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Incrimination of an environmental source of a case of Legionnaires' disease by pyrolysis mass spectrometry

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SUMMARY

Following a case of Legionnaires' disease *Legionella pneumophila* of the same serogroup was isolated from the hot water system of the hotel which had been epidemiologically implicated as the source of the infection. Pyrolysis mass spectrometry (PyMS) was used to compare 11 isolates from the hotel water sample with the patient's strain. Epidemiologically unrelated isolates, both clinical and environmental, of the same serogroup and monoclonal antibody type of *L. pneumophila* were included in the same analysis, together with relevant reference strains. The patient strain was shown to be indistinguishable from seven of the hotel water isolates, but clearly different from other unrelated clinical isolates, the reference strains and some of the other environmental isolates. PyMS is a rapid and cheap method for inter-strain comparison for *L. pneumophila*.

INTRODUCTION

The epidemiology of outbreaks of Legionnaires' disease has been extensively studied. Whilst *Legionella pneumophila* can be isolated from many environmental samples, including some water supplies, it has often been difficult to prove a causal relationship between any one source and clinical isolates when an outbreak has occurred.

A number of typing schemes have been described for *L. pneumophila*. Monoclonal antibody (Mab) approaches subdivide serogroup 1 into subgroups [1] but these are still insufficiently discriminatory to trace individual strains within those groups. Although it is claimed that the recently described restriction fragment length polymorphisms (RFLP) is able to match strains to isolates [2] this is very expensive to perform and requires specialized facilities.

It has been suggested that pyrolysis mass spectrometry (PyMS) might discriminate between strains of *L. pneumophila* [3]. In the present study PyMS was used for the inter-strain comparison of a clinical isolate of *L. pneumophila* serogroup 1 with various environmental strains thought to be epidemiologically linked to the case.

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MATERIAL AND METHODS

Case history

An 83-year-old man developed Legionnaires' disease 3 days after returning home having stayed at an hotel in another city for 4 nights. He was admitted to hospital and a sputum sample obtained on the sixth day of illness yielded *L. pneumophila* serogroup 1. The patient was treated with erythromycin and made an uneventful recovery.

Following notification of the case, three 5 l water samples were obtained from the room which the patient had occupied in the relevant hotel. The samples from the shower and the cold water supply were both negative for legionellae, but the hot water system (temperature 41 °C) yielded *L. pneumophila* following concentration by filtration on both acid-treated [4] and heat-treated samples [5] with viable counts of $1{\cdot}2 \times 10^4$ c.f.u./ml and $1{\cdot}4 \times 10^4$ c.f.u./ml respectively. Five discrete colonies from the acid- and six from the heat-treatment cultures were purified and preserved separately.

All these environmental isolates were sent to PHLS Legionella Reference Unit, Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory, Colindale, London for conventional typing, as was the patient's strain, a duplicate of which was kindly sent to us by Dr R. J. Fallon, Ruchill Hospital, Glasgow. Preliminary typing results suggested that all the strains involved were *L. pneumophila* serogroup 1 Benidorm.

Pyrolysis mass spectrometry (PyMS)

The clinical isolate of *L. pneumophila* Sgp 1 Benidorm, the 11 isolates from the hotel water supply, 6 unrelated clinical isolates and 6 unrelated environmental isolates of the same subgroup (kindly supplied by Dr T. G. Harrison, CPHL, Colindale) were investigated by PyMS, together with the following reference strains of *L. pneumophila*: NCTC 12025 Sgp 1 Heysham; NCTC 12024 Sgp 1 Allentown; and NCTC 12006 Sgp 1 Benidorm O30E.

All the strains were subcultured onto a single batch of supplemented BCYE agar (Oxoid) and incubated at 36·5 °C for 48 h. Two samples of each strain, allocated PyMS numbers as shown in Table 1, were pyrolysed in triplicate on a Horizon Instruments PYMS 200X pyrolysis mass spectrometer (Horizon Instruments Ltd, Heathfield, Sussex, UK). The resulting spectrograms were used as the basis for inter-strain comparisons using the techniques and principles previously described [6].

PyMS data analysis

After iterative normalization, the replicate spectra of each subculture were labelled as distinct groups and analysed for within-group and between-group variation. The 30 mass ion peaks showing the greatest discrimination between groups were subjected to principal component (PC) and canonical variate (CV) analyses, as in previous reports [7, 8]. Two separate data sets were created, one of the odd PyMS numbers and the other of the even numbers. Thus, duplicate results were available for each strain. Analysing each data set separately, isolates that could be distinguished from a cluster of strains were identified as outliers on the

Table 1. *The strains of* L. pneumophila* *pyrolysed*

PyMS nos.	Strain no./name	Source
1, 2	LC1331	Random environmental
3, 4	LC1078a	Random environmental
5, 6	LC1050	Random environmental
7, 8	LC1069	Random environmental
9, 10	LC820a	Random environmental
11, 12	LC1237	Random clinical
13, 14	LC1235	Random clinical
15, 16	LC1052	Random clinical
17, 18	LC1127	Random clinical
19, 20	LC1051a	Random clinical
21, 22	LC1234	Random clinical
23, 24	M.A.	Patient isolate
25, 26	Acid A	Hotel water
27, 28	Acid B	Hotel water
29, 30	Acid C	Hotel water
31, 32	Acid D	Hotel water
33, 34	Acid E	Hotel water
35, 36	Heat A	Hotel water
37, 38	Heat B	Hotel water
39, 40	Heat C	Hotel water
41, 42	Heat D	Hotel water
43, 44	Heat E	Hotel water
45, 46	Heat F	Hotel water
47, 48	NCTC 12006	Reference strain O30E
49, 50	LC1185	Random environmental
51, 52	NCTC 12024	*L. pneumophila* Serogroup 1 Allentown
53, 54	NCTC 12025	*L. pneumophila* Serogroup 1 Heysham 1

* All strains were *L. pneumophila* serogroup 1 Benidorm unless otherwise stated.

ordination diagram (combined PCCV1 *v.* PCCV2) if their triplicate points did not overlap any from strains within the cluster. On sequential analyses these outliers were edited out of each data set until ordination diagrams showed that few, if any, triplicates overlapped. At this stage, any strain in which both subcultures had been differentiated from the remainder was removed from the total (combined) data set. The remaining strains were reanalysed using the data from both subcultures in a single combined data set, so that a measure of the reproducibility of the technique could be obtained. Thereafter, outlying strains were identified as those in which the duplicate triangles on the ordination diagram were quite separate from a cluster. The end point was a number of strains in which the groups resulting from the triplicate analyses of separate subcultures of the same strain were at least as different one from the other as those of different strains and which formed one or more discrete clusters on the ordination diagrams.

RESULTS

In the initial stages of the analysis the separate subcultures of the following strains were differentiated in both data sets: the 3 reference strains, all 6 unrelated clinical isolates and 3 of the 6 unrelated environmental strains, together with 1

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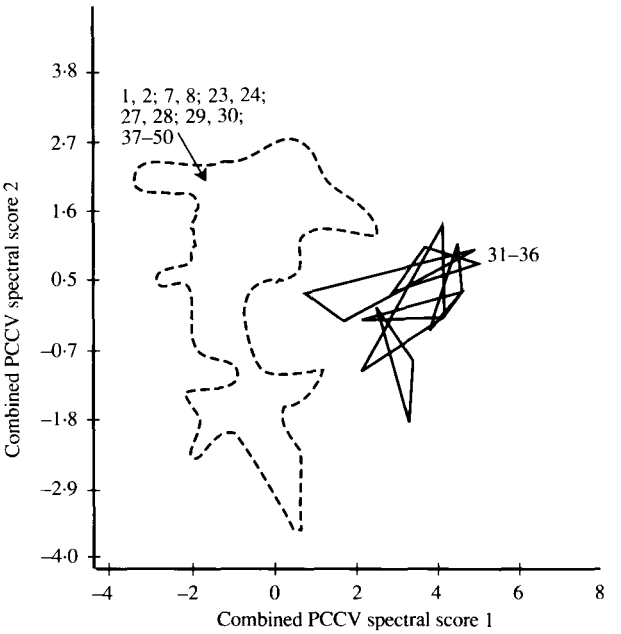

Fig. 1. Ordination diagram of spectral data of 14 strains of *L. pneumophila* Sgp 1 Benidorm. The axes represent the first two canonical discrete functions. The results of triplicate analysis of the same subculture have been joined together. The area delineated by the broken line encompasses all the data from the indicated strains.

Table 2. *Summary of strains of* L. pneumophila *serogroup 1 Benidorm indistinguishable by PyMS*

PyMS nos.	Strain no./name	Source
1, 2	LC1331	Random environmental
7, 8	LC1069	Random environmental
23, 24	M.A.	Patient isolate
27, 28	Acid B	Hotel water
29, 30	Acid C	Hotel water
37, 38	Heat B	Hotel water
39, 40	Heat C	Hotel water
41, 42	Heat D	Hotel water
43, 44	Heat E	Hotel water
45, 46	Heat F	Hotel water
49, 50	LC1185	Random environmental

isolate from the suspect water supply. When these strains had been edited from the total data set, the first analysis of the remaining 14 strains in duplicate revealed two clusters (Fig. 1). A small group of three strains was quite separate from the main cluster. Two of these had been isolated from the hotel's water supply after acid treatment and the other after heat treatment of the same sample.

The large cluster of 11 strains could not be differentiated further by PyMS and were considered to be very closely related (Table 2).

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DISCUSSION

PyMS demonstrated that the patient strain of *L. pneumophila* was indistinguishable from 7 of the isolates from the epidemiologically-incriminated hot water supply of the hotel. In addition, 3 unrelated environmental strains of *L. pneumophila* Sgp 1 proved to be very similar. In contrast, the patient's isolate was clearly different from the 6 unrelated clinical isolates, 3 unrelated environmental isolates, 1 of the isolates from the hotel's water supply and the reference strain of *L. pneumophila* Sgp 1 Benidorm, as well as 2 reference strains of other Mab subgroups of the same serogroup.

It is of interest that three of the isolates from the incriminated hotel water supply were not as closely related to the patient strain as the other seven and formed a distinct cluster on the final ordination diagram. Isolates from a single water sample with a uniform genotype on RFLP analysis may show considerable phenotypic variation [9]. The RFLP results are not available as yet for our strains but it will be interesting to compare these with the PyMS assessment of inter-strain relatedness.

It has been suggested that acid- and heat treatment of water samples may alter the phenotypic characteristics of legionellae [10] and repeated subculture is known to stabilize the Mab phenotypes of some strains of *L. pneumophila* [2]. The effects of acid- and heat treatments and repeated subcultures on the PyMS characterization of our strains of *L. pneumophila* are being studied currently. Meanwhile, we endorse the recommendation [9] that it is essential to preserve a representative number of colonies of legionellae from any water sample that yields this organism.

PyMS is a quick and reliable method of inter-strain comparison for many species, including streptococci [11], salmonellae [7] and staphylococci [8] and now, *L. pneumophila*, although the method does not yet offer formal type designations. To our knowledge this is the first occasion that PyMS has been used to trace an environmental source of clinical disease. The speed and reproducibility of the technique and its applicability to a wide range of different bacterial species make it an attractive method for microbial epidemiological studies.

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REFERENCES

1. Joly JR, McKinney RM, Tobin JO, Bibb WF, Watkins ID, Ramsay D. Development of a standardized subgrouping scheme for *Legionella pneumophila* serogroup 1 using monoclonal antibodies. J Clin Microbiol 1986; **23**: 768–71.
2. Saunders NA, Harrison TG, Haththotuwa A, Kachwalla N, Taylor AG. A method for typing strains of *Legionella pneumophila* serogroup 1 by analysis of restriction fragment length polymorphisms. J Med Microbiol 1990; **31**: 45–55.
3. Kajioka R, Tang PW. Curie-point pyrolysis mass-spectrometry of *Legionella* species. J Anal Appl Pyrolysis 1984; **6**: 59–60.
4. Bopp CE, Sumner JW, Morris GK, Wells JE. Isolation of *Legionella* spp. from environmental water samples by low pH treatment and use of a selective medium. J Clin Microbiol 1981; **13**: 614–719.

5. Dennis PJ, Green D, Jones BPC. A note on the temperature tolerance of *Legionella*. J Appl Bacteriol 1984; **56**: 349–50.
6. Aries RE, Gutteridge CS, Ottley TW. Evaluation of a low-cost automated pyrolysis mass spectrometer. J Anal Appl Pyrolysis 1986; **9**: 81–98.
7. Freeman R, Goodfellow M, Gould FK, Hudson SJ, Lightfoot NF. Pyrolysis-mass spectrometry (Py-MS) for the rapid epidemiological typing of clinically significant bacterial pathogens. J Med Microbiol 1990; **32**: 283–6.
8. Freeman R, Gould FK, Wilkinson R, Ward AC, Lightfoot NF, Sisson PR. Rapid inter-strain comparison by pyrolysis mass spectrometry of coagulase negative staphylococci from persistent CAPD peritonitis. Epidemiol Infect. In press.
9. Harrison TG, Saunders NA, Haththotuwa A, Hallas G, Birtles RJ, Taylor AG. Phenotypic variation amongst genotypically homogeneous *Legionella pneumophila* serogroup 1 isolates: implications for the investigation of outbreaks of Legionnaires' disease. Epidemiol Infect 1990; **104**: 171–80.
10. Colbourne JS, Dennis PJ, Trew RM, Berry C, Vesey G. Legionella and public water supplies. Water Sci Technol 1988; **20**: 5–10.
11. Magee JT, Hindmarch JM, Burnett IA, Pease A. Epidemiological typing of *Streptococcus pyogenes* by pyrolysis mass spectrometry. J Med Microbiol 1989; **30**: 273–8.