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Value of Serological Testing for Diagnosis of Legionellosis in Outbreak Patients

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Serum antibody detection tests and a urine antigen detection technique were compared in samples from 116 patients epidemiologically characterized as belonging to a legionellosis outbreak. Sera were tested by enzyme-linked immunosorbent assays (ELISAs) for immunoglobulin M (IgM) and IgG plus IgM and by immunofluorescent assays (IFAs) for IgG, IgM, IgA, and polyimmunoglobulin using commercial kits (Vircell); concentrated urines were tested with the Binax NOW *Legionella* test. ELISA for IgM, ELISA for IgG plus IgM, antigenuria detection, and IFA for IgM were able to diagnose 72.3%, 60.5%, 53.3%, and 51.4%, respectively, of patients. Antigenuria was present in 53.8% of first samples, ELISA detected IgM in 29.7%, ELISA detected IgG plus IgM in 7.9%, and IFA detected IgM in 3.9%. Ten antigenuria-negative first samples tested serologically positive, 9 of them to IgM by ELISA. Despite the single source of the samples included in the study, detection of IgM using a sensitive technique such as ELISA seems to be a suitable complement to antigenuria detection for the diagnosis of legionellosis.

Legionellosis classically has two distinct clinical presentations: Legionnaires' disease, a severe form of pneumonia clinically indistinguishable from other types of pneumonia, and Pontiac fever, a flulike illness. Most cases of legionellosis are caused by *Legionella pneumophila* serogroup 1 (7, 21).

Direct methods of diagnosis include culturing, direct fluorescent staining, and antigen detection in urine. While the first two methods display low and variable sensitivities (7), the latter has become a reference technique in most laboratories, enabling easy and early diagnosis of legionellosis (3, 10, 20). Indirect immunofluorescence is the most common method for serological diagnosis. Although serology yields good sensitivity and specificity data (1, 11, 23, 24), the delay in the development of a measurable antibody response constitutes a major drawback for diagnosis in the acute patient (5). Immunoglobulin M (IgM) detection is widely used in infectious serology, since IgM appears earlier in the course of a disease; however, despite its reported validity for the diagnosis of legionellosis (2, 6, 19, 27), its use is not widespread and some authors consider it of limited value (15, 20). The enzyme-linked immunosorbent assay (ELISA) technique, which generally shows higher sensitivity and better characteristics in terms of both automation and objective measurement than immunofluorescence does, has not been thoroughly studied for the detection of IgM antibodies in legionellosis.

This work presents a comparison of serological tests for antibody detection (ELISA and immunofluorescence) and an antigen detection technique in urine, using samples from 116 patients epidemiologically characterized as belonging to a legionellosis outbreak.

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MATERIALS AND METHODS

Patients. Sera (208) and urine samples (107) from 116 patients were included in the study. First samples, taken at hospital admission, were available from 95 patients and were collected within 1 week after the onset of symptoms. All patients belonged to the Legionnaires' disease outbreak that occurred in July 2001 in Murcia, Spain, which was caused by an *L. pneumophila* serogroup 1 strain and has been described elsewhere (8). The association of all patients with this outbreak was confirmed by the Epidemiology Service of the Regional Health Council of Murcia on the basis of epidemiological studies, including a review of the clinical history and an epidemiological questionnaire for each patient. All patients were diagnosed with pneumonia, presenting new infiltrates upon examination by chest radiology, together with one or more of the following clinical signs and symptoms: nonproductive cough, arthromyalgia, vomiting, diarrhea, confusion, headache, high fever (above 39°C), and hyponatremia. Patients with antecedents of admission to hospital during the 6 weeks previous to the onset of symptoms were excluded. No cases of Pontiac disease were recorded: each patient showed one or more chest infiltrates together with a respiratory syndrome. Age and sex distributions of patients were as follows: 90 were male and 26 were female, with ages between 23 and 86 years (mean age, 60.4). Eighty patients were hospitalized, and 36 were treated as outpatients. The study also included 400 blood donor sera from a geographical area where no legionellosis outbreak had been reported in recent years.

Direct test. Legionella antigen was detected in urine using the Binax NOW *Legionella* Urinary Antigen Test (Binax Inc.) following the manufacturer's instructions and with modifications previously described (4): samples were heated for 5 min in a bath at 100°C, centrifuged at 3,000 rpm for 10 min, and concentrated 25 to 50 times by ultrafiltration (Minicon B15; Millipore) before testing.

Serological tests. Two commercial ELISA kits, a *Legionella pneumophila* serogroup 1 ELISA kit for IgM and a *Legionella pneumophila* serogroup 1 to 6 ELISA kit for IgG plus IgM (both from Vircell S. L., Granada, Spain), were used according to the manufacturer's protocol. The kits include microplates coated with lipopolysaccharide from *Legionella pneumophila* either from serogroup 1 or from serogroups 1 to 6. For IgM testing, IgG was removed by treatment with sorbent directly into the well. Bound antibodies were revealed with either an IgM-specific or an IgG-plus-IgM-specific peroxidase conjugate. These assays use single 1/20 dilutions of sera and include cutoff calibrators to score samples as negative or positive (with 10% gray zones above and below the cutoffs). Samples with uncertain results were retested: if such a sample produced the same result, it was scored as uncertain.

Immunofluorescent assays (IFA) were performed with the following commercial kits according to the manufacturer's instructions: *Legionella pneumophila* IFA for IgM (Vircell S. L.) and *Legionella pneumophila* IFA for IgG (Vircell

TABLE 1. Results with different techniques for legionellosis in 116 patients

Result	No. of patients						
	Antigenuria detection	ELISA for IgM	ELISA for IgG + IgM	IFA for IgM	IFA for IgG	IFA for IgA	IFA for poly-immunoglobulin
Positive	57	81	66	54	45	19	54
Negative	50	31	55	51	26	36	18
Uncertain		2	3	1		1	
Not done	9	2	2	10	45	60	44
% Sensitivity (95% confidence interval)	53.3 (43.4–63.0)	72.3 (63.1–80.1)	60.5 (45.2–63.6)	51.4 (41.4–61.3)	63.4 (51.1–74.5)	34.5 (22.2–48.6)	75.0 (63.4–84.5)

S. L.). IgA and polyimmunoglobulin IFAs were carried out on commercial *Legionella pneumophila* IFA slides (Vircell S. L.) with commercial fluorescein isothiocyanate-labeled IgA-specific or IgA-plus-IgG-plus-IgM-specific conjugates (Sigma, St. Louis, Mo.). Briefly, serum dilutions were reacted onto acetone-fixed *Legionella pneumophila* serogroup 1 (Philadelphia strain) for 30 (IgG and polyimmunoglobulin) or 90 (IgM and IgA) minutes. Interferences from IgG and rheumatoid factor were avoided by sorbent treatment in the IgM and IgA determinations. Fluorescein isothiocyanate-specific conjugates were incubated for 30 min. IgG or polyimmunoglobulin titers of ≥1/256 and IgM or IgA titers of ≥1/96, as well as titers exhibiting seroconversion (fourfold rise in titer), were considered positive.

Statistical analysis. The significance of the discordance between the assays was measured by McNemar's test with Yates' correction with the help of a DAG_Stat spreadsheet (18). Differences between population groups were analyzed by the chi-square test.

RESULTS

Comparison of techniques for patient classification. The ability of each test to correctly score patient status is shown in Table 1. IgG, IgA, and polyimmunoglobulin IFAs are shown as not done when a second serum sample, taken 4 weeks after the first in order to study seroconversion, was not available. Calculated sensitivities [number positive/(number positive + number negative)] are shown in the table. Serological tests, except IgM and IgA IFA, showed greater sensitivities for patient classification than did antigenuria. Thirty-five patients testing negative for antigenuria were positive by serological tests. It should be noted that many patients could not be evaluated for IgG, polyimmunoglobulin, and IgA due to the lack of suitable second samples as mentioned above. As a whole, 97 out of 116 patients (83.6%; 95% confidence interval, 75.6% to 89.8%) were positive by one or more tests used in this study. The antigenuria test and ELISA for IgM together scored 94 positive patients. Table 2 compares results for antigen detection with serological test results: tests with large numbers of nonevaluable cases are not included. Only IgM ELISA showed a significant ($P < 0.01$) difference with antigenuria detection.

No differences between sex groups were observed in the assays. The proportion of patients showing positive results for IgM by ELISA was significantly higher ($P < 0.001$ by the chi-square test) in patients <65 years old (90.9% positive patients) than in patients ≥65 years old (48.1% positive patients). The difference from the antigenuria test (75.7% positive for <65-year-old patients versus 51.9% positive for ≥65-year-old patients) was not significant ($P > 0.05$). Yet, while both outpatients and hospitalized patients presented the same proportion of positive results for IgM by ELISA (73.5% versus 71.8%), antigenuria-positive results were significantly more frequent ($P < 0.001$ in a chi-square test) in hospitalized patients (62.5%) than in outpatients (25.9%).

Comparison of techniques in first samples. Results for antigenuria detection and serological testing in the first samples from 95 patients are shown in Table 3. Antigen detection in urine samples displayed the greatest sensitivity (53.8%). Ten antigenuria-negative samples tested serologically positive: of these, nine presented IgM levels detectable by ELISA for IgM. The results for these samples are shown in Table 4: seroconversion (by any of the IFAs) was demonstrated in six of the seven patients of this group for whom a second sample (taken more than 4 weeks later) was available. Of the serological tests, ELISA for IgM proved the most sensitive (29.7%), although it detected a significantly lower proportion of positive first samples than samples positive for antigenuria (Table 5).

Evaluation of specificity of ELISA for IgM in blood donor samples. In blood donor samples, IgM ELISA scored 11 positive and 1 uncertain result out of 400 sera, showing a specificity of 97.0%.

TABLE 2. Comparison of serological tests versus antigenuria detection for diagnosis of legionellosis in 116 patients[a]

Antigenuria status	No. of patients								
	ELISA for IgM			ELISA for IgG plus IgM			IFA for IgM		
	+	−	ND	+	−	ND	+	−	ND
+	44	11	2	40	14	3	36	17	4
−	31	17	2	23	25	2	15	29	6
ND	6	3	0	3	6	0	3	5	1

[a] ND, not done or uncertain. *P* values were calculated by McNemar's test with Yates' correction and were as follows: for ELISA for IgM, 0.0034; for ELISA for IgG plus IgM, 0.1884; and for IFA for IgM, 0.8597.

DISCUSSION

Although culturing remains the gold standard for the diagnosis of legionellosis, its sensitivity may be limited in clinical routine laboratories (7, 20). In our work, legionellae were isolated in only 8 out of 46 cultured samples (data not shown). Over recent years, antigenuria detection has emerged as a reference method for the diagnosis of Legionnaires' disease (12), especially for that caused by *L. pneumophila* serogroup 1, since it enables early diagnosis: antigen has been detected in urine as soon as 1 day after the onset of symptoms (14). This method has been reported to show very good sensitivity values

TABLE 3. Results for legionellosis diagnosis in first samples from 95 patients

Result	No. of patients						
	Antigenuria detection	ELISA for IgM	ELISA for IgG plus IgM	IFA for IgM	IFA for IgG	IFA for IgA	IFA for polyimmunoglobin
Positive	49	22	6	3	1	1	0
Negative	42	52	69	73	75	74	76
Uncertain		1				1	
Not done	4	20	20	19	19	19	19
Positive proportion (%)	53.8	29.7	7.9	3.9	1.3	1.3	0

in the detection of serogroup 1 infections (10, 13, 17). In the present study, antigen detection was by far the most sensitive technique for detecting legionellosis in first samples drawn at patient admission; its sensitivity was slightly over 50%, thus broadly agreeing with recent surveys reporting a sensitivity of 60 to 70% (16, 22, 26). In a previous study of samples from different patients belonging to the same outbreak, a sensitivity of 69.6% was recorded for the Binax NOW antigenuria test performed on concentrated samples (9). This higher sensitivity value is probably attributable to the inclusion only of samples from patients with positive diagnoses of legionellosis by serology and/or culturing; a positive correlation between test sensitivity and clinical severity has been documented (26). In our study, the proportion of antigenuria-positive results was significantly lower in outpatients, plausibly corresponding to less-severe cases, than in hospitalized patients. The election of an epidemiological criterion to select patients in the present study probably shows a broader clinical spectrum of patients, with less-severe cases included in the analysis, although each of them presented a pneumonia syndrome.

Among serological tests, only IgM detection by ELISA presented a sensitivity (30%) worth mentioning in first samples. The value of IgM detection as an early serological marker of Legionnaires' disease has been reported previously (2, 6, 27); however, these studies made no comparisons with antigenuria detection. The fact that the ELISA technique showed greater sensitivity than the immunofluorescence assay was unsurprising, having already been reported in previous comparisons (23, 25). The difference in sensitivities between the two techniques is particularly marked in first samples, where IgM levels are still too low to be detected by a less sensitive technique such as IFA. Although IgM detection in these samples was significantly less sensitive than in the antigenuria test, it should be noted that nine patients with negative results in first urine samples displayed positive IgM levels with the ELISA. In order to ensure the specificity of this test, a survey was carried out on 400 blood donor samples; the ELISA for IgM yielded a specificity of 97%. Consequently, a positive result in the ELISA for IgM should be considered as presumptive: confirmation by highly specific antigenuria detection (20) or seroconversion would be advisable. The proportion of IgM-positive results was significantly lower in the group of older patients, probably reflecting the natural decline of the immune system in the elderly, since the direct antigen detection method did not show such a difference.

For the patient series as a whole, serological techniques—with the exception of the IFA for IgA—displayed greater sensitivity than antigen detection; 35 patients would have been scored as negative if serological testing had not been carried out. In a recent study, sensitivities of 66% and 92.5% were reported for antigen detection and IFA, respectively (16). Taking into account the large number of patients not evaluable in some IFAs due to the lack of second suitable samples, ELISA for IgM proved to be the best of the serological tests, showing a high statistical significance ($P < 0.01$) when compared with antigenuria detection. Thirty-one of the patients testing positive only to serological tests were positive by ELISA for IgM. This technique showed greater sensitivity than ELISA for IgG plus IgM, particularly in first samples (29.7% versus 7.9%; $P < 0.01$). Measurement of low IgM levels may be more effective using a test set up to specifically measure this class of immunoglobulin than in one that must detect both IgG and IgM.

Nineteen patients (16%) were negative in all laboratory tests. Seven of them were patients with single samples collected very early after the appearance of symptoms, when serological responses had still not developed. The negative responses in

TABLE 4. Results for legionellosis diagnosis in 10 first samples testing negative for antigenuria but positive by serology

Technique	Result for patient no.:									
	020[a]	164	189	410	436	538	539	668	762	789
Antigenuria detection	−	−	−	−	−	−	−	−	−	−
ELISA for IgM	+	+	−	+	+	+	+	+	+	+
ELISA for IgG plus IgM	+	−	+	−	−	−	−	−	−	+
IFA for IgM	+	−	−	−	−	+	−	−	−	−
IFA for IgG	+	−	−	−	−	−	−	−	−	−
IFA for IgA	+/−	−	−	−	−	−	−	−	−	+
IFA for polyimmunoglobulin	−	−	−	−	−	−	−	−	−	−
Seroconversion	NA[b]	+	+	NA	+	NA	+	−	+	+

[a] Time of collection was not accurately recorded for this sample
[b] NA, second sample not available.

TABLE 5. Comparison of ELISA for IgM verus antigenuria detection in first samples[a]

Antigenuria status	No. with following result by ELISA for IgM		
	+	−	ND
+	12	26	11
−	9	23	10
ND	1	3	0

[a] $P = 0.0068$ in McNemar's test with Yates' correction. ND, not done or uncertain.

the remaining 12 patients may be due to the low sensitivities of the assays used or the lack of responsiveness on the part of the patients, but they may also reflect a weakness of the epidemiological criterion used to select the patients. On the other hand, this criterion has enabled the inclusion of a larger selection of patients, showing a clinical picture probably closer to reality.

It should be noted that all patients belonged to a single epidemic outbreak caused by the same *Legionella pneumophila* strain. Therefore, it is not possible to know whether the conclusions drawn from these data can be extended to sporadic, nosocomial, or travel-associated cases. An evaluation of the ELISA for IgM using unknown or blinded samples would provide valuable additional information.

Although antigen detection remains the best method for the early diagnosis of legionellosis, the results obtained here support the suggestion made by other authors (12, 16) that other laboratory methods, including those aimed at detecting antibodies, may provide a valuable complement. Of these, IgM detection by ELISA proved here to be the best alternative.

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