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Single-Antigen Immunofluorescence Test for Chlamydial Antibodies

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A simple method is presented for producing large numbers of inclusions of *Chlamydia trachomatis* serotype L2 in cell cultures on slides for immunofluorescence antibody staining. Preliminary results with a total of 1,241 human sera from different groups were consistent with findings by earlier methods: 82% of chlamydia-positive men with nongonococcal urethritis had antibodies at titers of ≥8, with a geometric mean titer (GMT) of 44.9; 68.5% of chlamydia-negative men with nongonococcal urethritis had titers of ≥8, with a GMT of 38.6; 27% of male blood donors had titers of ≥8, with a GMT of 19.6; 95.0% of chlamydia-positive women had titers of ≥8, with a GMT of 80.3; 67.0% of chlamydia-negative partners of men with nongonococcal urethritis had titers of ≥8, with a GMT of 47.0; 50.2% of control women had titers of ≥8, with a GMT of 27.7% and 8.1% of children (aged 1 to 15 years) had titers of ≥8, with a GMT of 17.8.

The recent observations of the common incidence of *Chlamydia trachomatis* infections call for antigenic tests more sensitive than the complement fixation (CF) test, which apparently is of use in systemic chlamydial diseases only (4). The micro-immunofluorescence test (13) is sensitive and specific, but technically cumbersome and available in only a few laboratories. Modifications of the micro-immunofluorescence test involve use of pooled antigens (11, 14), of a single antigen (serotype L2 [10]), or of inclusions in cell cultures of antigens, such as serotype E (7) or serotypes L1 and L2 (1; P. Terho, in preparation).

We present here a simple method for producing large inclusions of serotype L2 on objective slides for the immunofluorescence antibody test (IFAT) and results of preliminary screening with this test.

MATERIALS AND METHODS

Cells. The KoMu cell line derived in this department from dog kidney (submitted for publication) was kindly supplied by N. Oker-Blom. It grows in large epithelioid-like cells in monolayers that show good contact inhibition and can be subcultured weekly at a ratio of 1:10 to 1:12. The growth medium was Parker medium 199 supplemented with 10% tryptose phosphate broth, 10% fetal bovine serum, and 50 μg of gentamicin per ml.

Preparation of slides. Objective slides were kept overnight in Haemosol (Merz and Dade, Berne, Switzerland), rinsed thoroughly with tap water and distilled water, and stored in absolute ethanol. On the day of inoculation, the slides were flamed and put into plastic petri dishes (14-cm diameter), using four slides (76 by 26 mm) per dish. The cells were trypsinized and dispersed into 4 to 5 volumes of growth medium, and an inoculum was added which, in final dilution, contained (per milliliters) about 10^5 inclusion-forming units of *C. trachomatis* serotype L2 (434 Bu; obtained from P. Terho, Department of Virology, University of Turku) grown conventionally in irradiated McCoy cells (3). Thereafter, the petri dishes were seeded with 50 ml of cell suspension to ensure the submersion of slides. After 48 h at 37°C in a humidified 5% CO_2 atmosphere, the slides were removed, washed with phosphate-buffered saline without calcium or magnesium (PBS), fixed with methanol at −20°C, air dried, and stored at −20°C.

IFAT. The slides were thoroughly dried by air under pressure and divided into 32 squares (8 by 4) with a thin brush and nail polish. Sera were diluted with loops in amounts of 0.025 ml in PBS on microplates, and 10 μl of dilutions of 1:8, 1:32, 1:128, and 1:512 was transferred to the corresponding square with a Finnpipette device (Finnpipette Ky, Helsinki, Finland). After a 30-min incubation at 37°C in a humidified atmosphere, the slides were rinsed three times with PBS, and anti-human immunoglobulin G-fluorescein isothiocyanate conjugate (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) was added.

After further incubation for 30 min, the slides were washed three times with PBS, and once with distilled water, air dried, mounted with Veronal buffer at pH 8.5, and observed under a Reichert Zetopan fluorescence microscope. In positive samples, large, brightly fluorescent inclusions comparable in size to those obtained in irradiated McCoy cells (3) were seen against the cell background when 10X objective was used. The titer was extrapolated from the intensity noted in the last reactive serum dilution. Positive control serum was titrated in twofold steps.

CF test. The CF test was done in a microtitration

system with group antigen extracted from *C. trachomatis* with acetone and ether and kindly supplied by C. Mordhorst (Statens Seruminstitut, Copenhagen, Denmark). A yolk sac control antigen was always included.

Sera. Human sera, stored at −20°C, were from the following groups: (i) 193 men with nongonococcal urethritis (NGU) and positive *C. trachomatis* isolations, examined at an outpatient department for venereal diseases; (ii) 394 men with NGU and negative isolation results, examined at the same clinic; (iii) 152 healthy control men (blood donors; mean age, 33 years) with no history of chlamydia isolation; (iv) 58 women (including 45 partners of the NGU men described above) with positive isolation results, examined at a gynecological outpatient clinic (patients with pelvic inflammatory diseases were excluded); (v) 91 isolation-negative NGU partners, examined at the same clinic; (vi) 183 control women, consisting of blood donors, women attending a family planning clinic and with no isolation history, and chlamydia-negative patients without pelvic inflammatory disease, examined at the same gynecological outpatient clinic (all of the women were of fertile age); (vii) 160 children, aged 1 to 15 years, who had been sent to the department on suspicion of viral illnesses; and five paired sera from suspected cases of psittacosis to study cross-reactivity with *C. psittacii*.

Statistical significance was evaluated by the chi-square test.

RESULTS

IFAT titers in different groups. The distribution of titers in the different groups, the cumulative percentages of low (≥8) and high (≥64) titers, and geometric mean titers (GMTs) are presented in Table 1. The men with chlamydia-positive NGU had significantly more antibodies ($P < 0.01$) and more high titers ($P < 0.001$) than did the chlamydia-negative NGU men. However, the GMT of chlamydia-positive men was of the same magnitude as that of the chlamydia-negative men. Control men had antibodies in low and high titers significantly less frequently than did men with NGU ($P < 0.001$), and the GMT of seropositive men was half that of the men with NGU. The high-titer positive sera tended to be found among blood donors over 30 years of age (11.5 versus 1.0% among those under 30 years of age [$P < 0.05$]).

Similarly, chlamydia-positive women had significantly greater occurrence of antibodies in both low ($P < 0.001$) and high titers ($P < 0.001$) as compared with chlamydia-negative NGU partners. On the other hand, the difference of the latter group from the control women group was not as significant statistically in the high-titer ($P < 0.01$) and especially in the low-titer ($P < 0.025$) groups. Compared with the male groups, women had higher GMTs and a higher incidence of antibodies, except in the comparison between chlamydia-negative NGU men and chlamydia-negative NGU partners.

Of the 160 children, only 13 had titers of ≥8, and three had titers of ≥64. Only two of these children had a diagnosis not attributable to a possible *C. psittacii* infection (varicella, gastroenteritis). The others were suffering from pneumonia (four cases), acute respiratory infection (six cases), and prolonged fever (one case).

Comparison of IFAT with CF test. Of the adult sera, 467 were titrated by both methods (Table 2). The percentages of sera positive only in the IFAT and only in the CF test were 34.5 and 4.5, respectively. Of those positive in IFAT, two (0.6%) had higher titers in the CF test, in 5.8% the titers were identical, and in the remainder the IFAT titer was higher.

IFAT in suspected *C. psittacii* infections. The results of IFAT with patients with suspected *C. psittacii* infections are presented in Table 3. IFAT was positive in all cases.

DISCUSSION

The method described above represents a simple means for large-scale production of objective slides for IFAT. The slides can be stored at

TABLE 1. *Distribution of titers obtained with the single-antigen chlamydia IFAT in different groups studied*

Group	No. studied	No. with a titer of:											GMT[a]
		<8	8	16	32	64	128	256	512	1,024	≥8 (% of total)	≥64 (% of total)	
Men, with NGU and chlamydia isolation-positive	193	37	14	35	25	44	26	7	3	2	156 (82.0)	82 (42.4)	44.9
Men, with NGU and chlamydia isolation-negative	394	124	50	47	60	47	44	12	10		270 (68.5)	113 (28.7)	38.6
Men, controls	152	111	15	8	11	5	2				41 (27.0)	7 (4.6)	19.6
Women, chlamydia isolation-positive	58	3	4	6	6	12	14	7	4	2	55 (95.0)	39 (65.5)	80.3
Women, NGU partners and chlamydia isolation-negative	91	30	12	7	11	10	11	6	4		61 (67.0)	31 (34.0)	47.0
Women, controls	183	91	29	13	18	17	10	5			92 (50.2)	32 (17.5)	27.7
Children, 1 to 15 years of age	160	147	7		3	3					13 (8.1)	3 (2.6)	17.8

[a] GMT of patients with antibodies of titers of ≥8.

TABLE 2. *Correlation of IFAT with CF test titers in 467 adult sera*

CF test titer	IFAT titer								
	<8	8	16	32	64	128	256	512	1,024
<8	*133*[a]	39	35	29	33	20	4	1	
8	14	*6*	14	14	19	17	5	2	1
16	6	2	*8*	12	19	12	4	1	
32	1			*4*	3	5	3		
64					*0*			1	

[a] Italicized numerals are the number with equal titers on both tests.

TABLE 3. *IFAT with C. trachomatis serotype L2 in sera from five patients with suspected C. psittacii infection*

Patient	Age (yr)	Sex	History	Day of illness	Titer with:	
					CF test	IFAT
SK	62	♀	Pneumonia	2	8	128
				9	32	256
HR	61	♀	Pneumonia	2	8	64
				16	128	512
ML	20	♀	Pneumonia	15	64	32
				27	64	32
AP	56	♂	Pneumonia	20	64	64
				32	64	64
BB	26	♀	Encephalitis (buried dead bird)	10	512	128
				20	1,024	256

−20°C for months before testing. The test is easily readable by low-power fluorescence microscopy due to the characteristic appearance of the large inclusions in the cell vacuoles. It has been estimated previously that the serotype L2 used in this test covers 95.5% of the *C. trachomatis* antibodies detected in micro-IFAT (10). The percentages of positive sera demonstrated in the groups studied (chlamydia-positive NGU men, chlamydia-negative NGU men, healthy control men, women excreting chlamydia, chlamydia-negative NGU partners, control women, and children of age 1 to 15 years) were consistent with earlier findings using technically more laborious methods (5–8, 10, 11, 13–15). The chlamydia-positive NGU men had significantly more antibodies and higher titers than did the chlamydia-negative NGU men. The difference between the latter, sexually promiscuous group and the healthy control men can be explained by the persistence of antibodies, by the booster effect of cross-reacting antibodies in multiple infections (5), or by the possibility that the conventional method of chlamydia isolation in McCoy cells is not sensitive enough.

Women tended to have antibodies more frequently and in higher titers than did men. This was observed a decade ago with the CF test (9) and later with the IFAT (5, 7, 8, 11, 14). The explanation may be that, in addition to having a larger area of infection, women often remain asymptomatic for longer periods of time, thus providing a larger and longer antigenic stimulus for antibody production. These findings resemble those obtained in serology with *Neisseria gonorrhoeae* (16).

Recently, Chandler et al. (2) showed that a majority of babies born to chlamydia excreters acquired the infection. In Finland the carrier rate among the patients of a gynecological outpatient clinic (J. Paavonen et al., Br. J. Vener. Dis., in press) was found to be about the same as that observed by Chandler et al., and children in this study had chlamydial antibodies. However, cross-reactions with *C. psittacii* were not excluded, because nearly all seropositive children had the clinical picture found in ornithosis, too. The cross-reactions with this agent, also noticed earlier in IFAT (7), should be kept in mind. *C. psittacii*, along with *C. trachomatis* strains not cross-reacting with serotype L2 (10), may have produced some of the reactions seen in the CF test only. The possible cross-reactions with gram-negative bacteria deserve attention. Certain strains of "*Bacterium anitratum*" (=*Acinetobacter* [12]), part of the normal commensal flora of women, are known to react one way with chlamydial group CF antigen. If wider cross-reactions occur, they could explain the frequent low titers found in women. The finding of occasional high titers in chlamydia-negative and control groups prompts further studies.

The IFAT described provides a sensitive method for screening large numbers of sera for chlamydial antibodies in epidemiological studies and offers considerable savings in time and equipment. Preliminary results indicate that it can also be applied in the serodiagnosis of some acute cases (J. Paavonen et al., Sex. Transm. Dis., in press).

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