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Improvement of Growth of *Chlamydia pneumoniae* on HEp-2 Cells by Pretreatment with Polyethylene Glycol in Combination with Additional Centrifugation and Extension of Culture Time

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The following adaptations led to improved growth of *Chlamydia pneumoniae* on HEp-2 cells compared to that by the standard method: monolayer preincubation with 7% polyethylene glycol (PEG), extension of incubation time to 7 days, and extension of incubation to 7 days in combination with centrifugation on days 3, 4, and 5. These adaptations resulted in approximate increases in numbers of inclusion-forming units (IFU) of 2-, 5-, and 69-fold, respectively. A combination of preincubation with PEG, prolonged incubation, and centrifugation on days 3, 4, and 5 increased the numbers of IFU >300-fold. This is therefore recommended as the optimal method for culturing *C. pneumoniae*.

Chlamydia pneumoniae has been recognized as an important pathogen causing respiratory tract infections. Direct isolation has proven to be difficult, and only a few laboratories have been able to isolate and propagate *C. pneumoniae* from patient samples. Therefore, serology is used for most clinical and epidemiological studies.

For improvement of isolation of *C. pneumoniae*, results with different cell lines have been compared. Some authors demonstrated that HL cells were more suitable than HeLa 229 cells, whereas others found that HEp-2 cells were even better for the isolation of *C. pneumoniae* (2, 4, 6, 7). Data on the influence of other parameters on the growth of *C. pneumoniae* are limited and not conclusive except that blind passage seems necessary (5). The use of polyethylene glycol (PEG) has proven to enhance the isolation rate of *Chlamydia trachomatis* (3). PEG is a high-molecular-weight molecule that reduces the dielectric constant and therefore increases the hydrophobicity of the cell membrane. This facilitates the fusion of cell membranes (1). Therefore, we investigated whether pretreatment with PEG, increased incubation time, and additional centrifugation, each separately and in combination, could improve the growth of *C. pneumoniae* on cell monolayers.

In preliminary experiments with different cell lines for comparing the growth of four different *C. pneumoniae* strains, the best results were obtained with HEp-2 cells. For further experiments, *C. pneumoniae* TW-183 and HEp-2 cells were selected. HEp-2 (CCL-23; American Type Culture Collection, Rockville, Md.) monolayers in flat-bottom tubes were used as such or pretreated with 100 μl of 7% PEG (average molecular weight, 3,350; Sigma Chemical Co., St. Louis) in Optimem (Gibco Life Technologies Ltd., Paisley, Scotland) for 1 h, after which 100 μl of a dilution of laboratory strain *C. pneumoniae* TW-183 (Washington Research Foundation, Seattle) was added and centrifuged at 1,200 × *g* for 1 h. Then, 1 ml of *Chlamydia* growth medium, i.e., Optimem supplemented with 4% fetal calf serum, gentamicin (25 μg/ml), vancomycin (25 μg/ml), amphotericin B (2.5 μg/ml), cycloheximide (0.5 μg/ml), and glucose (4.5 mg/ml), was added. In experiments where centrifugation at 1,200 × *g* on day 3 was required, the culture medium was refreshed. Each experiment was performed with two inocula, containing 2 to 20 inclusion-forming units (IFU) and 30 to 100 IFU, respectively. All experiments were performed in duplicate and repeated at least four times. Statistical analysis was performed on log-transformed IFU numbers by linear regression and the paired *t* test. The different methods are summarized in Table 1.

Table 2 summarizes the results of different culture methods with 30 to 100 IFU of TW-183 as inoculum. Comparable results were obtained with 2 to 20 IFU as inoculum (data not shown). With some methods, the number of IFU was so high that a very conservative approximation was made. Each parameter investigated—pretreatment with PEG, prolonged incubation time, and additional centrifugation—resulted in a significant increase of numbers of IFU of *C. pneumoniae*. Method 6 (Table 1) resulted in an ~69-fold increase in the number of IFU compared to the standard method (Table 2). Method 7 (Table 1) resulted in even higher numbers of IFU (data not shown). However, TW-183 is a well-adapted laboratory strain. Therefore, the results obtained may not be representative for *C. pneumoniae* strains in general and certainly not for fresh isolates. Therefore, method 7 (Table 1) was also compared with the standard method (method 1) for three

TABLE 1. Different culture methods tested with *C. pneumoniae* TW-183

Method no.	Day(s) centrifugation was performed	Length of culture (day)	Pretreatment of HEp-2 monolayer with PEG[a]
1	0	3	No
2	0	3	Yes
3	0	4, 5, 6, or 7	No
4	0, 3	5, 6, or 7	No
5	0, 3, 4	6 or 7	No
6	0, 3–5	7	No
7	0, 3–5	7	Yes

[a] Pretreatment for 1 h with 7% PEG.

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TABLE 2. Increase in number of IFU of *C. pneumoniae* by different methods

No. of IFU by standard culture[a] (mean $\log_{10}$ ± SEM)	Adapted culture			Fold increase in no. of IFU[b]	P[c]
	Day(s) centrifugation was performed	Length of incubation (day)	No. of IFU (mean $\log_{10}$ ± SEM)		
2.05 ± 0.15	0[d]	3	2.44 ± 0.11	2.4	0.006
1.90 ± 0.05	0	7	2.59 ± 0.17	4.7	0.033
1.93 ± 0.06	0, 3, 4, 5	7	3.77 ± 0.08	69.4	0.005

[a] Standard culture, centrifugation day 0 and incubation until day 3.
[b] Fold increase in absolute numbers is calculated by subtracting the mean $\log_{10}$ IFU obtained by the standard method from that obtained by the adapted method and taking the inverse $\log_{10}$.
[c] By the *t* test for paired samples.
[d] With 1-h pretreatment of HEp-2 monolayer with 7% PEG.

additional *C. pneumoniae* strains, which were passaged after mouse passage for only three or four times on HEp-2. The strains used were 2023, 2043 (American Type Culture Collection), and UZG1 from M. van den Abeele, University Hospital, Ghent, Belgium. All three strains were kindly provided by T. Ossewaarde, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. For these strains, an even greater increase in the number of IFU was seen when the combination of pretreatment with PEG, centrifugation on days 0, 3, 4, and 5, and culture for 7 days was compared with the standard culture (Table 3). Results are shown only for the inoculum with 2 to 20 IFU. A very high number of cells of the monolayer was infected with *C. pneumoniae* by the optimized method, often with more than one inclusion per cell. Infection of the monolayers with the larger inoculum (30 to 100 IFU) resulted in disruption of the monolayer, leaving multiple *C. pneumoniae* particles visible on the coverslip after staining. The supernatant of these cultures was subcultured and contained a very high number of IFU. High percentages of PEG were toxic for the monolayer, but pretreatment with 7% PEG in Optimem did not influence the cell viability and enhanced the recovery of *C. pneumoniae* by a factor of 2.

TABLE 3. Increase in number of IFU for three mouse-propagated *C. pneumoniae* strains

C. pneumoniae strain	No. of IFU (mean $\log_{10}$ ± SEM)		Fold increase in no. of IFU[c]	P[d]
	Standard culture[b]	Optimized method[a]		
UZG1	0.85 ± 0.09	3.65 ± 0.34	631	<0.001
2023	1.36 ± 0.12	3.89 ± 0.32	339	0.002
2043	1.04 ± 0.09	3.61 ± 0.34	372	0.001

[a] Pretreatment of HEp-2 monolayers with 7% PEG, for 1 h, centrifugation on days 0, 3, 4, and 5 (with refreshment of culture medium on day 3), and incubation until day 7.
[b] Centrifugation day 0 and incubation until day 3.
[c] Fold increase is calculated by dividing the mean number of IFU obtained by the optimized method by that obtained by the standard method.
[d] By the *t* test for paired samples.

The increase in numbers of IFU seen after prolonged incubation is probably due to infection of new HEp-2 cells by elementary bodies released after the first multiplication cycle. The further improvement in yield of IFU after additional centrifugation is most likely due to a better contact between released elementary bodies and HEp-2 cells. All methods combined gave an enormous increase in the number of IFU. Therefore, we propose the following as the best culture method. Pretreat three cell monolayers, each inoculated with a part of the patient sample, with 7% PEG. Then, fix one monolayer at day 3. If the result is negative, incubate the second monolayer for 7 days, centrifuge on days 3, 4, and 5, and refresh the culture medium on day 3. The third monolayer can be used for further cultivation, in order to obtain *C. pneumoniae* strains for further investigation. This method should be further evaluated on clinical specimens.

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