Epidem. Inf. (1987), 99, 241–247
Printed in Great Britain

Legionella pneumophila: comparison of isolation from water specimens by centrifugation and filtration

By R. J. BRINDLE, P. J. STANNETT and R. N. CUNLIFFE
Public Health Laboratory, Level 6/7, John Radcliffe Hospital, Headington, Oxford OX3 9DU

(*Accepted 13 May 1987*)

SUMMARY

A comparison was made between membrane filtration and centrifugation for the isolation of *Legionella pneumophila* from seeded water samples. Using samples of varying concentration, the optimum speed and time of centrifugation were determined and the relationship between the number of organisms present in the water and the proportion recovered was examined. Following this, sequential routine environmental waters were filtered and centrifuged in parallel.

Centrifugation and filtration using nitrocellulose filters were found to be comparable. The optimum speed and time of centrifugation was approximately 6000 *g* for 10 min. There was a constant proportion of viable organisms recovered irrespective of the concentration in the unspun samples.

INTRODUCTION

Membrane filtration is widely used as a method of isolating *Legionella* spp. from environmental specimens of water. The bacteria retained by the filter are resuspended in a smaller volume of sterile water. Following acid and heat pre-treatments which reduce the other flora commonly found in water systems, the suspension is plated out on to selective media. The filtration method becomes difficult to apply with very dirty water samples and those which contain certain biocides, as these do not filter very rapidly. The resuspension of the organisms depends on thorough shredding and mixing of the large filters used, and this is never likely to be completely successful. Furthermore, the whole system needs to be sterilized by immersion in boiling water between samples. Continuous flow and batch centrifugation have both been used, but there has not been adequate data comparing these methods with filtration (Edelstein, 1985).

An initial comparison of centrifugation and filtration, as performed in our laboratory, using seeded samples with different concentrations of organisms spun at 10800 *g* for 20 min, showed no apparent difference. It was decided therefore to determine the optimum spin speed and time that would give good recoveries without excessive machine wear, and examine the relationship between the number of organisms present in a sample and the proportion recovered, as this is important in estimating the numbers present in a sample. Once these were determined, routine laboratory specimens were tested in parallel by centrifugation and filtration and the results compared.

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

Table 1. *Centrifugation speeds and times*

Sample no.	Rev./min	RCF	Duration (min)	$\text{Log}_{10} \int \omega^2 dt$
1	8900	13400	40	9·28
2	8000	10800	30	9·04
3	7000	8280	20	8·75
4	7500	9450	10	8·41
5	5500	5130	10	8·20
6	4000	2730	10	7·96
7	3000	1530	10	7·72
8	2400	970	10	7·53
9	1800	540	10	7·28
10	1800	540	5	6·97
11	1400	325	5	6·76
12	1000	170	5	6·46
13	800	108	5	6·28
14	800	108	3	6·04
15	600	60	3	5·80
16	400	27	3	5·46
17	300	14	3	5·23
18	0	0	10	

The Sorvall RC-5C calculates the integral of the angular velocity $(\omega)^2$ with time (dt), which takes into account the acceleration and deceleration phases of the spin and displays RCF in *g* as an alternative to rev./min.

MATERIALS AND METHODS

Filtration

One litre samples of water to be tested for the presence of *Legionella* spp. by filtration were placed in a sterile filter system (Sartorius pressure filter holder with barrel, 142 mm diameter) and forced through a 450 nm pore-size cellulose nitrate filter (Sartorius or Millipore) under positive pressure (100 kPa). The filter was then removed to a sterile universal container, shredded and vortex-mixed with 20 ml of sterile water. The concentrate was heat- and acid-treated (Dennis, Bartlett & Wright, 1984; Bopp *et al.* 1981) and 100 μl spread on to selective Buffered Charcoal Yeast Extract (BCYE) agar (Edelstein, 1981). The plates were incubated at 37 °C in a humidified chamber and read at 3 and 4 days. The filtration funnel and filter holder were sterilized in boiling water. Any possible *Legionella* spp. were picked and subcultured on to blood agar and BCYE agar and identified using indirect fluorescence with rabbit polyclonal antisera (Division of Microbiological Reagents and Quality Control, PHLS) and monoclonal antibodies (Brindle, Stannet & Tobin, 1987).

Centrifugation

The centrifuge used in all the following experiments was the Sorvall RC-5C with a fixed-angle rotor (GS3) of nominal capacity 6 × 500 ml. The polypropylene centrifuge tubes were sterilized by rinsing in ethanol after thorough washing.

Determination of optimum speed and time of centrifugation

Seventeen spin speed-times which could be accommodated by the centrifuge were determined. Each one differed by 0·25 $\log_{10}$ of the integral of speed^2 × time

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

($\int \omega^2 dt$) in a range from a Relative Centrifugal Force (RCF) of 13400 *g* (8900 rev./min) for 40 min to 14 *g* (300 rev./min) for 3 min (Table 1).

The water samples were prepared on the day of testing with suspensions of *L. pneumophila* serogroup 1 grown up on BCYE agar over 48 h. Surface viable counts were performed, using the spread-plate method with a sterile glass rod as a spreader. Ten litres of sterile distilled water was prepared in four 2·5 l bottles and each bottle was inoculated with 2·5 ml of the suspension. The bottles were well shaken and 200 ml samples were transferred to sterile 500 ml centrifuge tubes and sealed, to give a total of 54 samples which were then subdivided up into 18 batches of 3 samples to be spun at the same speed-time. The tubes were marked to show the position of the pellet. After spinning, the supernatant was poured off and the deposit carefully removed and suspended in 2 ml of sterile water. Fifty microlitres of the concentrates and tenfold dilutions were spread on to two separate batches of media. Final colony counts were made after 4 days. This experiment was repeated four times with different concentrations of organisms. Not every spin was performed every time, as with low concentrations no isolation was made at the low speed-time spins, and at high concentrations the number of organisms present at the higher speed-time spins was too great to be read.

Determination of the relationship between inoculum and the number of organisms recovered

Seeded water samples were prepared as previously described with *L. pneumophila*. A concentration of approximately 5×10^6 c.f.u./l was prepared in sterile water and 14 twofold dilutions were made. One hundred microlitres of the original suspension and nine further twofold dilutions were spread on to BCYE agar and incubated at 37 °C. Two 200 ml volumes of each of the original suspension and the dilutions were centrifuged at 6100 *g* (6000 rev./min) for 10 min. Concentrates (100 ×) and tenfold dilutions of these were plated out as above, and both sets of plates read after 4 days incubation.

Comparison between cellulose nitrate filtration and centrifugation at 6100 g for 10 min

All routine specimens presented to the laboratory for testing for the presence of *Legionella* spp. over 6 weeks were both filtered and centrifuged in parallel by the methods described. Fifty specimens were tested.

RESULTS

Optimum speed of centrifugation

Combining the four experiments to determine the optimum speed and time of centrifugation showed that there was a slow fall-off in the numbers isolated from the highest speed-time spin, 13400 *g* for 40 min, to 5130 *g* for 10 min. There then followed a rapid decline in the number of organisms isolated until 170 *g* for 5 min and thereafter a gentle decrease until the slowest tested speed was reached. All the results were corrected for an inoculum of $1{\cdot}74 \times 10^6$, which was used in run number two. This enabled the results from the four runs to be combined, and allowed for the differences in concentration and any variations this might produce in the

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

Table 2. *Log_{10} c.f.u./50 µl of concentrate from each spin corrected for viable count of $1{\cdot}74 \times 10^6$ c.f.u./l (6·24 log_{10})*

Sample no.	Run number 1	2	3	4	Mean
1	—	2·45	2·56	2·63	2·55
2	—	2·86	2·62	2·56	2·68
3	—	2·22	2·29	2·48	2·33
4	—	2·79	2·53	2·50	2·61
5	—	2·62	2·47	2·44	2·51
6	—	2·04	2·49	2·35	2·29
7	1·77	1·63	—	2·00	1·80
8	1·74	1·93	—	1·81	1·83
9	1·57	1·27	—	1·52	1·45
10	1·47	1·54	—	1·22	1·41
11	1·21	1·36	—	1·22	1·26
12	1·04	0·98	—	—	1·01
13	1·18	0·88	—	—	1·03
14	0·90	1·15	—	—	1·03
15	1·03	1·05	—	—	1·04
16	0·92	0·96	—	—	0·94
17	0·82	0·88	—	—	0·85
C.f.u./l by surface-viable count	6·56	6·24	4·94	4·39	—

This table is presented as a graph in Fig. 1.

proportion and total number of organisms isolated. One result of the correction is that it exaggerates slightly the three phases detailed above. All the individual results of each experiment showed a similar trend as far as they went. There was one complete run, one with 6 high speed-time spins, one with 12 high to medium speed-time spins and one with 12 medium to low speed-time spins (Table 2).

All control spins (stationary rotor for 10 min, emptying and rinsing with 2 ml sterile water) produced either none or single colonies from the 50 µl plated out. A graphical display of the combined results is included (Fig. 1). The maximum recovery rate for each run varied from 4% for run two (with an inoculum of $1{\cdot}74 \times 10^6$ c.f.u./l) to above 30% for run four (with an inoculum of $2{\cdot}45 \times 10^4$ c.f.u./l).

Relationship between inoculum and recovery

The results of the examination of the recovery as a proportion of the initial count are shown in Table 3. The undiluted suspension contained $5{\cdot}6 \times 10^6$ c.f.u./l as calculated from the numbers of colonies present in the serial twofold dilutions. The number of colonies from the unspun suspension of each dilution was then compared with the number of colonies recovered from the concentrates of the spun suspensions and the proportion recovered calculated as a percentage. The numbers of c.f.u. for both the unspun and spun suspensions are plotted against the dilution in Fig. 2. Both plots have the same gradient (−1·06), which reflects their linear relationship and the accuracy of the dilutions. A perfectly accurate dilution plotted in this way would have a gradient of −1. Despite the close relationship

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

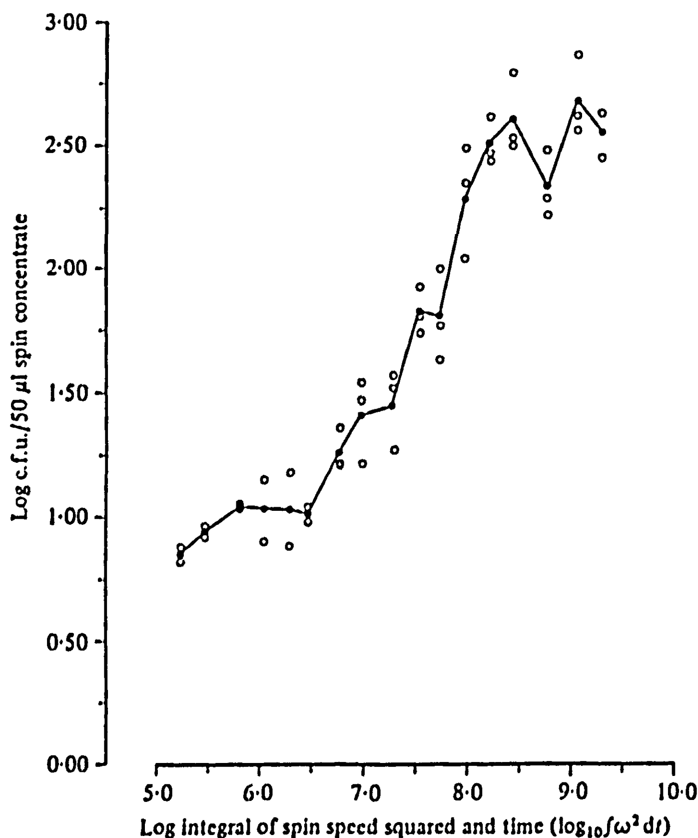

Fig. 1. Recovery of *L. pneumophila* against spin speed squared and time. (○—○), c.f.u./50 μl recovered for individual spins. (●—●) Mean c.f.u./50 μl for the four runs. Note that there is a rapid fall-off in the number of organisms recovered below a $\log_{10} \int\omega^2 dt$ of 8·2, which corresponds to 5000 *g* for 10 min, though good recoveries are still being maintained at 2700 *g* for 10 min.

Table 3. *The number of c.f.u./100 μl of the suspensions before and after centrifugation expressed as* $\log_{10}$

Log dilution	Unspun suspension	Spun suspension	Recovery (%)
0	Uncountable	3·65	—
0·3	2·45	4·00	35
0·6	2·11	3·56	28
0·9	1·77	3·49	52
1·2	1·44	3·09	45
1·5	1·07	2·66	39
1·8	0·92	2·61	49
2·1	0·63	2·31	48
2·4	0·24	2·04	63
2·7	0·00	1·37	23
3·0	—	1·17	—
3·3	—	0·84	—
3·6	—	0·93	—
3·9	—	0·45	—
4·2	—	—	—

Each is a twofold dilution, and this is reflected in the increase in log dilution by steps of 0·3 and by the appropriate decrease in the numbers of c.f.u./100 μl obtained from the unspun and spun suspensions. The undiluted unspun suspension contained $5{\cdot}6 \times 10^6$ c.f.u./l. Centrifugation was at 6100 *g* for 10 min.

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

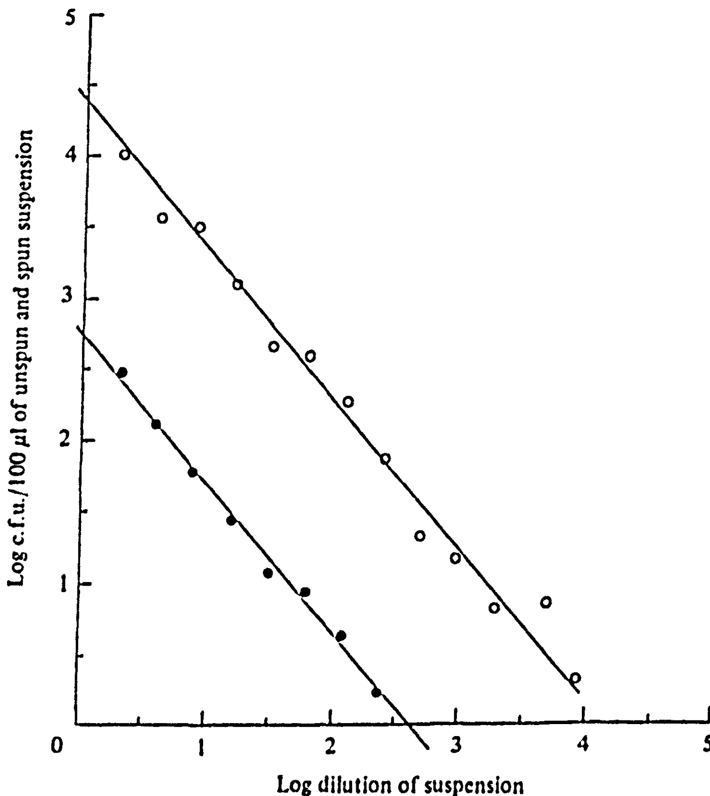

Fig. 2. Recovery of *L. pneumophila* from unspun (●—●) and spun (○—○) suspensions against dilutions of a suspension containing $5 \cdot 6 \times 10^6$ c.f.u./l. Note that the two curves are parallel. If there was a non-linear relationship between the number of organisms recovered and the inoculum size this would not be so.

Table 4. *Filtration compared with centrifugation of routine specimens*

Filtration	Centrifugation: Positive	Centrifugation: Negative	Total
Positive	11	0	11
Negative	2	37	39
Total	13	37	50

there is variation in the proportion recovered between the dilutions. There was a mean loss of 0·39 $\log_{10}$ or a recovery of about 40%.

Comparison between filtration and centrifugation using routine specimens

Of the 50 routine environmental specimens tested there were 11 specimens positive by filtration and 13 by centrifugation (Table 4). All those positive by filtration were also positive by centrifugation. There was variation in the numbers of organisms detected, but neither method was consistently higher in its estimation.

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press

DISCUSSION

The method of membrane filtration is essentially a modification of that described by Orrison, Cherry & Milan (1981). The most likely modification in general use is that shredding of the filter with scissors and mixing is used rather than a blender to fragment the filter. This change saves the need to sterilize the blender in between samples. The authors achieved a maximum loss of one $\log_{10}$, although others have failed to achieve recoveries of better than 5% (loss of 1·3 $\log_{10}$) using a variety of methods (Edelstein, 1985).

Centrifugation offers a saving on time and effort. It is apparent that it is as good a method as membrane filtration in terms of sensitivity and quantification. It is possible that the use of different filter materials would reduce the loss that occurs from electrostatic attraction of the organisms to the filter surface. It has been shown that below an RCF of about 5000 *g* for 10 min there is a significant loss of sensitivity. A reasonable compromise is 6100 *g* (6000 rev./min on the Sorvall RC-5C with a GS3 fixed-angle rotor) for 10 min. To obtain significantly higher recoveries would require much higher spin speeds, and it is possible that the destructive loss produced by these speeds would negate any such advantage.

It is recommended that any laboratory which is either testing water samples for *Legionella* spp. or contemplating doing so should consider using batch centrifugation rather than membrane filtration, especially if it already has available a suitable centrifuge.

REFERENCES

BOPP, C. A., SUMNER, J. W., MORRIS, G. K. & WELLS, J. G. (1981). Isolation of *Legionella* spp. from environmental water samples by low pH treatment and use of a selective medium. *Journal of Clinical Microbiology* 13, 714–719.

BRINDLE, R. J., STANNETT, P. J. & TOBIN, J. O'H. (1987). Monoclonal antibody typing of clinical and environmental isolates. *Epidemiology & Infection* 99, 235–239.

DENNIS, P. J., BARTLETT, L. R. & WRIGHT, A. E. (1984). Comparison of isolation methods for *Legionella* spp. In *Legionella*. Proceedings of the 2nd International Symposium (ed. C. Thornsberry, A. Ballows, J. C. Freely and E. Jakubowski), pp. 292–294. Washington, D.C.: American Society for Microbiology.

EDELSTEIN, P. H. (1981). Improved semiselective medium for isolation of Legionella from contaminated clinical and environmental specimens. *Journal of Clinical Microbiology* 14, 298–303.

EDELSTEIN, P. H. (1985). Environmental aspects of Legionella. *American Society for Microbiology News* 57, 460–467.

ORRISON, L. H., CHERRY, W. B. & MILAN, D. (1981). Isolation of *Legionella pneumophila* from cooling tower water by filtration. *Applied and Environmental Microbiology* 41, 1202–1205.

https://doi.org/10.1017/S0950268800067704 Published online by Cambridge University Press