J. Hyg., Camb. (1983), 91, 179–187
Printed in Great Britain

Isolation of *Legionella pneumophila* from water supplies: comparison of methods based on the guinea-pig and culture media

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(*Received* 23 *December* 1982; *accepted* 6 *May* 1983)

SUMMARY

The detection of *Legionella pneumophila* in water via guinea-pig intraperitoneal injection has been compared with direct isolation of these organisms on semiselective BCYEα media. Both techniques were of similar sensitivity, detecting as few as 10^2 *L. pneumophila* in 10 ml of water. However, at this concentration, detection using guinea-pig intraperitoneal injection was via the indirect parameters of antibody production and immunofluorescent microscopy. Isolation of *L. pneumophila* from guinea-pig tissue for further investigation required $> 10^7$ organisms injected and in this respect is relatively much less sensitive than direct isolation on semiselective BCYEα media. Since *L. micdadei* and *L. longbeachae* are inhibited by one of the selective supplements used, other supplements and techniques need to be developed in order to avoid possible inhibition of other *Legionella* species.

This work is based on simulated water samples containing strains of *L. pneumophila* defined in terms of pneumonic Legionnaires' disease in the guinea-pig. As such, this work serves as a guide to technique and the need to test large numbers of naturally infected water samples in parallel by techniques outlined in this work is emphasized.

INTRODUCTION

In the absence of suitable isolation media the intraperitoneal injection of guinea-pigs has become the accepted technique for the isolation of *Legionella pneumophila* from water supplies (Fliermans *et al.* 1981; Orrison, Cherry & Milan, 1981; Tobin, Swann & Bartlett, 1981). Guinea-pig inoculation allows separation of this fastidious, slow-growing group of organisms from less fastidious, faster-growing organisms which frequently occur in water samples. These samples usually contain variable numbers of *L. pneumophila* and a variety of contaminating organisms in varying concentrations. The guinea-pig's response also varies, in that it is conditioned by the number of *L. pneumophila* present. This may be expressed in terms of lethality, temperature, antibody production and demonstration of organisms in the tissues.

Improvements in media and selective procedures now indicate that the role of the guinea-pig should be evaluated in relation to these improved *in vitro* procedures in terms of sensitivity, reliability and time.

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MATERIALS AND METHODS

Organisms

One strain of *L. pneumophila* (74/81, serogroup 1) was isolated in our laboratory from naturally contaminated water supplies and was subcultured only three times before administration to animals. The other was the Philadelphia serogroup 1 type strain (NCTC 11192), which has an unrecorded passage history. The two strains have been shown to differ in pathogenicity for guinea-pigs when administered as a small particle aerosol (Fitzgeorge *et al.* 1983). Strain 74/81 was lethal for guinea-pigs and had an LD_{50} of 10^4 organisms, whereas the Philadelphia serogroup I type strain was not and had an LD_{50} of $> 10^6$ organisms. *L. micdadei* and *L. longbeachae* strains were kindly supplied by Mr A. Laverick, Public Health Laboratory, Nottingham. The fatty-acid profile, together with the morphological, cultural and biochemical features exhibited by these strains were characteristic of the species.

Growth and assay of bacterial suspensions were as detailed by Fitzgeorge *et al.* (1983). Doses indicated in the text refer to numbers of viable organisms injected.

Selective media

BCYEα medium was made selective by the addition of selective supplements developed from those formulated by Edelstein (1981) and designated in this paper as CCP and by Wadowsky & Yee (1981) designated GVPC.

CCP selective supplement consists of the following: cefamandole (Eli Lily and Co. Ltd) at 4 μg/ml, polymyxin B sulphate (Sigma Chemical Co. Ltd) at 79·2 USP iu/ml and cyclohexamide (Sigma Chemical Co. Ltd) at 80 μg/ml.

GVPC selective supplement consists of the following: glycine (BDH) at 3 mg/ml, polymyxin B sulphate (Sigma Chemical Co. Ltd) at 79·2 USP i.u./ml, vancomycin (Sigma Chemical Co. Ltd) at 5 μg/ml and cyclohexamide (Sigma Chemical Co. Ltd) at 80 μg/ml.

Immunofluorescent procedures

Antibody titres were evaluated and the presence of organisms in smears and tissue macerates were demonstrated by indirect immunofluorescence techniques recommended by the Division of Microbial Reagents and Quality Control (DMRQC), PHLS, Colindale, London.

Animals

Female Dunkin-Hartley guinea-pigs of category 4 health status (MRC, 1974), weighing 300–500 g were used and housed in groups of 4–6. Blood samples from normal guinea-pigs showed these animals to be free from antibody to *L. pneumophila*.

Clinical observations

Rectal temperatures were recorded at daily intervals following infection. Temperatures were considered elevated when they were $\geqslant$ 104 °F ($\geqslant$ 40 °C).

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Guinea-pig experimental procedures

Experimental procedures were based on the techniques used in this and other PHLS laboratories for the examination of water supplies (Dennis *et al.* 1982). Using this technique water samples were concentrated from 25 l to 30 ml by membrane filtration. However, for the purpose of these experiments, water samples of defined composition were required. Therefore, simulated water samples were prepared by suspending graded concentrations of *L. pneumophila* in sterile distilled water. Each sample was injected I.P. into four guinea-pigs, each receiving 10 ml. The guinea-pigs' temperatures were taken daily for 10 days. At 4 and 10 days following infection two guinea-pigs were sacrificed and blood, lung, liver and spleen were cultured and smears examined by an indirect immunofluorescence technique, the cut surfaces of the tissues being spread on BCYEα agar and on microscope slides.

To examine the connexion between bacteraemia and pyrexia, guinea-pigs were injected I.P. with 10^7 of *L. pneumophila*. Three animals were sacrificed and bled on days, 1, 2, 3, 4 following infection and viable counts were made on BCYEα agar.

Directed isolation of L. pneumophila *by centrifugation and cultivation on BCYEα agar*

Simulated water samples, 10 ml in volume, were subjected to centrifugation at 4000 ***g*** for 30 min at 10 °C. The supernatant was discarded and the sediment was resuspended in 0·1 ml of sterile distilled water. Viable counts were made on BCYEα agar before and after centrifugation.

RESULTS

Response of guinea-pigs injected I.P. with graded doses of L. pneumophila

Table 1 shows the response of guinea-pigs to I.P. injection of graded doses of *L. pneumophila* (strain 74/81) ranging from 10^1 to 10^9/10 ml. Approximately 10^8 organisms were required to cause death of guinea-pigs (this has been found to apply to four other *L. pneumophila* strains tested in our laboratory). When injected with 10^7 organisms the animals do not die but the organisms may be recovered from the tissue on necropsy at 4 days, but not 10 days following infection. Detection of the organism in smears by the indirect fluorescent antibody technique raises the sensitivity of detection considerably, approximately 10^2 organisms now being detectable.

As could be expected, antibody to *L. pneumophila* was not seen in 4-day serum samples but appeared in 10-day samples. As for tissue smears, antibody production was positive for as few as 10^2 organisms injected I.P.

Elevated temperatures occurred in response to as few as 10^2 organisms and this was as sensitive as antibody and detection of the organism in smears. Nevertheless, although indicative of infection this parameter cannot be regarded as a specific response to *L. pneumophila*. Elevated temperatures occurred predominantly over a period of 1–4 days and it is obviously not necessary to record temperatures after this period of time.

Although blood, lung, liver and spleen were cultured and examined via fluorescent microscopy, the spleen proved the most relevant organ to examine. This

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Table 1. *Response of guinea-pigs injected* I.P. *with* L. pneumophila *strain 74/81*

Dose	Death at 10 days	Cultured at		Tissue IFA at		Serum* antibody at		Temperature	
		4 days	10 days	4 days	10 days	4 days	10 days	1–4 days	5–9 days
10^1	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10^2	0/2	0/2	0/2	1/2	1/2	0/2	2/2	1/2	0/2
10^3	0/2	0/2	0/2	2/2	2/2	0/2	2/2+	2/2	0/2
10^4	0/2	0/2	0/2	2/2+	2/2+	0/2	2/2	2/2	0/2
10^5	0/2	0/2	0/2	2/2	2/2	0/2	2/2	2/2	0/2
10^6	0/2	0/2	0/2	2/2	2/2	0/2	2/2	2/2+	0/2
10^7	0/2	2/2	0/2	1/2	1/2	0/2	2/2	2/2	0/2
10^8	1/2+	2/2+	0/2	2/2	2/2	0/2	2/2	2/2	1/2
10^9	4/4	4/4	—	4/4	—	0/2	—	4/4	—

Fractions indicate number of guinea-pigs showing positive response over number used. + = level at which responses change from positive to negative in groups of guinea-pigs injected I.P. with *L. pneumophila* strain NCTC 11192.

* Antibody titres < 1/8 considered negative.

was positive more frequently than other organs and only once out of 36 examinations was it negative when other organs were positive.

Guinea-pigs similarly infected with the Philadelphia serogroup type I strain NCTC 11192 (of different pathogenicity when administered as a fine particle aerosol, Fitzgeorge *et al.* 1983), responded approximately as for strain 74/81. A comparison is shown in Table 1. Death occurred when approximately 10^8 organisms were injected and again the most sensitive detection parameters were those of serum antibody and detection of the organism in tissues by fluorescent microscopy. However, this strain did not produce pyrexia unless $> 10^6$ organisms were injected.

Guinea-pig response to I.P. *injection of* L. pneumophila *and water-borne contaminating organisms*

Guinea-pigs injected I.P. with graded doses of *L. pneumophila* mixed with three species of water-borne contaminating organisms (a coliform, pseudomonas and micrococcus species) each at a concentration of 10^5 organisms in 10 ml, responded as they did when injected with *L. pneumophila* (strain 74/81) alone. The contaminating organisms did not produce a temperature when injected alone and were not isolated from post-mortem material at 4 and 10 days following infection.

Bacteraemia and pyrexia in guinea-pigs following I.P. *injection with* 10^7 L. pneumophila

Fig. 1 shows that 10^7 *L. pneumophila* injected I.P. are detectable in the blood $1\frac{1}{2}$ h after injection at concentrations of 10^3–10^4/ml of blood. On days 1–4 following infection, almost invariably a bacteraemia and pyrexia coincided. The only exception was one animal with a very low level of organisms in the blood but with a normal temperature.

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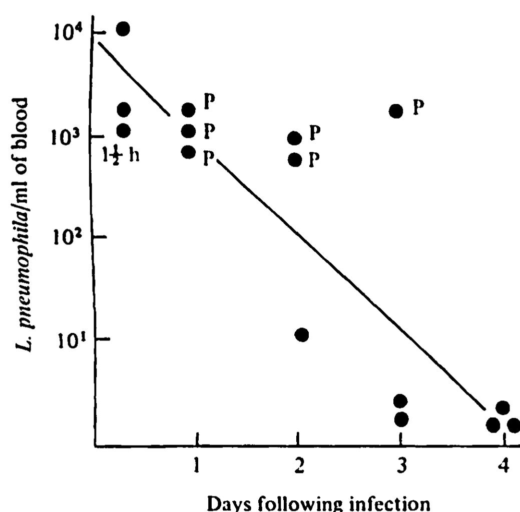

Fig. 1. Comparison of bacteraemia and pyrexia in guinea-pigs after I.P. injection of 10^7 *L. pneumophila* P = pyrexia.

Table 2. *Growth of* L. pneumophila, L. micdadei *and* L. longbeachae *on BCYEα agar with and without selective supplements*

Legionella species	Medium	Day on which growth appeared	Viable count
L. pneumophila (74/81)	BCYEα	3	$1{\cdot}6 \times 10^7$/ml
	BCYEα+GVPC	3·5	$1{\cdot}1 \times 10^7$/ml
	BCYEα+CCP	3·5	$1{\cdot}2 \times 10^7$/ml
L. micdadei	BCYEα	3	1×10^6/ml
	BCYEα+GVPC	4	9×10^5/ml
	BCYEα+CCP	No growth	No growth
L. longbeachae	BCYEα	3	$1{\cdot}6 \times 10^4$/ml
	BCYEα+GVPC	4	8×10^3/ml
	BCYEα+CCP	No growth	No growth

GVPC = glycine, vancomycin, polymyxin and cyclohexamide.
CCP = cephamandole, cyclohexamide and polymyxin.

Growth of Legionella *species on BCYEα medium with and without selective supplements*

When *L. pneumophila* (strain 74/81) was plated on to BCYEα agar, growth appeared on the 3rd day of incubation. The addition of selective supplements (GVPC, CCP) delayed growth by approximately ½ day and reduced the viable counts by 31 % and 25 % respectively (Table 2).

When this procedure ws repeated with *L. micdadei* on BCYEα agar supplemented with GVPC, growth was retarded by 1 day and the viable count reduced by 10 %. BCYEα agar supplemented with CCP prevented growth of this species. *L. longbeachae* responded in a similar manner to *L. micdadei* with retardation of growth of BCYEα agar plus GVPC supplement and a viable count reduction of 31 %. Again CCP supplement prevented growth (Table 2).

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Table 3. *Direct isolation of* L. pneumophila *from water on BCYEα agar with and without selective supplements*

Viable count (in 10 ml) before centrifugation: BCYEα	Recovered after centrifugation (%)		
	BCYEα agar	BCYEα agar + GVPC	BCYEα agar + CCP
4×10^1	27·5	18·2	20·6
4×10^2	70·8	46·7	53·1
$4{\cdot}2 \times 10^3$	76·5	50·5	57·4
$3{\cdot}4 \times 10^4$	81·2	53·5	60·9
$3{\cdot}4 \times 10^4$	89·5	60	67·2

GVPC = glycine, vancomycin, polymyxin and cyclohexamide.
CCP = cephamandole, cyclohexamide and polymyxin.

Both these selective media prevented the growth of the three water-borne contaminating organisms used in this work.

Direct isolation of L. pneumophila *from water samples*

Table 3 shows that, at the lowest concentration of *L. pneumophila* (41/10 ml) tested, 27·5%, 18·2% and 20·6% of the original number of organisms may be recovered after centrifugation, resuspended in 0·1 ml of distilled water and culture on BCYEα agar, BCYEα agar plus GVPC supplement and BCYEα agar plus CCP supplement respectively. The recovery rate is enhanced when greater numbers of organisms are present in the original sample.

DISCUSSION

This study provides strong evidence that direct plating is the method of choice for the isolation of *L. pneumophila* from water samples and that isolation by guinea-pig inoculation, as judged by some criteria, may no longer be necessary.

Isolation of *L. pneumophila* from guinea-pig tissue requires that the 10 ml of water sample injected contains $> 10^7$ of that organism and $> 10^8$ if recovery of the organism subsequent to death of the animal is required. Recovery of the organism is the preferred method of positively identifying an infected water, since the isolated organism may then be subjected to further tests. Direct isolation by centrifugation and cultivation on BCYEα agar may be made when the water sample contains as little as 10^2 *L. pneumophila* in 10 ml, an increase in sensitivity of 10^5–10^6. However, using indirect methods, detection of serum antibody and *L. pneumophila* organisms in tissue smears via fluorescent techniques, guinea-pig inoculation provides a similar order of sensitivity which may be acceptable on a survey basis.

Although *L. pneumophila* was often seen in smears from tissue (even in those from animals injected with quite low doses, i.e. about 10^2 of *L. pneumophila*) it was often not possible to cultivate these organisms (this required $> 10^7$ *L. pneumophila* injected). The apparent failure of these organisms to grow may be due to deficiencies in the media, to the organisms being dead, to non-specifity of the fluorescent technique used, or to inhibition of growth by tissue components (Lattimer *et al.* 1980). The growth medium used in this work (BCYEα, prepared

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as described by Edelstein, 1981) gives as a routine a viable count which is 20% of the total count. This 20% viability would not account for the 10^5–10^6 difference observed between demonstration of the organism and its cultivation. *L. pneumophila* observed microscopically in tissue at 4 days P.I. is still present in tissues from animals similarly infected at 10 days P.I. The organisms do not appear to be cleared or degraded by the animals' defence mechanisms. This would indicate that the organisms are probably still viable on the 4th day following infection. Apart from the controls normally incorporated in the indirect fluorescent antibody technique for the microscopical demonstration of organisms, detection of *L. pneumophila* by this method and by demonstration of antibody to this organism were closely parallel and tended to confirm specificity. Lattimer *et al.* (1980) observed that a high concentration of some tissue components exerts an inhibitory effect on the primary isolation and growth of *L. pneumophila*. In this work smears from the cut surface of organs were used and perhaps inhibitory tissue components were present in sufficient concentration to inhibit growth even though 'streaking' the inoculum may have lessened this effect to a small degree. A more satisfactory technique would perhaps be that of tissue maceration and dilution to lower the concentration of possible inhibitory substances before attempted cultivation. However, to do this on a survey basis, involving many samples, would be more laborious and time consuming. Nevertheless, differences in sensitivity have often been observed in the literature (Dennis *et al.* 1982, Lattimer & Ormsbee, 1891) and diagnosis by IF rather than by isolation of the organism often occurs in practise.

The guinea-pig efficiently eliminated the three test bacterial contaminants and this occurred without interference with the normal pattern of response to *L. pneumophila* infection. These organisms did not produce a pyrexia when injected alone.

Injection of a wide range of concentrations of *L. pneumophila* (10^2–10^9) produced pyrexia within 4 days of infection and results indicate that blood cultures are best attempted at these times, and that it is not necessary to record temperatures beyond the 4-day period.

Although injection of 10^7 *L. pneumophila* produced pyrexia which coincided with bacteraemia, when lower doses of organisms were given, *L. pneumophila* was not detected in the blood even though pyrexia did occur. This observation may well relate to the number of organisms available for culture and also shows that culture of blood alone is not sufficient for the detection of these organisms. The author's own experience with naturally infected water confirms that pyrexia may occur at any time up to 4 days following injection.

L. pneumophila strains of differing pathogenic potential appear to produce approximately the same response pattern in guinea-pigs when injected I.P. The main difference is the dose of *L. pneumophila* required to produce pyrexia. Nevertheless, both strains tested exhibited approximately the same LD_{50}.

Direct isolation of *L. pneumophila* from water samples by cultivation on BCYEα agar does not require that the guinea-pig is killed or infected by these organisms and possibly a different spectrum of *Legionella* species may emerge as a result. Testing in parallel, i.e. a combination of guinea-pig inoculation and direct cultivation methods, is required and being conducted in this laboratory to gain more information before the guinea-pig isolation technique is eventually discarded.

Whilst the antibiotic supplements used in this work did not unduly inhibit the

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growth of *L. pneumophila*, one of these supplements (CCP) did inhibit growth of *L. micdadei* and *L. longbeachae*. Direct isolation of legionella will obviously require that a range of supplements be available (or other selection techniques be developed), otherwise there may be a risk of suppressing some *Legionella* species. Again, parallel testing over a period of time would be advisable.

Edelstein, Snitzer & Finegold (1982) examined the guinea-pig response to infection with natural water samples containing *L. pneumophila* in terms of isolation by cultivation of post-mortem tissue on BCYEα agar and compared this *in vitro* direct isolation and selection techniques. They concluded that for less heavily infected water samples, the *in vitro* technique was the method of choice. However, these workers only examined one *Legionella* species in terms of the least-sensitive *in vivo* parameter, that of isolation of the organism from tissue and their approach was not quantitative.

The saving in time and cost and the increased sensitivity obtained using direct isolation methods is obvious, and time spent developing and proving these techniques, could be well repaid.

L. pneumophila strains used in this present work were selected because of a defined capacity to produce a bronchopneumonia in guinea-pigs when given as an aerosol (Fitzgeorge *et al.* 1983) unlike the strains which may be found in naturally infected water which presumably exhibit a range of virulence which would need definition upon successful isolation. Therefore this work serves only as a guide to isolation technique and the need to test a large number of naturally infected waters in parallel by techniques outlined in this paper, still remains.

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