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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.

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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

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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

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

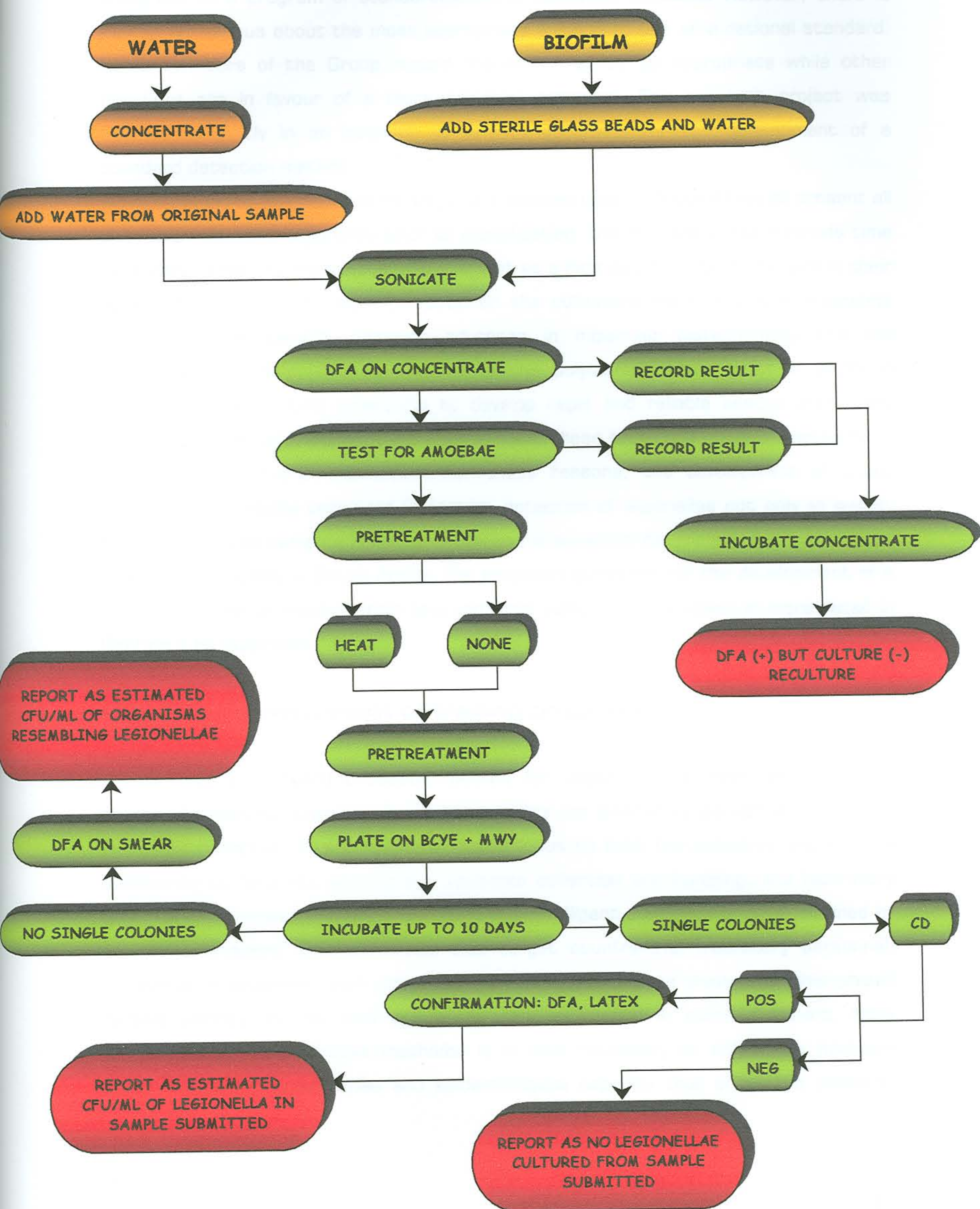

FIGURE 4.1: General procedure for proposed method

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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,

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.

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 $\geq 10^3$;

- ➪ *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;

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.

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.

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

➪ <u>The presence of legionellae in dental unit water lines</u>

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.

➪ <u>Legionella infections in HIV positive mine workers</u>

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.

➪ <u>Legionella infections in miners with tuberculosis</u>

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.

➪ <u>Legionella-like amoebal pathogens (LLAPs)</u>

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.

➪ <u>Molecular methods</u>

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.

➪ <u>Immunomagnetic separation techniques</u>

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.