Evaluation of detection methods for *Legionella* species using seeded water samples

C Bartie[1]* , SN Venter[2] and LH Nel[2]

[1] *Department of Immunology and Microbiology, National Centre for Occupational Health, PO Box 4788, Johannesburg 2000, South Africa*

[2] *Department of Microbiology and Plant Pathology, Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa*

Abstract

South African laboratories are currently using various methods in a non-standardised 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 min, 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.

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 minimise 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-standardised 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

* To whom all correspondence should be addressed.
☎(011) 720 -5734; fax: (011) 720- 6608; e-mail: bartid@health.gov.za
Received 12 January 2001; accepted in revised form 6 June 2001.

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 isolation and confirmation of *Legionella* species in environmental samples. 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.

Materials and methods

Seeding of samples

A type strain of *L. pneumophila* SG 1 was obtained from the American type culture collection (ATCC 33152). Fresh sub-cultures were prepared on BCYE medium before each experiment. Plates were incubated aerobically at 37°C for 3-5 d. 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 8 x 10^7 organisms per mℓ, 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.

Sample concentration

Sample concentration was conducted using 0.45 µm cellulose membrane filters. After concentration, the membranes were aseptically removed, cut into smaller pieces and placed into sterile containers with 10 mℓ sterile distilled water.

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 10 mℓ 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 6 000 g for 10 min. The sediment was resuspended in sterile distilled water. Thereafter, serial dilutions were made and inoculated onto αBCYE agar in duplicate.

Sample pretreatment

The sample concentrates were treated with acid or heat prior to inoculation onto the different agar media. Acid treatment was carried out as previously described (Bopp et al. 1981). For heat treatment, 1 mℓ of the sample concentrate was incubated at 50°C in a water bath for 30 min (Groothuis and Veenendal, 1983). After incubation, serial dilutions were made and agar inoculated using *Standard Methods*.

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 0.1 mℓ 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).

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.

Confirmation tests

Single *Legionella*-like colonies were tested for cysteine dependence (CD), by inoculation and incubation of αBCYE and nutrient agar plates until growth was observed on the BCYE agar. Colonies growing on αBCYE, but not on nutrient agar, were regarded as cysteine dependent (CD+) and reported as presumptive legionellae. The CD+ colonies were confirmed by DFA testing and/or latex agglutination.

The direct immunofluorescence test (DFA) was carried out 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.

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*).

Reporting and interpretation of results

Colony counts were performed on all agar media, for all of the dilutions. For the ISO and AS methods, counts of confirmed (i.e. 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 (i.e. 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 of between 30 and 300 were used. Where <30 colonies were observed in all dilutions, the number of colonies in the highest dilution was recorded.

Results and discussion

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 (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 0.2 µm 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 nitro-cellulose filters with a pore size of 0.45 µm that are commercially available and used by most laboratories in South Africa were used throughout the study.

Centrifugation is often used as an alternative for filtration, but differences in opinion regarding the optimal centrifugation speed and time have been reported (Brindle et al., 1987; Boulanger and Edelstein, 1995). Some workers prefer centrifugation at 6 000 x g for 10 min while others consider 3 000 g for 30 min to be more appropriate. Brindle et al. (1987) reported a good correlation between centrifugation at 6 000 g for 10 min and filtration through 0.45 µm pore size, nitro-cellulose filters, with centrifugation having the added advantage of saving on time and effort.

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 min, or by placing the concentrate in an ultrasound tank for a maximum of ten min. 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 min as being appropriate.

Different combinations of concentration and resuspension methods were compared. Results (not shown) 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. It was concluded that concentration by membrane filtration using nitro-cellulose filters with a pore size of 0.45 µm, followed by sonication for 10 min, would be the most appropriate method for the samples.

Direct plating on artificial media has been shown to be more sensitive than the animal inoculation methods initially used and is considered to be the gold standard for *Legionella* detection from environmental sources. αBCYE agar has been in use since the late seventies and is still the most commonly used agar medium for *Legionella* culture. To improve the selectivity of this medium, supplements have been added in various combinations and quantities as preferred by different workers. Since the early eighties, sample pretreatment with acid and/or heat has been incorporated in the majority of culture techniques (Bopp et al., 1981; Dennis et al., 1984). Despite extensive testing of these culture media and pretreatment methods in a number of countries, no culture medium or pretreatment method has yet been proven ideal for all samples in all conditions.

The data summarised in Table 1 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 BCYE plates (85.9, 98.7 and 89.7% for tap water, cooling water and make-up water respectively). The use of selective media resulted in a considerable decrease in organism recovery, depending on the culture medium used (Table 1). 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 might 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 1). In the absence of sample pretreatment, culture on BCYE 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

TABLE 1
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: make-up water

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.

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 (e.g. 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 the recovery of legionellae from the non-sterile seeded samples. In all the samples (sterile and non-sterile), the sample pretreatment steps required by the ISO and AS decreased the recovery of organisms significantly.

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 industrial and environmental water samples, not seeded with laboratory-adapted *Legionella* strains, is investigated.

Acknowledgements

This project was funded through a grant from the South African Water Research Commission.

Rreferences

BOPP CA, SUMNER JW, MORRIS GK and WELLS JG (1981) Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. *J. Clin. Microbiol.* **13** 714-719.

BEJ AK, MAHBUBANI MH and ATLAS RM (1991) Detection of viable *Legionella* pneumophila in water by polymerase chain reaction and gene probe methods. *Appl. Environ. Microbiol.* **57** 597-600.

BOULANGER CA and EDELSTEIN PH (1995) Precision and accuracy of recovery of *Legionella* pneumophila from seeded tap water by filtration and centrifugation. *Appl. Environ. Microbiol.* **61** 1805-1809.

BRINDLE RJ, STANNETT PJ and CUNLIFFE RN (1987) Legionella pneumophila: Comparison of isolation from water specimens by centrifugation and filtration. *Epidemiol. Inf.* **99** 241-247.

DEN BOER JW, YZERMAN EPF, SCHELLEKENS J, LETTINGA KD, BOSHUISEN H, VAN STEENBERGEN J et al., (2000) A large outbreak of Legionnaires' disease at a Dutch flower show. Presented at the 5th Int. Conf. on Legionella, 26-29 September 2000, Ulm, Germany.

DENNIS PJ, GREEN D and JONES BPC (1984) A note on the temperature tolerance of *Legionella. J. Appl. Bacteriol.* **56** 349-350.

GRABOW NA, KFIR R and GRABOW WOK (1991) A most probable number method for the enumeration of *Legionella* bacteria in water. *Water Sci.* **24** 143-147.

GROOTHUIS DG and VEENENDAL HR (1983) Heat treatment as an aid for the isolation of *Legionella pneumophila* from clinical and environmental samples. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **255** 39-43.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION (1996) Water Quality – Detection and Enumeration of *Legionella.* ISO/DIS11731.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION. STANDARDS AUSTRALIA (1991) Australian Standard. Waters – Examination for Legionellae. AS 3896-1991.

KUSNETSOV JM, JOUSIMIES-SOMER HR, NEVALAINEN AI and MARTIKAINEN PJ (1994) Isolation of *Legionella* from water samples using various culture methods. *J. Appl. Bacteriol.* **76** 155-162.

LYE D, FOUT GS, CROUT SR, DANIELSON R, THIO CL and PASZKO-KOLVA CM (1997) Survey of ground, surface, and potable waters for the presence of *Legionella* species by EnviroAmp® PCR *Legionella* kit, culture, and immunofluorescent staining. *Water Res.* **31** 287-293.

MAHBUBANI M, BEJ AK, DI-CESARE JL, MILLER R, HAFF L and ATLAS RM (1990) Detection of *Legionella* with polymerase chain reaction and gene probe methods. *Mol. Cell. Probes* **4** 175-187.

ROBERTS KP, AUGUST CM AND NELSON JD Jr (1987) Relative sensitivities of environmental legionellae to selective procedures. *Appl. Environ. Microbiol.* **53** 2704-2707.

STANDARD METHODS (1992) *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.

TALLIS G, GREIG J, ZWOLAK B, CARNIE J, HART W, TAN A and RYAN N (2000) A descriptive analysis of the Melbourne aquarium outbreak of Legionnaires' disease. Presented at the 5th Int. Conf. on Legionella, 26-29 September 2000, Ulm, Germany.