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Micro Cell Culture Method for Isolation of *Chlamydia trachomatis*

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Presterilized mictotiter plates (96 wells) with BHK-21 cells on 5-mm cover slips were successfully used for cell culture isolation of trachoma from 15 infected conjunctival scrapings.

After Tang's (16) successful isolation of the trachoma organism in eggs, his procedure became the method of choice for the isolation of *Chlamydia trachomatis* from ocular infections of humans. Growth was attempted in cell culture (1, 4, 10-12, 14) and, though successful, the method was not used routinely because of difficulty in serial passage of some strains. Since 1967 several workers have used cell culture for laboratory-adapted strains and primary isolates in preference to eggs because of increased sensitivity (5, 7, 9) due to cell irradiation (H. R. Dressler, F. B. Gordon, W. T. McQuilkin, A. L. Quan, and G. S. Huber, Bacteriol. Proc., p. 117, 1965; 8), increased glucose in the medium (N. A. Vedros and F. B. Gordon, Bacteriol. Proc., p. 134, 1963), centrifugation of the inoculum onto the cells (17), and the temperature at which the centrifugation is done (35 C) (2, 6, 15). The cell culture system is now routine for isolation of chlamydia from genital, ocular, and other clinical specimens.

The existing cell culture methods, which utilize individual flat-bottomed tubes and 12 to 13-mm round cover slips, limit the number of tubes which can be handled at one time because of the centrifugation step. For this reason, primary isolations of trachoma were tried using presterilized microtiter plates (Cooke Engineering Co., Alexandria, Va.) containing BHK-21 cells in monolayer on 5-mm round cover slips in 96 wells. Self-adhering microtiter plate sealers were used to maintain sterile conditions. This allowed a maximum of 384 (4 × 96) tests to be done with a single centrifugation.

Infected conjunctival scrapings were obtained from 15 owl monkeys (*Aotus trivirgatus*) 7 days after challenge with trachoma strain HAR-36. The specimens were stored in 0.5 ml of PGS (3) at −60 C until tested. These monkeys were known to be infected from duplicate conjunctival scrapings stained by immunofluorescence (13) on the same day (Table 1). The cells used for isolation were BHK-21 (courtesy of W. Blyth, Medical Research Council, the Medical School, Bristol, England) grown in suspension in Rubella medium (Flow Laboratories) as modified in this laboratory by T. R. Rota (Eagle minimal essential medium with Earle balanced salt solution containing 10% inactivated calf

TABLE 1. *Isolation of trachoma from serial dilutions of conjunctival scrapings from infected owl monkeys*

Monkey no.	Conjunctival inclusion rate in monkeys (%)[a]	Average no. of inclusions in BHK-21 monolayer cultures[b]				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1	8.8	83[c]	8	0	0	0
2	8.6	570	122	6	0	0
3	4.4	274	23	3	0	0
4	2.1	246	28	3	0	0
5	1.8	286	50	7	0	0.5[d]
6	6.3	155	14	0	0	0
7	1.1	343	26	3	0	0
8	34.6	1,590	420	24	7	0
9	1.5	1,222[c]	145	11	1	0
10	21.7	3,069	238	34	6	0
11	12.0	701	219	23	2	0
12	13.5	517[c]	51	3	0	0
13	1.0	428	22	1	0	0
14	2.9	672	85[c]	13	1	0
15	11.7	1,871	260	18	3	0

[a] Inclusions were detected by immunofluorescence using FITC-LGV. Expressed as the number of inclusions/number of cells × 100.

[b] Average number of inclusions from two cover slips at each sample dilution.

[c] Results from a single cover slip only. Cell sheet was incomplete on the second cover slip (4/150 cover slips in this experiment were unacceptable for this reason).

[d] A single inclusion identified in one of two cover slips.

serum, 10% tryptose phosphate, 100 × minimal essential medium nonessential amino acids [20 mg/liter], 100 × Eagle basal medium vitamins [20 mg/liter], and 15% glucose [20 ml/liter]). The same medium supplemented with 8.8% glucose, 50 μg of streptomycin per ml, and 100 μg of vancomycin per ml was used for all dilutions of the specimens and for the isolation.

Each of the cover slips in the 96 wells of the microtiter plate was seeded with 10^5 BHK-21 cells previously irradiated with 5,000 rads of ^{60}Co. After 48 h of incubation at 35 C, the 0.2 ml of Rubella medium covering the BHK-21 cells was removed, and 0.2 ml of the conjunctival sample, from an infected monkey serially diluted, was delivered into each of three wells containing the BHK-21 monolayers. An equal volume of inoculum from the same serial dilutions was also put into the yolk sac of three 8-day-old embryonated eggs for comparison infectivity titrations. Centrifugation of the inoculum in the microtiter plate was done at 35 C for 30 min at 1,000 × *g* in a horizontal head. A force of 2,000 × *g* ruptured the plates around the wells. After 48 h of incubation at 35 C, two of three cover slips were removed, washed with phosphate-buffered saline, mounted, and stained by a human lymphogranuloma venereum serum conjugated with fluorescein isothiocyanate (FITC-LGV). All of the inclusions on each cover slip were counted. Yolk sacs from the inoculated eggs were harvested individually at the time of embryo death or from sacrificed eggs 12 days after inoculation and were examined for elementary bodies by immunofluorescence. The same FITC-LGV reagent was used throughout for identification of inclusions in the monkey conjunctival scrapings, inclusions in the BHK-21 inoculated cells, and elementary bodies in the yolk sac smears. Only one passage in cell culture or eggs was done.

TABLE 2. *Comparisons of infectivity titrations by the microtiter method in BHK-21 cell monolayers and in embryonated eggs*

Monkey no.	Egg titration end point[a]	BHK-21 titration end point[a]	Increased sensitivity of cell culture compared with eggs (log)
1	10^{-2}	10^{-2}	None
2	10^{-3}	10^{-3}	
3	10^{-3}	10^{-3}	
4	10^{-3}	10^{-3}	
5	10^{-3}	10^{-3}	
6	10^{-1}	10^{-2}	1
7	10^{-2}	10^{-3}	
8	10^{-3}	10^{-4}	
9	10^{-3}	10^{-4}	
10	10^{-3}	10^{-4}	
11	10^{-3}	10^{-4}	
12	10^{-1}	10^{-3}	2
13	10^{-1}	10^{-3}	
14	10^{-2}	10^{-4}	
15	10^{-1}	10^{-5}	3

[a] The highest serial dilution of monkey conjunctival scraping which infected BHK-21 monolayers or the yolk sac of embryonated eggs as determined by immunofluorescent staining.

Isolations were obtained from all 15 conjunctival scrapings (Table 1). No inclusions were seen in normal, uninoculated BHK-21 cells in microtiter plates containing test samples or in cells inoculated with the conjunctival scrapings of the 15 monkeys prior to challenge. No bacterial contamination of test or control tubes was encountered, despite working on an open bench.

From comparison of infectivity titrations, the micro cell culture method was clearly more sensitive than eggs in the isolations of trachoma and required only 2 days for determination of infectivity, whereas the egg method required up to 12 days. Ten of the 15 samples were found to be positive at dilutions 10- to 1,000-fold greater in cell culture than in eggs (Table 2).

The micro cell culture method has also been used in this laboratory for antibiotic tests with the trachoma organism.

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