APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1983, p. 1119–1121
0099-2240/83/031119-03$02.00/0

Vol. 45, No. 3

Improved Facility and Sensitivity in the Use of Guinea Pigs for the Isolation of *Legionella pneumophila* from Cooling Tower Water

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Received 12 July 1982/Accepted 8 December 1982

The established criteria for the determination of the optimum time for the sacrifice of guinea pigs inoculated with samples of cooling tower water were found to be inadequate for the detection of low levels of *Legionella pneumophila*. By ignoring the requirement for fever and by sequentially sacrificing the infected guinea pigs on days 3 through 5 postinoculation, we simplified the procedure, and the sensitivity of detection was improved a great deal.

The use of guinea pigs for selective enrichment has been the traditional method for isolation of *Legionella pneumophila* and other *Legionella* species from environmental samples (7). Although alternative procedures have been developed for culturing of legionellae from potable water (1, 3, 8), air conditioning and industrial process cooling towers frequently contain other bacteria and fungi which can overgrow *L. pneumophila* in these selective enrichment procedures (A. Holden and E. D. Leinbach, unpublished data). In instances such as these, guinea pig inoculation is required for conclusive demonstration of the presence of viable bacteria of the genus *Legionella*.

The success of the guinea pig procedure is in the criteria used for timing the sacrifice of the animals inoculated intraperitoneally with a water sample suspected of containing legionellae. As originally described by Morris et al. (7), animals are sacrificed after the appearance of frank fever (0.6°C above the base line) for 2 consecutive days or after any rise in temperature accompanied by symptoms of illness. In the absence of pyrexia or clinical symptoms, sacrifice commonly has been delayed until days 5 to 10 postinoculation (2, 5–7). This procedure ties the ability to detect the presence of viable legionellae to the virulence of the strains found in the cooling tower water sample being analyzed. We suspected that under such conditions many samples containing viable legionellae at numbers lower than those required to produce overt illness in guinea pigs might be reported as negative for viable legionellae. This situation led us to a reexamination of the criteria for timing the sacrifice.

Male, barrier-sustained, Hartley strain guinea pigs (400 to 450 g; Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were facility adapted at a mean ambient temperature of 23 to 24°C for 10 days before inoculation. No antibiotics were present in either the feed or water. Mean rectal temperatures of these animals varied from 37.9 to 38.3°C during the 5 days preceding inoculation, with daily standard errors of the mean of up to 0.4°C. From studies involving over 100 animals, we concluded that a rectal temperature of 38.9°C for 2 consecutive days was required to distinguish between animals with frank fever and those showing random temperature fluctuations.

L. pneumophila (Philadelphia 1) was isolated from a spleen homogenate obtained from the Centers for Disease Control, Atlanta, Ga., subcultured on buffered charcoal-yeast extract agar containing α-ketoglutarate (BCYE) (3), suspended in nutrient broth containing 12.5% glycerol, and stored at −70°C. This seed pool was used to inoculate a BCYE plate. The legionellae from this plate were harvested after 48 to 60 h of growth at 37°C and then suspended in sterile distilled water to an optical density corresponding to approximately 10^9 CFU/ml. The *L. pneumophila* titer of the suspension was determined by serial dilution and plating on BCYE. Thus, all guinea pig inocula were prepared from a virulent strain of *L. pneumophila* which was passaged three times on BCYE, a procedure which does not affect the virulence of these bacteria for guinea pigs (4).

Inocula containing various levels of *L. pneumophila* were prepared by diluting the *L. pneumophila* stock suspension into sterile distilled water or into water obtained from an operating air conditioning cooling tower. This cooling tower water was naturally contaminated with 2×10^4 to 4×10^5 viable nonlegionellae per ml.

Portions of these samples (3 ml) were injected intraperitoneally into guinea pigs, and in most cases three guinea pigs were used per inoculum. Control animals inoculated with 3.0 ml of sterile water were included in all experiments.

The guinea pigs were sacrificed by CO_2 euthanasia. Cultures were prepared from the peritoneal cavity and from spleen homogenates as described by Morris et al. (7).

In a preliminary experiment, we found that when guinea pigs weighing 400 to 450 g were inoculated with our virulent Philadelphia 1 strain of *L. pneumophila* in distilled water, only one of three animals receiving 4.5×10^6 CFU/ml developed a fever (>38.9°C) for 2 consecutive days. At 4.5×10^7 CFU/ml, all three animals met this criterion, whereas at doses up to 4.5×10^5 CFU/ml, no 2-day fevers were noted.

The difficulties in attempting to detect levels of *L. pneumophila* below the fever dose are illustrated in Table 1. When guinea pigs received *L. pneumophila* inocula of 1.8×10^2 or 1.8×10^4 CFU/ml in cooling tower water naturally contaminated with 2×10^4 viable nonlegionellae per ml, none of the animals met the fever criterion for sacrifice, although several animals had frank fevers on single days during the experiment. When one animal from each group was sacrificed on day 5 postinoculation, *L. pneumophila* was isolated from both animals. However, if sacrifice was delayed until day 7 (7), none of the cultures, even those from animals that had fevers for 1 day, were positive for *L. pneumophila*. These data suggested that the detection of low levels of legionellae might be improved by earlier sacrifice of the experimental animals, even in the absence of fever.

The results of five separate experiments to test this hypothesis are summarized in Table 2. The samples were prepared, and the guinea pigs were inoculated as before with *L. pneumophila* at levels well below the fever dose. Although both sterile distilled water and cooling tower water (containing ca. 5×10^4 CFU of nonlegionellae per ml) were used as diluents, no significant differences in results were obtained. The data have been pooled in Table 2. For simplicity, the *L. pneumophila* inocula have been divided into a low range (<500 CFU per animal) and a middle range (between 1×10^3 and 5×10^5 CFU per animal).

As before, the probability of detection of *L. pneumophila* was very low if sacrifice was delayed until day 7 postinoculation, but it improved somewhat if animals were sacrificed on day 5. Detection was not improved by inoculation of the spleen homogenates in embryonated eggs (7); samples which were negative by culturing on BCYE were also negative in eggs. However, if animals were sacrificed on day 3 or day 4, without regard to fever, the probability of detection of *L. pneumophila* was high. Even at *L. pneumophila* levels of <500 CFU per animal (150 CFU/ml), the organism could be cultured from the spleen or peritoneum in 8 out of 11 samples when the guinea pigs were sacrificed on day 3.

In these experiments, the guinea pigs were able to clear virtually all nonlegionellae from the spleen and peritoneal cavity by day 3. In tests of more than 50 different samples of cooling tower water, we have found that very few nonlegionellae remain by day 3, as long as the levels of total viable bacteria in the inoculum do not exceed

TABLE 1. Rectal temperatures and recovery of *L. pneumophila* from guinea pigs inoculated with low doses of *L. pneumophila* in cooling tower water

Expt	Inoculum (CFU per animal)		Rectal temperature (°C) on indicated day postinoculation						*L. pneumophila* recovery from[a]:	
	L. pneumophila	Other bacteria	1	2	3	4	5	7	Peritoneum	Spleen
1	1.8×10^2	6.0×10^4	38.1	38.1	37.8	39.1[b]	38.6	—[c]	0[d]	+[d]
			38.7	38.9	38.1	37.9	38.0	38.3	0	0
			38.4	37.4	38.3	37.9	38.5	37.7	0	0
			38.1	38.3	38.5	38.4	37.8	37.9	0	0
2	1.8×10^4	6.0×10^4	38.3	38.9	39.1[b]	38.8	39.6[b]	—	+[d]	+[d]
			38.6	38.7	39.7[b]	38.5	39.3[b]	38.5	0	0
			39.5	38.2	39.8[b]	38.5	38.9	38.3	0	0
			38.1	38.6	38.9	38.3	38.5	37.8	0	0

[a] Peritoneal swabs or spleen homogenates were cultured on BCYE. 0, No legionella-like colonies isolated; +, confirmed colonies of *L. pneumophila* isolated.

[b] The guinea pig met the criterion for frank fever (>38.9°C).

[c] —, Not applicable.

[d] The guinea pig was sacrificed on day 5 postinoculation; all other guinea pigs were sacrificed on day 7 postinoculation.

TABLE 2. Effect of day of sacrifice on the recovery of *L. pneumophila* from guinea pigs and correlation with fever

Parameter	Inoculum range[a]	% Positive responders[b] on indicated day of sacrifice			
		3	4	5	7
Peritoneal culture	Low	63 (11)	43 (7)	12 (8)	14 (7)
	Middle	70 (10)	57 (7)	30 (10)	12 (8)
Spleen culture	Low	73 (11)	57 (7)	25 (8)	0 (7)
	Middle	80 (10)	71 (7)	30 (10)	12 (8)
Fever[c]	Low	18 (11)	0 (7)	0 (8)	29 (7)
	Middle	40 (10)	14 (7)	10 (10)	0 (8)

[a] Low range, <500 CFU of *L. pneumophila* per animal; middle range, 1×10^3 to 5×10^5 CFU of *L. pneumophila* per animal.

[b] Percentage of inoculated animals with detectable *L. pneumophila* or with fever. Numbers in parentheses indicate the numbers of animals inoculated.

[c] Rectal temperature of >38.9°C for at least 1 day postinoculation.

about 3×10^6 CFU per animal. With younger animals, which some investigators used (2, 4, 6), this may not be the case. Indeed, we chose to use more mature guinea pigs weighing between 400 and 450 g, because we thought that they would be more resistant to both respiratory infections caused by nonlegionellae, which would complicate the detection of legionella-induced fevers, and to nonlegionellae in the injected cooling tower water samples.

Although the probability of detection of *L. pneumophila* dropped markedly between days 4 and 5 (Table 2), we have found an occasional naturally contaminated cooling tower water sample to be culture negative for *L. pneumophila* on days 3 and 4 but culture positive on day 5. Thus, the data have led us to conclude that sequential sacrifice of guinea pigs on days 3, 4, and 5 postinoculation with a cooling tower water sample can improve the sensitivity of detection a great deal.

We are acutely aware of the need for a simple, inexpensive selection procedure for detecting *Legionella* species in environmental samples. However, it has been our experience that none of the available procedures is sufficiently selective to warrant abandonment of the guinea pig assay for cooling tower water. The methods described here eliminate the requirement for the development and detection of frank fever as the criterion for determining the time of sacrifice. By sequentially sacrificing guinea pigs weighing between 400 and 450 g on days 3, 4, and 5 postinoculation, we were able to isolate *L. pneumophila* from cooling tower water seeded with as few as 150 viable legionellae per ml, a level well below that required for detection of fever.

This work was supported by the Legionella Testing Service of the South Alabama Medical Sciences Foundation.

We thank Anthony Holden and Edsel P. Holden for technical assistance.

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