

Comparison of Phenol- and Heat-Killed Antigens in the Indirect Immunofluorescence Test for Serodiagnosis of *Legionella pneumophila* Group 1 Infections

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Received 26 March 1984/Accepted 12 July 1984

An antigen prepared with agar-grown *Legionella pneumophila* group 1 killed by 0.5% phenol and suspended in 0.5% yolk sac was examined for use in the indirect immunofluorescence test for legionellosis and compared with a heat-killed antigen. The serological results of the two antigens for single and paired sera agreed well. Morphological and staining characteristics were better for phenol-treated organisms. Electron microscopy observation showed an apparently well-preserved cell surface. The background antibody level among a healthy control population was very low (3.4% with titers of ≥ 16). Sera of patients with gram-negative bacteria infections (*Yersinia enterocolytica*, *Campylobacter jejuni*, *Salmonella typhimurium*, *Escherichia coli*, *Brucella melitensis*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, and *Chlamydia psittaci*) showed no cross-reactions with the phenol-killed antigen. The data suggest that phenol-killed antigen is sensitive and specific. This antigen is stable for at least 1 year.

The most commonly used diagnostic test for *Legionella* infection is still the indirect fluorescent antibody (IFA) test. Several factors, e.g. media, methods of killing, and strain differences, can influence the reactivity of *Legionella* antigens in surface antigen-dependent serological tests such as the IFA test (1, 7, 9). Ever since they were used in the Philadelphia epidemic of Legionnaires disease in 1976, antigens have been prepared by inactivating the bacteria with ether (9), heat (9), or Formalin (6). The ether-killed antigen was found to be unsatisfactory (9), and it still is controversial whether the use of heat or Formalin is the best method of inactivation for a more specific antigen; however, it has been demonstrated that results are comparable when the cutoff titer for a positive test result is lowered by one dilution factor (7).

In our laboratory, diagnostic material kindly supplied by the Biological Products Division of the Centers for Disease Control, Atlanta, Ga., was used for the diagnosis of patients clinically suspected of having legionellosis. The *L. pneumophila* group 1 antigen was prepared with Philadelphia 1 strain cultured on artificial medium, killed by heating at 100°C for 15 min, and suspended in 0.5% normal yolk sac (9).

We made some preliminary attempts to prepare IFA antigen for routine diagnostic use, with both *L. pneumophila* Philadelphia 1 strain and an *L. pneumophila* group 1 human strain isolated in Italy, by inactivation of bacteria with heat at 100°C for 15 min, Formalin 1%, or phenol 0.5% for 24 and 48 h. One reference *L. pneumophila* group 1 antiserum provided by the Centers for Disease Control and a few titered sera from patients have been checked against these suspensions. Results obtained with heat- and phenol-killed antigens were almost the same, whereas titers of sera checked with Formalin-killed antigen were slightly lower. Cells treated with phenol appeared to be more satisfactory because of their integrity and fluorescence intensity. For this reason, we decided to prepare our antigen by fixation with phenol and to use it in routine work in comparison with the Centers for Disease Control heat-killed antigen.

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MATERIALS AND METHODS

Strain and medium. *L. pneumophila* group 1 (strain Bari 161) isolated in Italy from the expectorate of a patient was used. Organisms were cultured on charcoal-yeast extract agar prepared in the laboratory.

Phenol-killed antigen preparation. Cell growth after 72 h at 35°C in air was scraped off, suspended in 0.5% phenol in distilled water at a density of ca. 10 McFarland standard turbidity, and allowed to remain for 48 h at room temperature. Charcoal-yeast extract agar plate was then streaked heavily and incubated for 5 days to test sterility. Suspensions were centrifuged, and the packed cells were resuspended in the same volume of 0.5% phenol with 0.05% sodium azide. The antigen titer was then determined. The working dilution was made in 0.5% normal yolk sac-phosphate-buffered solution (pH 7.6) containing 0.05% sodium azide and stored at 4°C.

Electron microscopy observations. A small drop of antigen, prepared as described above and suspended in 0.2 M ammonium acetate after the 48-h phenol treatment, was placed on a 400-mesh grid coated with a thin carbon film. After a few seconds of air drying, the specimen was negatively stained with 1% (wt/vol) sodium phosphotungstate, pH 7.0. Micrographs were taken with a Zeiss EM 10/C electron microscope.

Sera. Our work concerns 113 single and paired sera from patients with respiratory illness, which were submitted to this laboratory for legionellosis IFA testing. In addition, 54 sera of patients with bacteriologically diagnosed enterobacteria infections (*Yersinia enterocolytica*, 7 sera; *Salmonella typhimurium*, 4 sera; *Campylobacter jejuni*, 5 sera; and *Escherichia coli*, 15 sera from chronic cystitis) and other gram-negative bacteria infections diagnosed serologically (*Chlamydia psittaci*, 5 sera; *Coxiella burnetii*, 2 sera; *Brucella melitensis*, 2 sera; and *Mycoplasma pneumoniae*, 4 sera) or bacteriologically (*Pseudomonas aeruginosa*, 10 sera) have been tested against the phenol-killed antigen. The sera of 530 apparently healthy blood donors were also checked as negative controls.

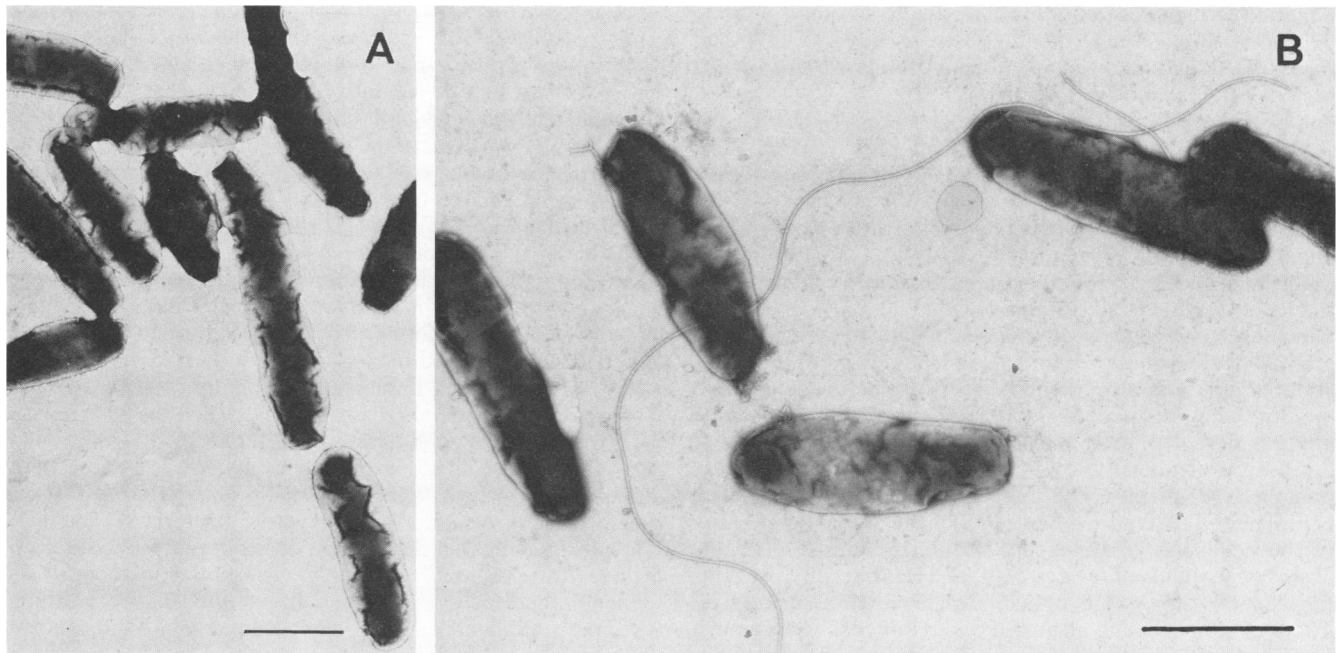


FIG. 1. Phenol-killed *L. pneumophila* negatively stained with 1% sodium phosphotungstate, pH 7.0. Electron micrograph magnifications, $\times 13,500$ (A) and $\times 20,000$ (B). Bars, 1 μm .

Serological assay. Sera to be tested were diluted in 0.01 M phosphate-buffered saline, pH 7.6. Indirect immunofluorescence testing was performed as usual (9).

RESULTS

General observations. As was observed preliminarily, the phenol-killed organisms showed a homogeneous, clear picture, with a regular outline showing a brightness of staining better than that of the heat-treated suspensions at the same experimental conditions. Background fluorescence was absent or very low. Electron microscopy observation of phenol-killed organisms showed an apparently well-preserved cell surface (Fig. 1).

Serological results. Eighty-three sera were nonreactive at a dilution of 1:16 with both antigens or only one antigen (Table 1). Seventy-nine of them (95.2%) were nonreactive with both antigens. Two sera reacted at a dilution of 1:16 only with the heat-killed antigen, whereas four sera showed titers of 16 (two sera) and 32 (two sera) only against the phenol-killed antigen.

Twenty-eight sera showed titers ranging from 16 to 4,096. Fifteen (53.6%) of them had the same titer against both antigens. Six (21.4%) sera had titers of one doubling dilution factor, and one serum had a titer of two doubling dilution factors higher against the heat-killed than the phenol-killed antigen. Four (14.3%) sera had titers of one doubling dilution factor, and two (7.1%) had titers of two doubling dilution factors lower against the heat-killed than the phenol-killed antigen.

Twenty-one of the previously described sera were acute and convalescent sera of eight patients with suspected legionellosis (Table 2). Four of these patients showed sero-conversion (a fourfold increase in titer to ≥ 128), and three had a significant (greater than or equal to fourfold) fall in titer against both antigens. One serum set had stable titers against both antigens.

Of sera from patients with gram-negative bacteria infection, all were nonreactive at a dilution of 1:16, except three cases with psittacosis and *Pseudomonas* and *E. coli* cystitis, which reacted at 16, and one case of *Y. enterocolytica*

TABLE 1. IFA titers of 113 human sera obtained with *L. pneumophila* group 1 phenol-killed antigen (strain Bari 161) compared with a heat-killed antigen (strain Philadelphia 1)

Titers obtained with phenol-killed antigen	No. of sera obtained with heat-killed antigen at titers of:									
	<16	16	32	64	128	256	512	1,024	2,048	4,096
<16	79	2								
16	2	5	1	1						
32	2	2	2							
64		1	1							
128					2	4				
256										
512							1			
1,024								3		
2,048								1	1	1
4,096								1		1

infection, which reacted at 32. Of sera from blood donors, 512 (96.6%) appeared to be nonreactive. Only 18 (3.4%) had titers ≥ 16 (8 had a titer of 16, 7 had a titer of 32, and 3 had a titer of 64).

DISCUSSION

The purpose of the present study was not to compare directly the phenol- and heat-killed antigens in sera from patients with infections other than legionellosis and from an apparently healthy population but only to evaluate the two antigens in routine laboratory practices on the diagnosis of suspected legionellosis patients.

Killing legionellae with phenol appears to be a suitable method of preparing IFA antigen which is specific, sensitive, and, in our laboratory experience, stable for at least 15 months when suspended in 0.5% normal yolk sac-phosphate-buffered solution and maintained at 4°C.

Absence of, or very low, background fluorescence and a homogeneous picture with a regular outline of the organism is probably due to a low disruption of the antigenic integrity of the cell surface. Being liposoluble, phenol is probably absorbed by lipids of the cell wall, and this partially prevents the denaturing action upon the cell membrane proteins. Titers of sera checked against the two antigens were almost similar. Of 83 sera that were negative with both antigens or only one antigen, 95.2% were negative with both. One serum, which was positive at a dilution of 1:32 with phenol-killed antigen but negative at 1:16 with the heat-killed

antigen, was an acute serum of a legionellosis case (patient no. 3; Table 2). All serum sets showing seroconversion or a fall in titer with the phenol-killed antigen acted in the same way with the heat-killed antigen (Table 2). Also, two sets had persistent titers with both antigens.

Antigens involved in the serodiagnosis of legionellosis by the IFA technique are surface antigens. Discrepancies in titers could be due to differences between strains used. In fact, strain Bari 161 was of a different subtype from strain Philadelphia 1 by monoclonal antibody typing (I. D. Watkins, unpublished data). Differences observed are also presumably due to the different surface-borne antigens involved in the IFA test performed with antigens killed by different methods. Heat-killed bacteria retain cross-reactive antigens (3, 4, 8). In crossed immunoelectrophoretic studies, a heat-stable antigen of *L. pneumophila* resembled the lipopolysaccharide of other gram-negative bacteria (2).

Schramek et al. (5) extracted by phenol treatment of *L. pneumophila* a substance with lipid A activity cross-reacting with lipid A of some gram-negative bacteria. Preliminary results indicate that our antigen does not react at dilutions of 1:32 with sera of patients with gram-negative infections. The phenol inactivation of legionellae, followed by washing, probably eliminates a portion of the lipid A-like substance of the surface lipopolysaccharide, resulting in a lack of false-positives at low serum dilutions and, thus, in a high specificity. These preliminary data need further investigations with a larger number of sera.

The background antibody level to *L. pneumophila* group 1 in a healthy population tested with the phenol-killed antigen was extremely low (3.4% of titers ≥ 16). This implies that titers found in the sera of patients suspected of having legionellosis are presumably due to antibodies specific for *L. pneumophila* group 1 antigens.

On the basis of the results described here, it is not possible to state which of the two legionellae-killing methods is the best. However, phenol treatment appears to be a suitable method which allows quicker readability without uncertainty and produces a clear picture brightly stained with a very low background fluorescence.

ACKNOWLEDGMENTS

This work was supported by grant no. 82.02422.52 of the Italian National Research Council (CNR).

We thank Donato Fumarola, Istituto di Microbiologia, Università Bari, for providing the expectorate sample from which we isolated the *L. pneumophila* Bari 161 (group 1) strain; I. D. Watkins, Sir William Dunn School of Pathology, Oxford University, for subtyping that strain by monoclonal antibodies; and Giuseppe Arancia, Laboratorio di Ultrastrutture, Istituto Superiore di Sanità, Rome, for electron microscopy observations. We are also grateful to Paolo Merucci, Ospedale Regina Elena, Rome, and Paolo Pugina, Ospedale Civile, Rovigo, for providing the sera of patients with gram-negative bacteria infections.

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TABLE 2. Titers of paired sera from patients with respiratory illness compared by IFA test performed with phenol- and heat-killed antigens of *L. pneumophila* group 1

Reaction and patient no.	No. of days after onset	Titers of sera obtained with:	
		Phenol-killed antigen	Heat-killed antigen
Seroconversion			
1	10	<16	<16
	28	1,024	512
	49	4,096	1,024
2	18	16	16
	27	2,048	1,024
	55	2,048	4,096
3	10	32	<16
	24	2,048	2,048
	40	4,096	4,096
4	12	16	<16
	55	1,024	1,024
	96	1,024	1,024
Fall in titer			
5	17	512	512
	35	<16	<16
6	10	128	128
	230	<16	<16
7	17	128	256
	62	128	256
	240	16	16
Stable titers			
8	7	32	16
	25	32	16

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