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Isolation of Legionella pneumophila from water systems: methods and preliminary results

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Abstract

A preliminary survey of water systems in hospitals and hotels showed that Legionella pneumophila may be found in water storage and distribution systems as well as in the recirculating cooling water of air-conditioning plants. Altogether 42 isolates of L pneumophila were made from 31 establishments, six of which were associated with cases of legionnaires' disease but in 25 of which there was no known association with disease. In the six establishments implicated epidemiologically as the source of legionnaires' disease, these organisms were found in each of their water-distribution systems and also in the cooling water from each of the three with cooling towers. In establishments not associated with cases, water from three out of nine cooling towers, four out of 24 taps or showers, and one out of 15 storage tanks was found to contain legionellae. The organisms were isolated by guinea-pig inoculation and subsequent culture of their peritoneal fluid, liver, and spleen.

Finding L pneumophila in water systems in the absence of cases of legionnaires' disease should not at present be an indication for attempts at eradication.

Introduction

During the investigation of legionnaires' disease in a renal transplant unit in 1979, *Legionella pneumophila* serogroup 6 was isolated from two cases and from shower-bath mixers in the unit.[1] Early in 1980 *L pneumophila* serogroup 1 was isolated from water-storage tanks, taps, and showers in a hotel associated with cases of *L pneumophila* serogroup 1 infection.[2] Samples were collected from other buildings to determine whether *L pneumophila* could be found in establishments not known to be associated with legionnaires' disease. Serogroup 1 strains were soon isolated from such sites, which were then studied in detail to develop methods of sampling and isolation.

In the light of these findings a pilot survey was begun in 1980 to determine how frequently the bacterium could be found in water storage and distribution systems in hospitals and hotels. The recirculating water of air-conditioning systems was also sampled in view of convincing evidence from the USA of airborne spread of *L pneumophila* from contaminated cooling towers and evaporative condensers.[3] Establishments not known to be associated with legionnaires' disease were included, together with those implicated as sources by epidemiological investigation. We report the methods of isolation and identification of the bacterium and the early results of the survey.

Methods

SPECIMEN COLLECTION AND PREPARATION

Water samples were collected from cooling towers, showers, hot and cold water-bath and basin taps, and storage tanks in 28 establishments in England and Wales and three hotels abroad and sent to Oxford for examination. Samples of less than 1 l were collected in sterile bottles or small plastic containers and 100-200 ml aliquots centrifuged in universal (28 ml) containers at 3500 rpm for 30 minutes. The deposit was resuspended in 10-20 ml distilled water. Larger samples were collected in sterile plastic containers. Usually 5-10 l was collected from cooling towers and 15-25 l from other sources. Organisms from samples of more than 1 l were concentrated by passing the samples through 142 mm diameter membrane filters (pore size 220-450 nm) under positive pressure of 0·28 kg/cm² (4 lb/sq in) from an air pump. The filters, connecting tubing, and rubber bungs were heated at 100°C for 15 minutes or at 65 C overnight between each specimen. Other work had shown that heating at 65 C killed legionellae within 45 minutes. Two 25 l containers and their filters were pressurised in parallel from a single pump, and an adjustable air vent limited the pressure. Specimens of 5 l or less were transferred to a 5 l pressure vessel. At least 10 l of the 25 l samples was filtered, using a second membrane if the first became blocked.

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After filtration the membrane was transferred to a sterile, wide-mouthed plastic pot (90 ml capacity) and cut into small pieces with sterile scissors. Distilled water (30 ml) was added and the pot shaken vigorously. Those concentrates for inoculation into guinea-pigs within 48 hours were held at room temperature, but for longer storage were held at −70°C.

GUINEA-PIG INOCULATION

Guinea-pigs were inoculated in pairs. A 1 ml sample of blood was taken by cardiac puncture from each of the 4-6-week-old animals in each pair, and each was then injected intraperitoneally with 5-10 ml of the resuspended deposit. Rectal temperatures were taken daily for six or seven days; 39·5°C or over was regarded as feverish. Most animals subsequently shown to be infected with legionellae developed a temperature on the third day after inoculation. Some feverish guinea-pigs showed no sign of illness, but others had ruffled fur, watering eyes, and wasting around the flanks. Of these last animals, some recovered, but in others the temperature fell after two days to less than 37°C and the animal died or was killed if it was in extremis. Guinea-pigs with fever lasting two days were usually killed; whenever the animal's condition allowed, however, one of each pair was kept for serological investigation.

Liver and spleen were removed post mortem, together with any free peritoneal fluid. In guinea-pigs which had become obviously ill, peritonitis with adhesions, exudate, and several ml purulent peritoneal fluid were found.[4] In feverish but otherwise symptomless animals little abnormal was found except for an enlarged spleen. Guinea-pigs in which fever lasted less than two days were bled four weeks later, and these sera together with the preinoculation samples were tested for legionella serogroup antibodies by the indirect immunofluorescence antibody technique.[5] One member in each of 67 guinea-pig pairs was given cortisone acetate subcutaneously at the time of inoculation, but this did not appear to enhance the isolation rate.

EXAMINATION OF GUINEA-PIG SPECIMENS

Peritoneal fluid samples were stained by Gram's method, counter-staining with neutral red for 15-20 minutes.[1] Legionellae could usually be seen in moderate numbers, both free and within phagocytes. These peritoneal fluid samples were also stained specifically by immunofluorescence using rabbit or guinea-pig antisera to individual legionella serogroups 1-6, as this technique showed more organisms than were visible by Gram's stain: samples in which no organisms were visible by Gram's stain were not tested by immunofluorescence. Smears of freshly cut spleen surface were also examined by indirect immunofluorescence staining.

A presumptive identification of legionellae could often be made at this stage. Confirmation by culture with final identification of isolates using immunofluorescence and gas-liquid chromatography[6] was always done.

CULTURE

Aliquots of peritoneal fluid were cultured on a solid agar medium, prepared locally by Dr R G Mitchell,[6] or Horwitz and Silverstein's modification of charcoal yeast-extract medium.[7] The cut surface of spleen was smeared on to a plate of either medium and the inoculum streaked to avoid any inhibitory effect.[8] The media were incubated in a candle jar or CO_2 incubator at 35-37°C and examined daily for one week and periodically thereafter for a further week. Though colonies of legionellae were sometimes visible after 48 hours of incubation, they usually required three to eight days to appear.

If no growth occurred on direct culture within a week a homogenate of guinea-pig spleen (or liver) was inoculated into eggs. Spleen or liver was homogenised with distilled water in a Griffiths tube and 0·1 ml of a 10% solution inoculated into the yolk sacs of four 6-7-day-old fertile eggs. These were incubated at 39°C and candled daily. Any embryos dying within three days were discarded. Yolk-sac smears of those dying at four to 10 days were examined by Gram's stain and immunofluorescence. Yolk sacs were cultured by smearing a small portion on to solid media described above whether or not bacteria had been seen by Gram's stain.

SEROGROUPING BY IMMUNOFLUORESCENCE

All isolates from solid media were serogrouped by indirect immunofluorescence[9] using an antigen prepared by heating an opalescent suspension of organisms in distilled water at 100°C for 10 minutes. This antigen was further diluted to give about 100-200 organisms per high-power field when spotted on to Teflon-coated slides (Hendley-Essex SM.010). The slides were fixed in acetone for 10 minutes and stored at −20°C. Antisera were prepared in rabbits and guinea-pigs using heat-killed *L pneumophila* serogroups 1-6 grown on solid media. After preliminary grouping with sera at working dilutions any isolate giving a positive result was titrated to titre in parallel with a known strain of the same group.

GAS-LIQUID CHROMATOGRAPHY

Cultures on slopes of modified charcoal yeast-extract medium were sent to the Bacterial Metabolism Research Laboratory, Colindale, for whole-cell fatty-acid analysis by gas-liquid chromatography.

Results

A total of 31 hotels and hospitals were studied. Altogether 42 isolations of *L pneumophila* were made in these establishments, six of which were associated with cases of legionnaires' disease but in 25 of which there was no known association with disease (table). All isolates had the characteristic profile of *L pneumophila* on gas-liquid chromatography. In the six establishments implicated epidemiologically as the source of legionnaires' disease, these organisms were found in each of their plumbing systems and also in the cooling water from each of the three with cooling towers. In establishments not associated with cases, water from three out of nine cooling towers, four out of 24 taps or showers, and one out of 15 storage tanks was found to contain legionellae.

Isolation of Legionella pneumophila from establishments (January to December 1980)

Specimens source	Establishments examined							
	Hospitals				Hotels			
	Disease associated		No known disease		Disease associated		No known disease	
	No	Positive	No	Positive	No	Positive	No	Positive
Cooling towers	3	3	2	0	—	—	7	3
Showers/taps	4	4	7	2	2	2	17	2
Water tanks	3	0	1	0	1	1	14	1
Humidifiers	3	0	—	—	—	—	1	0

Serogroup 1 *L pneumophila* was found in five of the case-associated establishments and serogroup 6 in the other. Serogroup 1 was found in the cooling-tower water of two of the other establishments not associated with cases, and *L pneumophila* of other serogroups, not yet finally defined, was found in their water systems. Isolations were made from hot, cold, and mixed water samples. Quantities of water specimens containing one guinea-pig infectious dose varied from 20 ml upwards.

Samples giving serologically positive responses without *L pneumophila* isolation are not included here, though a close association was found between fever, serological response, and isolation in pairs of animals inoculated with the same specimen.

Discussion

The injection of environmental specimens into guinea-pigs is a satisfactory but time-consuming method of isolating *L pneumophila* strains, but until a suitable selective medium is devised it remains the best way of extracting them from such contaminated material. Reports from the USA show that *L pneumophila* may be recovered from lakes, creeks, and mud as

well as cooling-tower water.[10 11] This study shows that the organism may also be found in water-distribution systems within hospitals and hotels. The findings so far suggest that infected water systems may be associated with cases in the absence of air-conditioning equipment in the establishment concerned. When both the water-distribution system and air-conditioning cooling-water system are infected the relative importance of each needs further clarification.

We plan to extend the survey to determine how frequently *L pneumophila* may be found in various types of plumbing systems as well as in humidification and heat-exchange recirculating systems. The principal objective of the project is to identify factors which permit establishment of the organism, with a view to devising inexpensive and effective control measures. At this stage it is clear that, in view of the apparently ubiquitous nature of *L pneumophila*, its demonstration in water systems in the absence of associated cases should not be an indication for active measures of eradication by methods whose long-term efficacy is as yet unknown. Spending large sums of money on these in an attempt to prevent a very occasional hazard may divert resources needed for other, more frequent and equally serious conditions.

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Remedial therapy after stroke: a randomised controlled trial

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Abstract

Of 1094 patients with a confirmed stroke admitted to Northwick Park, a district general hospital, 364 (33%) died while in hospital, 215 (20%) were fully recovered when discharged, and 329 (30%) were too frail or too ill from diseases other than stroke to be considered for active rehabilitation. Only 121 (11%) were suitable for intensive treatment. They and 12 patients referred direct to outpatients were allocated at random to one of three different courses of rehabilitation. Intensive was compared with conventional rehabilitation and with a third regimen which included no routine rehabilitation, but under which patients were encouraged to continue with exercises taught while in hospital and were regularly seen at home by a health visitor. Progress at three months and 12 months was measured by an index of activities of daily living. Improvement was greatest in those receiving intensive treatment, intermediate in those receiving conventional treatment, and least in those receiving no routine treatment. Decreasing intensity of treatment was associated with a significant increase in the proportions of patients who deteriorated and in the extent to which they deteriorated.

Probably only a few stroke patients, mostly men, are suitable for intensive outpatient rehabilitation, but for those patients the treatment is effective and realistic.

Introduction

Remedial therapists spend much of their time[1] rehabilitating patients disabled by strokes, though this has not been convincingly shown to improve chances of recovery. If rehabilitation is ineffective therapists' efforts are wasted and patients inconvenienced. If, on the other hand, rehabilitation is effective more investment in it might be justified.

Three randomised controlled trials[2-4] on the effectiveness of rehabilitation after stroke have been inconclusive, possibly because of their small numbers. Garraway *et al*[5] have recently shown that patients admitted to a special stroke unit fared better than those admitted to medical units, but their trial was concerned largely with the effects of inpatient management, and the advantage was not sustained on longer follow-up.[6] This paper compares the effectiveness of three intensities of outpatient rehabilitation.

Patients and methods

All 1094 patients with a recent confirmed stroke who were admitted to Northwick Park Hospital from October 1972 to September 1978 were considered for the trial. Of these, 364 (33%) died while in hospital and 215 patients (20%) made a full recovery while in hospital, in terms not only of day-to-day activities but also of limb function and speech. The remaining 515 patients were considered for the trial. The main criterion for entry was that the patient should