Microbiological investigations into an outbreak of Pontiac fever due to *Legionella micdadei* associated with use of a whirlpool

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Abstract

In the investigation of a large outbreak of non-pneumonic legionellosis at a leisure complex in Lochgoilhead, Scotland all direct cultures of environmental samples were initially negative for legionellae. Legionellae readily infect appropriate protozoa under suitable conditions, and following immunofluorescence to select specimens for special study, *Legionella micdadei* was isolated from whirlpool water via co-cultivation with *Acanthamoeba polyphaga*. *L. micdadei* was also isolated, along with host amoebae, from the whirlpool filter. The use of amoebae in the isolation of legionellae from environmental (and other) sources can be of great value, especially if specimens shown by indirect immunofluorescence to contain legionellae fail to yield legionellae on routine culture.

In 1988 a large outbreak of non-pneumonic legionellosis (Pontiac fever)¹ occurred at a leisure complex at Lochgoilhead in the West of Scotland.² The index case in this outbreak presented with Legionnaires' disease, had radiologically confirmed pneumonia, and showed a significant rise in antibody titre to *Legionella micdadei*. The presence of *Legionella* species antigen was shown in urine from this patient by Dr S Toma of Toronto. Sera from most of the patients in the outbreak showed an antibody response to *L. micdadei*. Environmental samples were taken to determine the possible source of infection. Because previous experience had shown that direct immunofluorescence of water deposits was misleading due to crossreactions of polyclonal antisera with many different bacteria, the deposits were screened by the indirect fluorescent antibody technique (IFAT), which was more specific due to the higher dilutions of antisera used.³

Methods

The purpose built leisure complex, which was a hotel annexe, comprised a swimming pool with a whirlpool spa, together with a curling rink. The complex contained toilet and changing facilities and was connected to the hotel by a lounge, which opened through a door directly on to the swimming pool and the whirlpool spa. Air to the complex was drawn from outside across the roof space above the curling rink to

the swimming pool area where it was heated before being discharged into the pool area. The air in the pool area was recirculated, and in both the swimming pool and curling rink areas was dehumidified by a small number of portable dehumidifiers, together with a large dehumidifier in the swimming pool area. Air from the pool area escaped to the outside via the lounge, this movement being assisted occasionally by exhaust fans set in the lounge windows. Most of the heat from the compressor associated with the curling rink refrigeration plant was used to heat the water in the swimming pool, but there was also a small evaporative condenser associated with this plant. The water supply to the hotel and leisure complex came from two nearby mountain streams and was said to be of a satisfactory bacteriological standard.

ENVIRONMENTAL SPECIMENS

Six days after the peak of the outbreak 5 litre water samples were taken from the whirlpool spa, the swimming pool, and cold water break tanks for the water supply to the hotel. Four showers in the complex were sampled, 500 ml being taken from each and also 500 ml of water from the overflow channel in the swimming pool. Smaller quantities of water were taken from the four dehumidifiers by the ice rink and two samples from the pool-side dehumidifier, as well as condensation from the windows separating the swimming pool from the curling rink. Swabs and scrapings of material were also taken from the dehumidifiers and the ducting between the main dehumidifier and the discharge vents, which were located over the windows separating the swimming pool area from the curling rink, and also from the ducting bringing a small amount of make-up air from the outside. A 2.5 litre sample of water was taken from the pond underneath the evaporative condenser situated outside the complex. In all, 45 specimens were taken. Twenty five days after disinfection of the pool systems with sodium hypochlorite samples from the edge and centre of the swimming pool and whirlpool spa filters were obtained.

SAMPLE HANDLING

The 500 ml water samples were centrifuged at 1500 × g for at least 20 minutes. Larger water samples were filtered through two 142 mm in diameter nylon membrane filters in series (0.45 µm average pore size (APD) (Ultipor) filter, followed by a 0.22 µm APD (Sartorius) filter).

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After filtration of a sample both membrane filters were cut into small pieces, placed in a universal container together with 20 ml of filtrate, and vortexed to resuspend the deposit. Swabs and material scraped from dehumidifiers, ducts, and the evaporative condenser as well as samples from the air filters associated with the whirlpool and swimming pool, were extracted in 10 ml of sterile distilled water. Smears of the deposits were made on 12 well (6 mm) Teflon coated slides (Henley, Essex) which were then air dried and gently heat fixed before examination by the IFAT. Samples of filter bed material from the swimming pool and the whirlpool spa were similarly examined. All water deposits and swabs were cultured.

CULTURE MEDIA FOR LEGIONELLAE

These were ACES buffered charcoal yeast extract agar (BCYE) with α ketoglutarate.⁴ Selective BCYE was made as described by Dennis,⁵ except that both low (1 mg/l) and high (5 mg/l) concentrations of vancomycin were used because high concentrations tend to be inhibitory, masking the presence of small numbers of legionellae. MWY medium⁶ (Oxoid, CM655 + SR110 + SR118) was also used.

Cultures were made from unheated water deposits as well as deposits heated at 50°C for 30 minutes. Cultures were inoculated with 0.1 ml of an undiluted suspension of deposit or swab material as well as 1/10 and 1/100 dilutions of the material in sterile distilled water. Cultures were incubated in an atmosphere of 5% (v/v) carbon dioxide in air at 36°C for 14 days. They were examined by plate microscopy on alternate days during this period for the typical "cut-glass" colonies of *Legionella* sp.

Pairs of guinea pigs were inoculated intraperitoneally with 2 ml of resuspended water deposit from the humidifier in the swimming pool area and also with sludge from the evaporative condenser. Individual guinea pigs were inoculated with water deposits which showed the presence of *L. micdadei* by fluorescence microscopy. One animal in each pair was killed either at three or five days after inoculation, irrespective of the presence of fever or symptoms. Single animals were killed three days after inoculation. Peritoneal swabs, liver, and spleen, were cultured on BCYE. The second animal in a pair was bled after six weeks and serum examined by the IFAT for antibody to *L. micdadei*.

Polyclonal antiserum to *L. micdadei* was prepared by inoculating a rabbit with a boiled suspension of a 72 hour culture of *L. micdadei*

(Tatlock strain) as previously described.⁷ Other antisera were prepared in rabbits or guinea pigs with either formalin or heat-killed suspensions of the species and serogroups of *Legionella* shown in table 1.

INDIRECT IMMUNOFLUORESCENCE FOR LEGIONELLAE

This was performed by standard technique⁸ using antiserum pools and then single polyclonal antisera. Deposits were reacted with antisera for 20–30 minutes at 36°C and after washing, with the appropriate fluorescein isothiocyanate conjugated species antiglobulin. Slides were examined by incident ultraviolet light illumination. The antisera used in the IFAT were raised to the legionellae listed in table 1. The specificity of a positive result was checked by titrating the strength of reaction with doubling dilutions of antiserum on a 12 well slide bearing the "positive" water deposit compared with a slide bearing a 1% formol saline suspension of the prototype strain of known legionella.

Identification of *L. micdadei*

Blue colonies on MWY medium that had a "cut glass" appearance under low power magnification were examined by Gram stain and subcultured on to horse blood agar as well as cysteine deficient BCYE. The colonies were examined by long wave ultraviolet light illumination for bound and diffusible pigment. Isolates were examined for their ability to hydrolyse hippurate and to produce β lactamase. Multi-well slides were inoculated with a light suspension of the organism in 1% formol saline and examined in parallel with a slide inoculated with a 1% formol saline suspension of the prototype strain with the IFAT using serial dilutions of specific hyperimmune antiserum to *L. micdadei*.

Isolation of *L. micdadei* via co-cultivation with amoebae

The methods outlined below were mainly based on those previously described⁹ for increasing the number of legionellae in pre-treated clinical specimens. These methods were partly devised to cope with the possibility that the organism responsible for the outbreak could have been a legionella-like amoebal pathogen (LLAP) which shared antigens with *L. micdadei*. LLAPs are bacteria which infect amoebae in the same manner as legionellae but do not grow on BCYE at 35°C. (Abstract presented by T J Rowbotham, Fifth International Conference on the Biology and Pathogenicity of Free Living Amoebae; Brussels, 1989.)¹⁰

The amoeba used for this work was *Acanthamoeba polyphaga* Linc Ap-1, which was originally isolated (together with *L. pneumophila* sero-group (SG) 1) from the pond of a cooling tower, epidemiologically implicated in an outbreak of legionellosis. A *polyphaga* was used as it was known to be a good host for several *Legionella* sp, including *L. pneumophila* SG-1 and *L. micdadei*, as well as LLAP-3, a bacterium isolated in 1986 from the sputum of a case

Table 1 *Legionella* antisera used

<i>L. anisa</i>	<i>L. hackeliae</i>	<i>L. parisiensis</i>
<i>L. birminghamensis</i>	<i>L. israelensis</i>	<i>L. pneumophila</i> SG 1–14
<i>L. bozemanii</i> SG 1 and 2	<i>L. jamestownensis</i>	* <i>L. quateriensis</i>
<i>L. brunensis</i>	<i>L. jordanis</i>	<i>L. rubrilucens</i>
<i>L. cherrii</i>	* <i>L. londoniensis</i>	<i>L. saintelensis</i>
<i>L. cincinnatiensis</i>	<i>L. longbeachae</i> SG 1 and 2	<i>L. santacrucis</i>
<i>L. dumoffii</i>	<i>L. maceachernii</i>	<i>L. spiritisensis</i>
<i>L. erythra</i>	<i>L. micdadei</i>	<i>L. steigerwaltii</i>
<i>L. feeleyi</i> SG 1 and 2	<i>L. moravica</i>	<i>L. wadsworthii</i>
* <i>L. geestiae</i>	* <i>L. nautarum</i>	* <i>L. worsleiensis</i>
<i>L. gormanii</i>	<i>L. oakridgensis</i>	

*Proposed new species.¹⁸
SG = serogroup.

of persistent pneumonia (T J Rowbotham, Brussels, 1989). *A. polyphaga* Linc Ap-1 also lacks bacterial symbionts, grows well in PYG broth,⁹ and was available in large quantities as it was in routine use in the Leeds laboratory.

Aliquots of whirlpool water (0.8 ml) (normally 1.0 ml) and filter materials (0.1 ml) were decimally diluted in sterile distilled water and incubated at 35°C with 1 ml volumes (1×10^5 trophozoites) of axenic *A. polyphaga* Linc Ap-1 which had been grown in PYG broth and washed three times in Page's amoebal saline before use.⁹ After about one hour 0.2 ml of a suspension (OD 0.3 at 540 nm) of *Klebsiella aerogenes* NCTC 7427 killed by ultraviolet light was added to each mixture, either in bijou bottles or 15 mm wells of a Cell Cult sterile multiwell dish (Sterilin Ltd, Middlesex).

The cultures were examined, after 48 hours for primary cultures and from 24 hours for secondary cultures, for amoebae full of motile bacteria. Suspensions containing infected amoebae were treated at room temperature with an HCl-KCl solution⁹ for 29 minutes to reduce the numbers of extracellular bacteria before vortexing for one minute and plating out on MWY medium. To reduce further the numbers of such bacteria in cultures from filter materials, the acid washed amoebae were washed three times with amoebal saline before vortexing.⁹ In addition, 0.1 ml aliquots of the original water concentrate (dilutions 10^{-1} to 10^{-4}) and filter materials (dilutions neat to 10^{-7}) decimally diluted in sterile distilled water were spread directly on to MWY medium.

Isolation, purification, and identification of amoebae

For the whirlpool water concentrate and each sample of filter material six lawns of live *K. aerogenes* on Stoianovitch's malt extract, yeast extract agar⁹ (amoebal agar) were inoculated with a single diagonal streak of deposit (about 0.1 ml/lawn). The lawns incubated in resealable plastic bags at room temperature and 30, 35, 37, 42, and 45°C were microscopically examined frequently over the next three weeks for the growth of amoebae. Amoebae were transferred to and purified on further lawns of *K. aerogenes*, and similar amoebae isolated at different temperatures were compared over a range of temperatures. Techniques frequently used at Leeds for the isolation and purification of amoebae by encouraging the infection of unwanted amoebae with stock strains of legionellae, and the outgrowth of the desired amoebae, were not required or used in this investigation. Stock strains of amoebae were established from single or small numbers of microscopically similar cysts or trophozoites and stored at room temperature on slopes of amoebal agar inoculated with *K. aerogenes*. Amoebae were identified by their morphological and growth temperature characteristics.¹¹

Interaction of L. micdadei with amoebae isolated from the filters

To each of the 25 compartments, 100 × 100 mm, Petri dishes (Sterilin) 1.5 ml of amoebal agar was added. After drying to remove excess

moisture half the compartments were flooded with one drop/compartment of a suspension of live *K. aerogenes* (OD 0.2 at 450 nm); the remaining compartments were flooded with single drops from 10 ml of the same suspension inoculated with a wire stabbed into a single colony of *L. micdadei*. The *L. micdadei* strain, originally obtained by direct plating on MWY from whirlpool water, had been grown on BCYE for three days immediately before use. The dishes were incubated at 37°C overnight and dried briefly to remove any excess moisture. Suspensions of cysts (except for *Vannella platypodia*, which is not known to produce cysts) were prepared in amoebal saline from cultures, set up an appropriate number of days before, and inoculated on to the centre of pairs of lawns (one with *L. micdadei* and one without). That each well had been inoculated with amoebae was confirmed by examining each lawn microscopically through the base of the plate with a long working distance × 40 objective (total magnification 400 times). Thirteen strains of amoebae, representing all the species isolated from both filters, were grown at six different temperatures on lawns of live *K. aerogenes* with and without *L. micdadei*. The growth or fate of the amoebae was recorded at 24, 30, 48, 72, and 96 hours, and again after seven days (table 2).

Results

In the IFAT small brightly fluorescing single coccobacilli or single short bacilli were seen only in specimens stained with *L. micdadei* antiserum. The positive sites were the dehumidifier ducting, the whirlpool spa (both filter bed material and a concentrated sample of whirlpool water), and the water in the overflow channel at the side of the swimming pool. Very scanty bacilli were also seen in material from the swimming pool filter and the condensate from the curling rink dehumidifier. The specificity of the staining of *L. micdadei* was confirmed in that the titre of the *L. micdadei* antiserum was identical by the IFAT with both the control and test specimens. Cultures in Glasgow of water specimens and guinea pig material on selective and non-selective BCYE media yielded no legionellae. Guinea pigs bled after six weeks showed no antibody to *L. micdadei*. Samples of water from the sites which showed large numbers of fluorescing bacteria with the IFAT were also examined at the Leeds laboratory.

L. micdadei was isolated in Leeds from concentrated whirlpool water by co-cultivation with *A. polyphaga* trophozoites and by direct plating on MWY medium (Oxoid Ltd). Subsequently *L. micdadei* was also isolated in Glasgow by direct plating on MWY medium. The approximate numbers of viable *L. micdadei* expressed as number/ml of unconcentrated whirlpool water from the three determinations were ≥80, 80, and 4, respectively. In Leeds inhibition of *L. micdadei* by other bacteria on the direct isolation plates was noted, and no legionellae were isolated on the more crowded plates. *L. micdadei* was not isolated by direct

Table 2 Isolation, growth rate, and temperature range of amoebae from whirlpool (WP) and swimming pool (SP) filters, and their ability to act as hosts for *L. micdadei* at various temperatures (°C)

	From WP or SP				Growth and fate of amoebae at six temperatures						First amoebae full of motile <i>L. micdadei</i> seen after			
Amoeba	Growth rate (S, M, or F)				Room temperature	30	35	37	42	45	Hours	at °C		
Acanthamoeba														
<i>A. castellanii</i>	SP	35	M		■ .	■ ■	■ ■ ■	...	...	...	24	35		
<i>A. polyphaga</i>	WP	35	F		■ .	■ ■ ■	■ ■ ■	+	...	...	30	30	35	
	SP	RT	F		■ .	■ ■ ■	■ ■ ■	...	...	...	30	30	35	
	SP	35	F		■ .	■ ■ ■	■ ■ ■	...	...	...	30	30	35	
	SP	37	F		■ .	■ ■ ■	■ ■ ■	L ■ . .	...	...	24	35		
<i>A. culbertsoni</i>	WP	42	F		(L) ■ . .	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	24	37		
	WP	42	F		+(+) ■ . .	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	24	35	37	42
	WP	45	F		(+) ■ . .	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	24	35	37	42
	SP	45	F		(L) ■ . .	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	■ ■ ■	24	35	37	42
Hartmannella														
<i>H. cantabrigiensis</i>	SP	RT	M		■ . .	■ ■	■ ■ ■	■ ■	■ ■	...	24	37	42	
<i>H. vermiformis</i>	WP	RT	F		■ . .	■ ■ ■	■ ■ ■	■ ■ ■	...	...	24	37	42	
Vannella														
<i>V. platypodia</i>	SP	RT	S		■ . .	■ ■	■ ■ ■	■ . .	...	...				
	SP	RT	S		■ . .	■ ■	■ ■ ■	■ . .	...	...				

(Plates examined at 24, 30, 48, 72, and 96 hours).

Amoebal growth: . . . none, ■ . . poor/slow, ■ . moderate, ■ . good.

Growth rate: S = slow, M = moderate, F = fast.

Infection: + rare, ++ several, +++ numerous, amoebae full of motile *L. micdadei*, L = lysed. (For *A. culbertsoni* at room temperature (21–22°C). (+) numerous infected amoebae at seven days, (L) amoebae lysed at seven days).

L. micdadei strain originally isolated from WP water by direct plating. Whirlpool temperature = 36°C.

plating from the whirlpool or swimming pool filter samples on any medium in either laboratory. The filter samples had a much higher content and variety of bacteria. At Leeds *L. micdadei* was isolated via amoebae from both whirlpool filter samples, but from neither of the swimming pool filter samples. Despite repeated culture on both BCYE and MWY media in Glasgow of waters which had been stored for long periods at room temperature, no additional sites yielded *L. micdadei*. Subcultures of *L. micdadei* from the whirlpool water grew well on BCYE at 35°C and 37°C. No growth occurred at room temperature, 42°C, or 45°C. Growth at 30°C was slow. *L. micdadei* readily infected *A. culbertsoni* over a wider range of temperature (table 2) and at 42°C under microaerophilic—closed jar partially evacuated and refilled with the following gas mixture: nitrogen 80%, hydrogen 10%, carbon dioxide 10%—conditions but not at 37°C under anaerobic conditions, although the amoebae continued to grow. Unexpectedly, hartmannellae also acted as hosts for *L. micdadei* at temperatures above those for amoebal growth (table 2).

Ciliates, green algae, and cyanophytes were not seen in the filter samples or in the concentrated whirlpool water. A few live amoebae were seen in the swimming pool filter samples, but rare cysts were only seen in one sample. Large numbers of roughly spherical cysts ranging in diameter from 10–14 µm (mean from 50 cysts 11.5 × 12.0 µm) consistent with *A. culbertsoni* were present in the whirlpool filter samples, but not trophozoites. No amoebae were seen in, or isolated from, concentrated whirlpool water, but a variety of free living

amoebae were isolated from both the swimming pool and whirlpool filters (table 2). *V. platypodia* was the only amoeba isolated which could not be infected with *L. micdadei* (table 2).

On the original isolation plates for amoebae, *H. vermiformis*, was isolated from only one whirlpool filter sample. *H. vermiformis* was also isolated at the same time from the same sample, on *K. aerogenes* killed by ultraviolet light at 37°C, on which *A. culbertsoni* grew poorly but consumed up to four cysts of *H. vermiformis* per amoeba.

The large numbers of interfering, often spreading, bacteria in the whirlpool filter samples hampered the isolation of *L. micdadei* from serial co-cultivations of those samples with *A. polyphaga*, although the co-cultivations contained numerous infected amoebae. The ability of *A. culbertsoni* isolated from one of the whirlpool filter samples to act as a host for *L. micdadei* at 45°C (table 2) and of infected amoebae to travel across a lawn of bacteria away from the inoculum pool, was used during the final isolation of *L. micdadei* from the whirlpool filter samples. Ciliates were not noted in the liquid co-cultivations with *A. polyphaga* Linc Ap-1.

Discussion

Outbreaks of Pontiac fever, which was first described in 95 of 100 of the staff at a public health building in Pontiac, Michigan, in 1968,¹ have been caused by *L. pneumophila* SG 1,¹ SG 6,^{12,13} and *L. feeleyi* SG 1,¹⁴ but Lochgoilhead is the first reported outbreak caused by *L. micdadei*. Sera from the index case and from many of the 187 patients whose sera were examined by the IFAT showed a significant (≥

fourfold) rise in, or high titre (≥ 256) of, antibody to *L. micdadei*, and there was no doubt that the outbreak was due to this organism. Whirlpool spas have been implicated in several outbreaks of Pontiac fever^{12 13 15} and therefore it was not surprising to find a legionella in the whirlpool spa, particularly as the routine bromination of the spa had been unsatisfactory.² What was surprising was that *L. micdadei* was not isolated on the BCYE media used in Glasgow, despite the fact that these media had been found to support the growth of laboratory cultures of all serogroups and species of legionella listed in table 1, as well as a number of strains isolated directly from clinical specimens or the environment. Oxoid MWY medium grew *L. micdadei* from the whirlpool water on repeated occasions, although, curiously, the growth of some other laboratory adapted strains of *Legionella* sp was much less luxuriant on this medium than on BCYE. The reasons for the difference in performance between the media have not been elucidated but the likelihood that MWY would be suitable for the isolation of *L. micdadei* was noted by Edelstein.⁶ On MWY medium the colonies of *L. micdadei* were characteristically blue in colour as well as showing a typical "cut glass" appearance under the plate microscope. No other species of *Legionella* was shown. The IFAT with *L. micdadei* antiserum was of cardinal importance in selecting appropriate specimens for culture. Confirmation of the specificity of the test by titration of the *L. micdadei* antiserum in parallel with control slides bearing *L. micdadei* antigen was most valuable.

Co-cultivation of specimens with *A. polyphaga*, a technique which has been shown frequently at the Leeds laboratory to be the only way in which legionellae could be isolated from both clinical and environmental specimens (unpublished data),⁹ was central to the isolation of *L. micdadei* from the whirlpool water and it was only later that culture on MWY was successful. The whirlpool temperature was 36°C, a temperature at or above the maximum growth temperature for many of the free living amoebae isolated (table 2).

Disinfection conditions permitting, *L. micdadei* could have grown in both the whirlpool and swimming pool filters. No amoebae were seen in, or isolated from, the concentrated whirlpool water sample. This, coupled with a larger number of *L. micdadei* and cysts consistent with *A. culbertsoni* seen in the whirlpool filter samples, plus the isolation of *L. micdadei* from 0.1 ml volumes of the whirlpool filter samples diluted 10 or 100-fold but not from the neat or diluted swimming pool filter samples, implicates the whirlpool filter as the site of proliferation for *L. micdadei* and its host amoebae. The automatic dosing mechanism for whirlpool disinfection was faulty, whereas the manual addition of disinfectant to the swimming pool seems, despite unsatisfactory readings, to have been effective. How difficult it is to maintain whirlpools free from legionellae and their potential hosts is underlined by the isolation of both from the whirlpool filters 25 days after hyperchlorination. This agrees with the observations of Kilvington¹⁶ that legionellae can be found in amoebal cysts which are

resistant to chlorine at the concentrations normally recommended for disinfection of water systems. It may well be that at Lochgoilhead *L. micdadei* survived disinfection of the filters by being inside amoebal cysts.

The operating temperatures of the whirlpool suggest that *A. culbertsoni*, the most numerous of the amoebae isolated from the whirlpool filter, was the host for *L. micdadei* in this outbreak. The identification of *A. culbertsoni* made on established criteria is uncertain in view of the increasing numbers of *Acanthamoeba* sp now being recognised, which has made definitive speciation of acanthamoebae more difficult.¹⁷

Our findings show that preliminary screening by the IFAT is of great value in determining where exhaustive attempts at culture are worth while and also that one formulation of legionella culture medium may not be adequate for all situations. Co-cultivation with amoebae, however, can be a most rewarding part of the investigation of material from any outbreak of legionellosis where conventional cultural techniques have failed to show the presence of viable legionellae.

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