Evaluation of sensitivity of two serological tests for diagnosing pneumonia caused by *Legionella pneumophila* serogroup 1

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SUMMARY The sensitivity of an indirect immunofluorescence antibody test (IFAT) and a rapid microagglutination test (RMAT) for the diagnosis of Legionnaires' disease caused by *Legionella pneumophila* serogroup 1 was evaluated using serum specimens from 119 patients with bacteriologically confirmed infections. The sensitivity of both assays was found to be about 80%. In addition, antibody titres suggestive of *L pneumophila* infection were found in 40% of patients in the first week after admission to hospital. These data show that both assays can be used with confidence in the early diagnosis of Legionnaires' disease.

We have previously described two assays for the serodiagnosis of Legionnaires' disease caused by *Legionella pneumophila* serogroup 1. These are an indirect immunofluorescence antibody test (IFAT) using formalised yolk sac antigen prepared from the Pontiac strain of *L pneumophila*¹ and a semiautomatable rapid microagglutination test (RMAT) using a formalin killed suspension of bacteria grown on solid media.² These assays are used routinely within the Public Health Laboratory Service and other clinical diagnostic laboratories in the United Kingdom, as well as in some similar laboratories in other European countries. Results have been published which show that these tests have good specificity and are reproducible,³ but to date the sensitivity of the assays has only been determined using sera from a small number of patients with bacteriologically confirmed Legionnaires' disease.

This study reports the results obtained by examining sera from a large series of patients in whom Legionnaires' disease had been bacteriologically confirmed. In addition to providing an accurate assessment of the sensitivity of each assay, it was intended that such a study would also show features of Legionnaires' disease which lead to problems in its serodiagnosis.

Material and methods

PATIENTS

Patients were included in the series provided that they had a pneumonic illness clinically compatible with a diagnosis of Legionnaires' disease and that such a diagnosis was confirmed either by culture of *L pneumophila* serogroup 1 from clinical specimens or by showing the organism by immunofluorescence antibody staining, and that at least one serum specimen was available for the estimation of antibody titres.

Specimens were obtained from 119 patients who fulfilled these criteria—92 from the Hôpital Claude-Bernard and 27 from the Central Public Health Laboratory (CPHL). The diagnosis had been established by culture in 103 cases, by fluorescence antibody demonstration of the organism in lower respiratory tract specimens in 15 cases, and by fluorescence antibody demonstration of the organism in sputum in one case (table 1). Where possible, the following informa-

Table 1 Evidence of infection in 119 patients with Legionnaires' disease

<i>Isolation of Legionella pneumophila serogroup 1 from:</i>	
Respiratory tract specimen	100
Necropsy lung tissue	3
<i>Fluorescence antibody demonstration of organism in either:</i>	
Lung biopsy tissue, bronchoalveolar lavage specimens, transtracheal aspirates, or formalin fixed necropsy lung tissue	15
Fluorescence antibody demonstration of organism in sputum	1

tion was obtained for each patient:

- 1 The date of onset of symptoms (this was usually provided by the patients and is therefore somewhat subjective).
- 2 The date of admission to hospital (in the case of a patient who acquired Legionnaires' disease nosocomially the date of onset was used in place of the date of admission). A case was considered to be nosocomial if the onset of symptoms occurred 12 or more days after admission to hospital for an unrelated reason.
- 3 The dates on which blood specimens were taken.
- 4 The outcome of the illness (fatal or not).
- 5 Whether the patient was immunocompromised or not. The definition of an immunocompromised patient (used in this study) was as follows: a patient with generalised malignant disease or acquired immune deficiency syndrome (AIDS), or with a clearly shown primary immune defect, or a patient who was undergoing treatment with immunosuppressive drugs with one of the following regimens: steroids ≥ 3 mg/kg/day for 15 days or more or, ≥ 2 mg/kg/day for 30 days or more; cyclosporin A (conventional regimen); cyclophosphamide (conventional regimen); azathioprine (conventional regimen); combined chemotherapy for cancer, leukaemia, or lymphoma.

SEROLOGY

The IFAT and RMAT were performed as previously described,^{1,2} with the exception that the bacteria used for preparation of the RMAT antigen were grown on buffered charcoal yeast extract agar.⁴ Patients' serum specimens were examined by IFAT in either the Hôpital Claude-Bernard, or the CPHL. A selection of sera were examined in both centres to ensure that the results obtained were equivalent. The RMAT was performed only at CPHL.

Interpretation of serological results On the basis of serological results patients were considered to be:

- (i) Serologically negative for Legionnaires' disease (if the antibody titres of all available specimens were < 16 in the IFAT and < 8 in the RMAT).
- (ii) Serologically diagnostic for Legionnaires' disease (if the criteria used previously to establish the diagnosis of Legionnaires' disease were met^{1,2}—that is, for the IFAT, a four-fold or greater rise in titre to ≥ 64 between paired sera, or a single or standing titre of ≥ 128 ; for the RMAT, a four-fold rise in titre to ≥ 16 between paired sera or a single or standing titre ≥ 32).
- (iii) Serologically positive (but not diagnostic) for Legionnaires' disease (if any specimens gave a titre of ≥ 16 in the IFAT, a titre of ≥ 8 in the RMAT, but the diagnostic criteria in (ii) were not met).

Results

EPIDEMIOLOGICAL FEATURES OF THE PATIENT POPULATION

Of the 119 patients, 89 (74.8%) were men and 30 (25.2%) were women, giving a male:female ratio of 3:1. The mean age was 52.7 years (range 2–87 years, known for 109 patients), 53.5 years for men (range 18–87 years, known for 83 of 89 patients), and 50.2 years for women (range 2–87 years, known for 26 of 30 patients).

In this series 54 of 107 (50.5%) patients were classified as immunocompromised and 53 of 107 (49.5%) were apparently not immunocompromised (table 2). The 12 remaining patients could not be classified.

Of the 119 cases, 34 were nosocomial and 75 were community acquired (10 cases could not be classified). Thirty of the 34 nosocomial cases were immunocompromised. In one case data were unavailable. The fatality rate in this series was very high as 46 of 109 (42.2%) patients died (10 patients not known). It was noted that nine of 28 (32.1%) women had a fatal outcome as opposed to 37 of 81 (46.2%) men ($\chi^2 = 1.56$, $p > 0.1$, not significant).

SENSITIVITY OF IFAT AND RMAT

Three hundred and ninety seven serum specimens were available for examination by IFAT from the 119 patients. Only one specimen was available from 22 patients. From the results obtained (table 3) it can be seen that sensitivity of the IFAT with respect to a diagnostic result was 79.0%. In addition, a further

Table 2 Underlying illness of 54 immunocompromised patients with Legionnaires' disease

Malignancies:	
Leukaemia	14 (12)
Lymphoma	6 (3/5)*
Other malignancies	7 (3/6)*
Non-malignant illnesses treated with steroids in combination with cyclophosphamide, cyclosporin A, or azathioprine:	
Renal or heart or liver transplantations	11
Systemic lupus erythematosus	1
Rheumatoid arthritis	1
Polyarteritis nodosa	2
Goodpasture's syndrome	1
Non-malignant illnesses treated with steroids alone:	
Chronic bronchitis or asthma	4
Systemic lupus erythematosus	2
Rheumatoid arthritis	1
Brain oedema	1
Not stated	1
Others	
AIDS	2

Figures in parentheses indicate No of patients treated with steroids or cytotoxic drugs.

*Data not supplied for one patient.

Table 3 Sensitivity of IFAT

Test result	No of patients (%)		
Four-fold rise	71 (59.7)	} Diagnostic	94 (79.0)
Single specimen	13 (10.9)		
Standing titre	10 (8.4)		
A two or four-fold rise to <32	5 (4.2)	} Positive but not diagnostic	12 (10.1)
Single specimen	5 (4.2)		
Standing titre	2 (1.7)		
		Negative	13 (10.9)
Total			119 (100)
Positive			106 (89.1)

10.1% of specimens gave positive but not diagnostic results. The remaining 10.9% of specimens gave negative results.

Specimens were not available from four of 119 patients for examination in the RMAT. Thus 376 serum specimens from 115 patients were tested. The sensitivity of the RMAT, with respect to a diagnostic result, was 81.7%; a further 7.0% of the results were positive but not diagnostic; and 11.3% were negative (table 4).

If the IFAT and RMAT results are considered together diagnostic titres were obtained in 86.9%; positive but not diagnostic titres in 4.4%; and negative titres in 8.7% of the 115 cases (table 5).

Specimens from 16 of the 119 patients gave negative results in one or both assays. Three were positive (two diagnostic) by IFAT and negative by RMAT, while one was diagnostic by RMAT and negative by IFAT. Ten cases were negative in both assays and two were IFAT negative and not tested in the RMAT. Thus of the 115 patients tested in both assays, the RMAT and IFAT showed concordance in 111 (96.5%) cases.

TIMING OF THE ANTIBODY RESPONSE

The dates of both onset of illness and collection of the first serum specimen were known for 100 patients, and the average interval of time between these was 7.3 days (range one to 28). Similarly, the average time between admission and collection of the first serum

Table 4 Sensitivity of RMAT

Test result	No of patients (%)		
Four-fold rise	72 (62.6)	} Diagnostic	94 (81.7)
Single specimen	13 (11.3)		
Standing titre	9 (7.8)		
Two-fold rise	2 (1.8)	} Positive but not diagnostic	8 (7.0)
Single specimen	3 (2.6)		
Standing titre	3 (2.6)		
		Negative	13 (11.3)
Total			115 (100)
Positive			102 (88.7)

Table 5 Sensitivity obtained using combination of IFAT and RMAT

Test result	No of patients (%)		
Four-fold rise	82 (71.3)	} Diagnostic	100 (86.9)
Single specimen	15 (13.0)		
Standing titre	3 (2.6)		
Two-fold rise	1 (0.8)	} Positive but not diagnostic	5 (4.4)
Single specimen	2 (1.8)		
Standing titre	2 (1.8)		
		Negative	10 (8.7)
Total			115 (100)
Positive			105 (91.3)

specimen was four and a half days (range 0–25, 101 known). Seventy seven of these specimens were taken in the first week zero to six days after admission and 31 (40%) of these were positive in the IFAT, including nine of the 29 taken within 48 hours of admission (zero to one day). Sixty nine specimens taken in the first week were also examined in the RMAT and 31 (45%) were positive, including nine of the 27 taken within 48 hours. Although most of these early positive results fell into the category of "positive but not diagnostic," a significant number were diagnostic—16% and 23% for the IFAT and RMAT, respectively.

FACTORS AFFECTING THE ANTIBODY RESPONSE

The data were analysed to see if any epidemiological features correlated with a particular result being obtained in the serological assays (for example, were serum specimens from older patients more likely to be negative than those from younger patients?).

Patients were banded by age and the proportions of positive and negative results in each category compared. No significant differences were found. Similarly, analyses according to the sex of patient and whether they were immunocompromised or not showed no significant differences.

The outcome of illness was known for 109 of the 119 patients. Of the 46 patients who died, negative IFAT results were obtained in 10 instances, while only three negative results were obtained with specimens from 63 patients who survived. This is a significant difference (χ^2 Yate's correction = 6.15, $p < 0.05$).

A possible explanation for the difference in outcome might be that the only specimens available from the patients who died were collected soon after onset of Legionnaires' disease, before antibody was present at detectable titres. To determine whether this was the case the following analyses were performed:

1 The timing of death in relation to onset of illness was compared for seronegative and seropositive patients. The mean number of days between onset and death was 12.7 days (range four to 30, $n = 8$) for

IFAT seronegative patients and 15.5 days (range five to 31, $n = 19$) for IFAT seropositive patients.

2 The number of days between onset and the collection of last available specimen from IFAT seronegative patients who died was compared with the time from onset to the collection of the first serum specimen to give a positive result from IFAT seropositive patients who died. The mean for seronegative patients was 10.2 days (range four to 20, $n = 9$) and was 11.5 days (range four to 35, $n = 30$) for seropositive patients.

3 The number of days between onset and the last available serum specimen (taken within a month of onset) from seronegative patients (irrespective of the outcome of illness) was compared with the time from onset to the collection of the first serum specimen to give a positive IFAT result from seropositive patients (irrespective of the outcome of illness). The mean intervals were 9.6 days (range three to 20, $n = 11$) and 12.0 days (range three to 35, $n = 89$), respectively.

None of the differences observed between the groups approached significance, but as some of the groups contained only a few patients it is hard to draw any conclusions from these results.

Discussion

If a serological assay is to provide results helpful in the diagnosis of a patient's illness it must be rigorously evaluated. Variables such as the test sensitivity and specificity must be determined and from these the predictive values calculated.⁵

The most important test variable in the case of an illness of low prevalence, such as Legionnaires' disease, is the specificity. This has been determined previously for both the IFAT and the RMAT, as used in this study, with regard to infections caused by *L pneumophila* serogroup 1.³ It was shown that the specificity was excellent for both assays being 100% for a diagnostic result in the IFAT and 99.6% for the RMAT. In addition, it was shown that both the IFAT and RMAT were highly specific (about 97%) even with respect to positive, but not necessarily diagnostic, results.

The high specificity of these two assays indicates that considerable confidence can be placed in the validity of the diagnosis where specimens from a patient give serodiagnostic results. Unless the sensitivity has been determined, however, the percentage of patients who have Legionnaires' disease but fail to give serodiagnostic results will be unknown.

Although many serological assays have been described for the diagnosis of Legionnaires' disease, few have been adequately evaluated. Where attempts have been made to determine the sensitivity, these

have often been of limited value as the serum specimens examined were from patients whose illness had been diagnosed using another poorly evaluated serological test.⁶

Wilkinson *et al*⁷ evaluated an IFAT that uses a different type of antigen and different diagnostic criteria from those used in our IFAT: sera from patients from six well documented outbreaks of legionellosis were examined. This use of epidemic sera provided an alternative source of evidence for the patients' illness and this epidemiological approach was the best method available at the time. Inevitably the estimate of sensitivity was biased as the definitions of a case of Legionnaires' disease used in such epidemiological studies often included the consideration of serological results.

In the study presented here the estimate of the sensitivity of the IFAT and RMAT were determined using sera from 119 patients in whom Legionnaires' disease was diagnosed either by culture or demonstration of the organism in patients' specimens, or both of these methods. The results show that the IFAT has a sensitivity of 79.0% and the RMAT 81.7% with respect to a diagnostic result. From these results the predictive value of a diagnostic result in the RMAT is calculated to be between 67 and 91%, assuming the prevalence of Legionnaires' disease caused by *L pneumophila* serogroup 1 is 1–5% of patients with pneumonia. As the specificity of the IFAT is calculated as 100% the predictive value of a positive result is also 100%, irrespective of the sensitivity.⁵

In the case of both assays, although specimens from about 20% of the patients failed to give diagnostic results, positive titres were shown in about half (10% of the total) of these. While these results are not conclusive, they can be good indicators of a case of Legionnaires' disease (table 5). Where a combination of both the IFAT and RMAT were used, the sensitivity of a diagnostic result rose from 79% and 81.7%, respectively, to 86.9%. This increase is accounted for largely by a decrease in the percentage of positive (but not diagnostic) results. The reliability of a diagnosis based on such positive results is affected by the prevalence of Legionnaires' disease in the population being examined. If the prevalence of Legionnaires' disease is 1–5% the predictive value of any positive IFAT or RMAT result is 23–60%.

The concordance seen between the IFAT and RMAT was very high (96.5%), but discrepant results were seen in four instances. In one of these (case 1, table 6) one specimen was taken five days after onset of symptoms. This specimen had an IFAT titre of 128 but was negative in the RMAT. Subsequent analysis showed the specific antibody to be only of the IgG immunoglobulin class. This probably accounts for the discrepant results—being an agglutination assay, the

Table 6 Summary of patients whose sera gave negative results in either IFAT or RMAT

Case No	Fatal*	IFAT	RMAT	Days specimen taken†
1	Yes (not known)	Diagnostic	Negative	5 (IgG only)
2	No	Diagnostic	Negative	1, 2, 7, 31
3	Yes (23)	Positive	Negative	12, 15
4	No	Negative	Diagnostic	3, 10, 218
5	Yes (4)	Negative	Negative	3
6	Yes (15)	Negative	Negative	3, 8
7	Yes (6)	Negative	Negative	4
8	Yes (12)	Negative	Negative	5
9	Yes (11)	Negative	Negative	7
10	Yes (30)	Negative	Not tested	8, 9, 10, 13, 16
11	Yes (11)	Negative	Negative	10
12	No	Negative	Negative	3, 37
13	Yes (9)	Negative	Negative	≤ 9
14	Not known	Negative	Not tested	Not known
15	Yes (not known)	Negative	Negative	11, 20
16	Yes (22)	Negative	Negative	10, 19

*Figures in parentheses indicate the number of days after the onset of symptoms when the patient died.

†Days after onset.

RMAT is inefficient at detecting this class of antibody.⁸

Of particular interest are the specimens from case 4 (table 6). A diagnostic rise between the first specimen (three days after onset) and the second specimen (10 days) was shown by RMAT. These sera were negative by IFAT, and a third specimen (218 days) was negative in both assays. It is possible that had an appropriately timed third specimen been available a seroconversion would have been shown by IFAT, but this result does indicate that a RMAT diagnostic IFAT negative result is not necessarily a false positive result, as had been assumed previously.²

Specimens from 10 patients were negative in both assays. Attempts were made to determine any features which distinguished this group from the other patients. The seronegative patients did not differ significantly in age, sex, and in whether they were immunocompromised or not from the remaining patients. It was found, however, that patients who died were more likely to have been seronegative than patients who survived. One possible explanation for this observation could be that appropriately timed serum specimens were not available from some of the patients with a fatal outcome. Although analysis of the data presented here does not support this explanation, it should not be discounted as the numbers of patients in the seronegative group were small and hence only major differences in the timing of serum specimens would have been detected.

It can be seen from the epidemiological data that the series of patients selected was a biased sample from the general population of patients with Legionnaires' disease. Although the age range and male:female ratios were typical,⁹ the mortality was considerably higher than usual.⁹ Over 50% of the patients were also immunocompromised. This,

together with the fact that 67% (of those reported) required mechanical ventilation, suggests that the patients in this population were more severely ill than is typical in Legionnaires' disease. This is not surprising as it is probable that a severely ill patient will be investigated with greater rapidity and more intensively than those with milder disease, and hence *L. pneumophila* will be isolated more often.

As discussed above, patients who died were more often seronegative than those who survived. As the percentage mortality in this series of patients exceeded that which would usually be expected, it may be that the percentage of seronegative patients was also excessive. Thus the sensitivities calculated here may be underestimates of the true values.

This study was not undertaken to investigate the timing of the antibody response in legionella infections, but the data presented illustrate an important point. In both assays about a third of the specimens taken from patients within 48 hours of admission to hospital were positive, and within a week of admission this figure had risen to over 40%. This clearly shows that when appropriate specimens are taken an early indication of a legionella infection can be obtained.

Conflicting results have been reported on the value of serology, especially in patient management in cases of Legionnaires' disease. There are two main explanations for this. Firstly, different techniques and antigen preparations have been used. Secondly, serological criteria established for the diagnosis of Legionnaires' disease caused by *L. pneumophila* serogroup 1 have been extended to the many other serogroups and species now known, without any real assessment of the specificity or sensitivity of the serological tests using these different antigens.

We have previously shown that the IFAT using for-

molised yolk sac antigen and the RMAT are highly specific. We have now shown that these tests have a similar sensitivity of about 80%, even in very severe cases and in immunocompromised patients. Overall, the sensitivity and specificity of these assays are such that they can be used with confidence in the early diagnosis of Legionnaires' disease. In our hands about 40% of patients with Legionnaires' disease confirmed by culture caused by *L pneumophila* serogroup 1 do have antibodies likely to indicate such an infection during the first week after admission to hospital.

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