. J. Clin. Microbiol. Infect. Dis.* 18 (12): 852-858.

Springston J. (2000). Legionella lives. *Engineering Systems.* http://www.esmagazine.com/es/cda/articleinformation/features/bnp_fetures_item/0,2503,3615,00.html

Standards Australia AS 3896 (1991). Australian Standard: Waters – examination for legionella.

States S.J., Conly L.F., Kuchta J.M., Oleck B.M., Lipovich M.J., Wolford R.S., Wadowski R.M., McNamara A.M., Sykora J.L. and Keleti G. (1987). Survival and multiplication of Legionella pneumophila in municipal drinking water systems. *Appl. Environ. Microbiol.* 53 (5): 979-986.

Steele T.W., Lanser J. and Sangster N. (1990). Isolation of *Legionella longbeacheae* serogroup 1 from potting mixes. *Appl. Environ. Microbiol.* 56 (1): 49-53.

Steinert M., Engelhart H., Flügel M., Wintermeyer E. and Hacker J. (1995). The Lly protein protects *Legionella pneumophila* from light but does not directly influence its intracellular survival in *Hartmanella vermiformis*. *Appl. Environ. Microbiol.* 61 (6): 2428-2430.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Steinert M., Emödy L., Amann R. and Hacker J. (1997). Resuscitation of viable but non-culturable *Legionella pneumophila* Philadelphia JR32 by *Acanthmoeba castellanii*. *Appl. Environ. Microbiol.* 63 (5): 2047-2053.

Steinmetz I., Rheinheimer C. and Bitter-Suermann (1992). Rapid identification of legionellae by a colony blot assay based on a genus-specific monoclonal antibody. *J. Clin. Microbiol.* 30 (4): 1016-1018.

St John G. and Steinman H.M. (1996). Periplasmic copper-zinc superoxide dismutase of *Legionella pneumophila*: role in stationary-phase survival. *J. Bacteriol.* 178 (6): 1578-1584.

Stone B.J. and Abu Kwaik Y. (1998). Expression of multiple pili by *Legionella pneumophila*: identification and characterisation of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect. Immun.* 66 (4): 1768-1775.

Stout J.E. (1998). Culture methodology for Legionella species. HC Special Report 308. HC Information Resources, Fallbrook. CA.

Stout J.E., Lin Y.S., Goetz A.M. and Muder R.R. (1998). Controlling Legionella in hospital water systems: experience with the superheat-and-flush method and copper-silver ionisation. *Infect. Control Hosp. Epidemiol.* 19 (12): 911-914.

Strebel P.M., Ramos J.M., Eidelman I.J., Tobiansky L., Koornhof H.J. and Küstner H.G.V. (1988). Legionnaires' disease in a Johannesburg teaching hospital. Investigation and control of an outbreak. *South African. Med. J.* 73: 329-333.

Struelens M.J., Maes N., Deplano A., Bornstein N. and Grimont F. (1993). Comparison of methods for subtyping *Legionella pneumophila*. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Susa M., Hacker J. and Marre R. (1996). De novo synthesis of *Legionella pneumophila* antigens during intracellular growth in phagocytic cells. *Infect. Immun.* 64 (5): 1679-1684.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Szewzyk R., Allestam G. and Stenström T. (1991). Improved recovery of Legionella from water samples by use of black membrane filters. *Zbl. Hyg.* 192: 258-263.

Szewzyk U., Szewzyk R., Manz W. and Schleifer K. (2000). Microbiological safety of drinking water. *Ann. Rev. Microbiol.* 54: 81-127.

Ta A.C., Stout J.E., Yu V.L. and Wagener M.M. (1995). Comparison of culture methods for monitoring Legionella species in hospital potable water systems and recommendations for standardisation of such methods. *J. Clin. Microbiol.* 33 (8): 2118-2123.

Tarp B., Jensen J.S., Ostergaard L. and Andersen P.L. (1999). Search for agents causing atypical pneumonia in HIV-positive patients by inhibitor-controlled PCR assays.

Technical Memoranda: Minimising the risk of Legionnaires' disease. (TM13:1991). The Chartered Institution of Building Services Engineers, Delta House, 222 Balham High Road, London SW12 9BS.

Terranova W., Cohen M.L. and Fraser D.W. (1978). 1974 Outbreak of Legionnaires' disease diagnosed in 1977. Lancet Vol II, July 15: 122-124.

Tesh M.J., Morse S.A. and Miller R.D. (1983). Intermediary metabolism in *Legionella pneumophila:* utilisation of amino acids and other compounds as energy sources. *J. Bacteriol.* 154 (3): 1104-1109.

Thacker W.L., Benson R.F., Hawes L., Gidding H., Dwyer B., Mayberry W.R. and Brenner D.J. (1991). *Legionella fairfieldensis* sp. nov. isolated from cooling tower waters in Australia. *J. Clin. Microbiol.* 29 (3): 475-478.

Thorpe T.C. and Miller R.D. (1981). Extracellular enzymes of *Legionella pneumophila. Inf. Imm.* 33 (2): 632-635.

Tiefenbrunner F., Arnold A., Taraboi E., Cernek U. and Emde K. (1993). Comparison of different detection methods for isolation of *L pneumophila* from water supplied of Alpine

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

hotel resorts. In: Barbaree J.M., Breiman R.F. and Dufour A.P. (eds). Legionella. Current status and emerging perspectives. American Society for Microbiology, Washington, DC.

Tobiansky L., Drath A., Dubery B. and Koornhof H.J. (1986). Seasonality of Legionella isolated from environmental sources. *Isr. J. Med. Sci.* 22 (10): 640-643.

Tobin J.O., Watkins I.D., Woodhead S. and Mitchell R.G. (1986). Epidemiological studies using monoclonal antibodies to *Legionella pneuophila* SG 1. *Isr. J. Med. Sci.* 22: 711-714.

Tompkins L.S. and Loutit J.S. (1993). Detection of Legionella by molecular methods. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella: Current Status and Emerging Perspectives. American Society for Microbiology. Washington, DC. Pp 163-168.

Tyndall R.L. and Domingue E.L. (1982). Cocultivation of *Legionella pneumophila* and free-living amoebae. *Appl. Env. Microbiol.* 44 (4): 954-959.

Vandenesch F., Surgot M., Bornstein N., Paucod J.C., Marmet D., Isoard P. and Fleurette J. (1990). Relationship between free amoeba and Legionella: studies in vitro and in vivo. *Zbl. Bakt.* 272: 265-275.

Vesey G., Dennis P.J., Lee J.V. and West A.A. (1988). Further development of simple tests to differentiate the legionellas. *J. Appl. Bacteriol.* 65: 339-345.

Viswanathan V.K., Edelstein P.H., Pope C.D. and Cianciotto N.P. (2000). The *Legionella pneumophila* iraAB locus is required for iron assimilation, intracellular infection and virulence. *Infect. Immun.* 68 (3): 1069-1079.

Wadowski R.M. and Yee R.M. (1985). Effect of non-Legionellaceae bacteria on the multiplication of *Legionella pneumophila* in potable water. *Appl. Env. Microbiol.* 49 (5): 1206-1210.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Wiater L.A., Dunn K., Maxfield F.R. and Shuman H.A. (1998). Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. *Infect. Immun.* 66 (9): 4450-4460.

Wiese A., Brandenberg K., Ulmer A.J., Seydel U. and Muller-Loennies S. (1999). The dual role of lipopolysaccharide as effector and target molecule. *Biol. Chem.* 380 (7-8): 767-784.

Wilkinson H.W., Drasar V., Thacker W.L., Benson R.F., Schindler J., Potuznikova B., Mayberry W.R. and Brenner D.J. (1988). *Legionella moravica* sp. nov. and *Legionella brunensis* sp. nov. isolated from cooling tower water. *Ann. Inst. Pasteur Microbiol.* 139 (4): 393-402.

Williams H.N., Paszko-Kolva C., Shahamat M., Palmer C., Pettis C. and Kelley J. (1996). Molecular techniques reveal high prevalence of Legionella in dental units. *J. Am. Dent. Assoc.* 127 (8): 1188-1193.

Winn W.C. (1988). Legionnaires' disease: historical perspective. *Clin. Microbiol. Rev.* 1 (1): 60-81.

Wintermeyer E., Flügel M., Ott M., Steinert M., Rdest U., Mann K.H. and Hacker J. (1994). Sequence determination and nutational analysis of the lly locus of *Legionella pneumophila*. *Infect. Immun.* 62 (3): 1109-1117.

Wireman J.W., Schmidt A., Scavo C.R. and Hutchins D.T. (1993). Biofilm formation by *Legionella pneumophila* in a model domestic hot water system. In: Barbaree J.M., Breiman R.F. and DuFour A.P. (eds). Legionella, Current Status and Emerging Perspectives . American Society for Microbiology. Washington, DC.

Worksafe Australia (1989). Legionnaires' disease and related illnesses. National Occupational Health and Safety Commission. Australian Government Publishing Service, Canberra.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Wright J.B., Ruseska I., Athar M.A., Corbett S. and Costerton J.W. (1989). Legionella pneumophila grows adherent to surfaces in vitro and in situ. *Infect. Control Hosp. Epidemiol.* 10 (9): 408-415.

Yamamoto H., Klein T.W., Shinomiya H., Nakano M. and Friedman H. (1992). Infection of macrophages with *Legionella pneumophila* induces phosphorylation of a 76-kDa protein. *Infect. Immun.* 60 (8): 3452-3455.

Yamamoto H., Sugiura M., Kusunoki S., Ezaki T., Ikedo M. and Yabuuchi E. (1992). Factors stimulating propagation of legionellae in cooling tower water. *Appl. Environ. Microbiol.* 58 (4): 1394-1397.

Yu V.L. (1990). *Legionella pneumophila* (Legionnaires' disease). In: Mandell, Douglas and Bennet (eds). Principles and Practice of Infectious Diseases. Third Edition. Churchill Livingstone Inc.

Yu V.L. (1998). Resolving the controversy on environmental cultures for Legionella: a modest proposal. *Inf. Control Hosp. Epid.* 19 (12): 983-897.

Zähringer U., Knirel Y.A., Lindner B., Helbig J.H., Sonesson A., Marre R. and Rietschel E.T. (1995). The lipopolysaccharide of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. *Prog. Clin. Biol. Res.* 392: 113-139.