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Indirect Hemagglutination Test for Chlamydial Antibodies

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An indirect hemagglutination (IHA) test is described for chlamydial antibodies in psittacosis diagnostic sera; for this test tanned sheep erythrocytes sensitized with a deoxycholate extract of *Chlamydia psittaci* grown in Vero cell monolayers were used. Adaptation of the IHA test to the Microtiter system decreased sensitivity; nevertheless, the Microtiter-IHA test was more sensitive than the complement fixation test. Lymphogranuloma venereum antibodies also were detected by using antigen extracted from *C. psittaci*.

The indirect hemagglutination (IHA) test introduced by Boyden (2) in 1951 and shown by Stavitsky (12) to be specific, sensitive, and simple to perform has been successfully applied to diagnoses of several microbial infections. However, the test has not been used extensively for psittacosis serology. An IHA test for psittacosis described by Benedict and O'Brien (1) in 1958 seemed to be of limited value. Of 29 persons previously infected with psittacosis and possessing complement fixation (CF) titers, 31% were negative by IHA. Of 29 individuals with no known exposure to *Chlamydia* and negative by the CF test, 10% were IHA positive. Turner and Gordon (14) reported that rabbit antisera to a trachoma isolant and to an inclusion blenorrhea isolant agglutinated erythrocytes sensitized with antigen from either strain or from the mouse pneumonitis agent. Vedros (15) used the IHA test with rabbit antisera to differentiate *C. psittaci* strain 6BC from four *C. trachomatis* strains. Dhir et al. (4) described an IHA test with rabbit antisera and erythrocytes sensitized by polysaccharide from three trachoma strains; evidence was presented that polysaccharide was responsible for chlamydial group reactivity.

The present report describes an IHA test for chlamydial antibodies and compares the IHA titers of diagnostic sera with the CF titers.

MATERIALS AND METHODS

Antigens. *C. psittaci* strain RA 642, isolated from a parrot by intracerebral inoculation of mice, was used to produce erythrocyte-sensitizing antigen (ESA) after four serial passages on Vero cell cultures. For ESA production, we inoculated Vero cell monolayers in 32-oz Brockway bottles with a $10^{4.5}$ $TCID_{50}$ of strain 642 in 10 ml of Eagle medium (6) having 2% fetal bovine serum (EM). The inoculum $TCID_{50}$, which was determined by titration on cover slip monolayers of Vero cells, corresponded to $10^{7.8}$ mouse intracerebral LD_{50} (11).

After holding the monolayers inoculated for ESA production for 2 hr at 37 C, we added 40 ml of EM to each and continued their incubation. Medium was collected on days 4 and 6; the final harvest was on day 11 when approximately 70% of the cells showed cytopathic effects.

The collected medium was clarified by light centrifugation, and the organisms were pelleted from the clarified medium by centrifugation at 12,000 $\times$ *g* for 45 min. They were washed once and resuspended in pH 7.2 buffered saline solution (BSS; 0.15 M NaCl and 0.01 M phosphate) one-twentieth the original volume.

Antigen was extracted from organisms collected from the 5th, 6th, and 7th Vero cell passages by shaking with 1% sodium deoxycholate (Fisher Scientific Co.) for 4 hr in a 56 C water bath. Particulate residues were removed by centrifugation and discarded; the supernatant fluid containing ESA was dialyzed against BSS at 25 C for 48 hr.

Antigen for CF tests and for IHA inhibition experiments was produced in embryonated chicken eggs by the Center for Disease Control (CDC) Biological Reagents Section. The antigen titer was 64 by the CF test.

Sera. Human sera used in the tests were specimens submitted to CDC for diagnosis of psittacosis, lymphogranuloma venereum (LGV), and infections unrelated to *Chlamydia*. Psittacosis hyperimmune sera were produced in rabbits and guinea pigs with organisms grown and concentrated as those destined for deoxycholate extraction. Each animal received 2 ml divided equally for intravenous, intramuscular, intraperitoneal, and foot pad inoculation weekly for 7 weeks; sera were collected 10 days after the last

inoculation.

LGV strain JH obtained from the American Type Culture Collection was propagated in yolk sacs (10) of 7-day-embryonated chicken eggs; the harvested organisms were partially purified by fluorocarbon treatment (6) and used to produce rabbit immune serum as described for psittacosis antiserum.

Before being tested, sera were inactivated at 56 C for 30 min and adsorbed at 37 C for 30 min with half their volume of packed sheep erythrocytes to prevent nonspecific agglutination.

Erythrocyte sensitization. Within 2 weeks of collection, sheep erythrocytes were washed, tanned, and sensitized by the method of Stavitsky (12) with two modifications: NaH_2PO_4 was substituted for KH_2PO_4 in the buffered solutions and tannic acid was routinely used at a concentration of 1:40,000 rather than 1:20,000. The optimal dilution of antigen for sensitizing cells was determined by IHA block titration of each lot with positive sera; the optimal dilution was usually 1:10. Erythrocytes were washed, tanned, and sensitized on the day used; their stability was not determined.

Serological testing. The tube IHA test was done as described by Stavitsky (12). Sensitized cells were used at a 2.5% concentration; sensitivity was decreased by more concentrated suspensions, and more dilute suspensions were nonspecifically agglutinated. For the Microtiter-IHA test, a 0.4% erythrocyte suspension was optimal; 0.05 ml was added to 0.5 ml of twofold serum dilutions in wells of plastic Microtiter U-plates. The highest serum dilution producing a sheet of completely agglutinated cells was considered the end point. Controls included: (i) the lowest serum dilution tested and tanned but unsensitized erythrocytes; (ii) serum diluent and sensitized erythrocytes; (iii) positive serum (1:10) and erythrocytes sensitized with medium processed from uninfected Vero cells; and (iv) titration of known positive and negative sera.

The CF tests were done by the Laboratory Branch CF Microtiter method (3).

RESULTS

Production of erythrocyte-sensitizing antigen. Experiments were done to determine whether cells, as well as medium, should be processed for ESA. After the final harvest of medium from infected monolayers, the cells were disrupted by three freeze-thaw cycles in BSS. Cellular debris was removed by light centrifugation, and the supernatant fluid was deoxycholate extracted for ESA. Tanned erythrocytes treated with the extract were negative by the IHA test with known positive sera; the extracts were also negative for psittacosis CF antigen. In contrast, the organisms from the medium of these monolayers yielded ESA with a titer of 10 and CF antigen with a titer of 8.

Technical considerations for test performance. The optimal concentration of tannic acid for tanning sheep erythrocytes was 1:40,000 (Table 1). Untanned cells were not sensitized when treated with antigen.

Titers of tests read after 3 hr of incubation at 25 C remained unchanged after an additional 24 hr. Erythrocytes in tests incubated at 4 C settled so slowly that serum controls remained unreadable for at least 24 hr. Although settling was rapid at 37 C, agglutination occurred in antigen controls. Incubation for 3 hr at 25 C was optimal.

To determine the temperature for storing ESA, we placed samples of an antigen lot at 25, 4, and −35 C. After various time periods, tanned erythrocytes were sensitized by the samples at a 1:20 dilution, optimal for the antigen before storage. Antibody titers of sera with antigen stored at −35 C for 9 weeks (the longest period used) were identical to titers of the same sera tested with the antigen before storage. Lower titers were obtained when these sera were tested with the antigen samples stored at 25 and 4 C for 3 weeks.

Reproducibility of the IHA test was indicated by this experiment. Serum samples, which were tested repeatedly over a 9-week period with erythrocytes sensitized by antigen stored at −35C, varied no more than twofold in titer.

Comparison of serum titers by CF and IHA. Of 25 CF-positive sera tested, from patients with suspected chlamydial infection, all showed higher antibody titers in tube IHA than in CF tests (Table 2). In two instances both acute and convalescent sera were available. By CF, the titers of the convalescent sera were fourfold higher than those of the corresponding acute sera; the titers also showed fourfold rises by the IHA test. Paired sera from an additional patient with suspected psittacosis were negative by the CF test but had a titer of 10 by IHA. All of 15 presumably normal sera were negative by both of the tests.

TABLE 1. *Indirect hemagglutination (IHA) titers of two sera with sensitized erythrocytes tanned with various acid concentrations*

Tannic acid concn	IHA titers of sera	
	Serum A (immune)	Serum B (nonimmune)
1:5,000	<10	<10
1:10,000	40	<10
1:20,000	160	<10
1:40,000	640	<10
1:80,000	160	<10
None	<10	<10

TABLE 2. *Indirect hemagglutination (IHA) and complement fixation (CF) titers of chlamydial antibody in positive human sera*

Serum	Clinically suspected infection	CF	Macrotiter IHA	Microtiter IHA
1-S1	Psittacosis	64	2,560	640
S2	Psittacosis	256	10,240	2,560
2-S1	Psittacosis	8	20	ND[a]
S2	Psittacosis	32	80	ND
3	Psittacosis	8	20	10
4	Psittacosis	8	20	10
5	Psittacosis	8	40	20
6	Psittacosis	8	40	20
7	LGV[b]	8	80	40
8	LGV	8	80	20
9	LGV	8	80	20
10	LGV	8	80	40
11	LGV	8	80	40
12	LGV	8	80	40
13	LGV	8	80	40
14	LGV	8	80	80
15	LGV	8	320	ND
16	LGV	16	80	80
17	LGV	32	160	ND
18	LGV	32	2,560	320
19	LGV	32	2,560	320
20	LGV	128	1,280	320
21	LGV	512	2,560	640
22	LGV	512	5,120	2,560
23	LGV	512	>5,120	5,120
24-S1	Psittacosis	<8	10	10
S2	Psittacosis	<8	10	10

[a] Not done.
[b] LGV = lymphogranuloma venereum.

Titers of sera from rabbits and guinea pigs immunized with psittacosis or LGV organisms were at least fourfold greater in IHA tests than in CF tests; preimmune sera were negative by both tests.

Although most titers were lower by the Microtiter test than by the tube IHA test, all exceeded the CF titers (Table 2). Paired sera showing a fourfold titer rise by CF and tube IHA tests also showed the rise by the Microtiter test.

All sera negative by tube IHA tests were also negative by Microtiter tests.

IHA inhibition. To further establish specificity of the IHA test, blockage of sensitized-cell hemagglutination with positive sera was attempted by prior incubation of the sera with equal volumes of psittacosis CF antigen for 1 hr at 25 C. Hemagglutination was completely blocked; negative control antigen did not affect the serum titers.

DISCUSSION

Antigen for the IHA test was easily extracted from *C. psittaci* organisms with deoxycholate. Sufficient antigen was consistently produced, by a single Vero cell monolayer grown in a 32-oz bottle, to sensitize enough erythrocytes for tests in more than 400 tubes or 2,500 Microtiter wells. Use of tissue culture obviates the need for antibiotic-free embryonated chicken eggs, not always readily available.

The CF titers of 14 of the sera listed in Table 2 were 8; of these, 13 had relatively low IHA titers of 10 to 80. Titers below 16 by the CF test are often considered (8) not diagnostically significant; sera from 2 to 15% of asymptomatic persons may have CF titers of from 8 to 16 (10). Although final assessment of the significance of low titers by the sensitive IHA test will be dependent on more data, low IHA titers may prove no more diagnostic than low CF titers.

Benedict and O'Brien (1) obtained a chlamydial group-specific IHA antigen by centrifugation at 100,000 $\times$ *g* of infected chicken embryo allantoic fluid first clarified at 35,000 $\times$ *g*. Our attempts to produce antigen in this manner from infected allantoic fluid or Vero cell medium were unsuccessful. In addition, we detected no psittacosis antigen in the supernatant fluids collected after pelleting the organisms by centrifugation at 35,000 $\times$ *g*, even when the supernatant fluids were concentrated 50-fold. Intact organisms and deoxycholate-extracted cell walls of *C. psittaci* sensitized tanned erythrocytes only inconsistently.

Chlamydial antigen extracted by deoxycholate is group specific by the CF test (9). It also appears to be group specific by the IHA test because, in the present study, erythrocytes sensitized with the antigen were agglutinated by sera from patients with clinically diagnosed LGV (Table 2) and from animals immunized with LGV organisms. Although psittacosis is readily distinguishable clinically from LGV, a species-specific IHA test could, on occasion, be diagnostically useful. In addition, antigens specific for species or subgroups would be helpful both in epidemiological studies and in classifying chlamydial strains not infrequently isolated from a wide variety of hosts (13). Vedros (15) successfully produced IHA antigens differentiating the 6BC strain of *C. psittaci* from four *C. trachomatis* strains. Significant progress in isolating and characterizing trachoma antigens was reported recently by

Dhir et al. (4). Continued effort to produce antigens for differentiating members of the genus *Chlamydia* seems desirable.

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