

Optimizing Culture of *Chlamydia pneumoniae* by Using Multiple Centrifugations

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Three methods for the recovery of *Chlamydia pneumoniae* from spiked nasopharyngeal and blood specimens, including extended culture and additional centrifugations, were compared. Additional centrifugations and a 7-day culture time resulted in a 500- to 5,000-fold increase in the number of detectable inclusion-forming units.

Chlamydia pneumoniae, an obligate intracellular bacterium, is a causative agent for pneumonia, sinusitis, pharyngitis, and other respiratory disease (2–4). In addition, *C. pneumoniae* has been associated with atherosclerotic cardiovascular disease (1, 8, 9).

The method currently used by most investigators for culturing *C. pneumoniae* was first described in 1988 (6). Several investigators have reported modifications to this protocol to improve the recovery of *C. pneumoniae* from patient or mock specimens. Tjhe et al. reported that pretreatment of the host cell monolayer with polyethylene glycol (PEG) before inoculation increases recovery of *C. pneumoniae* slightly and that extending culture time to 7 days with additional centrifugations on days 3, 4, and 5 in combination with PEG pretreatment improves recovery by over 300-fold (10). Kazuyama et al. reported that pretreatment of patient specimens with trypsin before inoculation increased inclusion formation by 3 to 4 logs, depending on the strain used (5).

This study is a comparison of the standard, PEG pretreatment, and trypsin pretreatment methods for the recovery of *C. pneumoniae* from specimens inoculated with known concentrations of *C. pneumoniae*.

HEp-2 cells were cultured as previously described (12). Dram shell vials containing confluent HEp-2 cell monolayers were inoculated with 10^5 *C. pneumoniae* elementary bodies (strain CM1) by centrifugation at $1,000 \times g$ for 1 h and cultured for 72 h as previously described (6, 11).

After 72 h, a glass coverslip from one shell vial was fixed with methanol and stained with Pathfinder *Chlamydia* Confirmation stain (Kallstad, Chaska, Minn.) as recommended by the manufacturer, and the inclusions were visualized with a fluorescent microscope. *C. pneumoniae*-infected HEp-2 cells were then harvested from the remaining shell vials, pooled, diluted to the desired concentrations, and used to infect mock specimens.

Nasopharyngeal (NP) swab samples were collected from four healthy volunteers (health status determined by personal data). Swabs were placed in 3 ml of M4 transport medium (Microbes, Lilburn, Ga.) and vortexed for 1 min, and the swabs were removed. The NP specimens were spiked with 1,000 inclusion-forming units (IFU) of *C. pneumoniae*-infected HEp-2 cells per ml for preliminary experiments in shell vials or 300 IFU/ml for subsequent experiments in 96-well trays. These

concentrations were used to attain an easily countable number of inclusions.

Venous blood was collected from four healthy volunteers, inoculated with *C. pneumoniae* as described above, aseptically transferred to Vacutainer CPT cell separator tubes (Becton Dickinson, Franklin Lakes, N.J.), and processed as recommended by the manufacturer. After washing, the mononuclear cells were resuspended in 3 ml of sterile normal saline.

All specimens were then sonicated at 50 Hz for 15 s and held on ice until needed.

The three culture methods were compared as follows. HEp-2 cell-seeded shell vials were divided into groups corresponding to each culture method. One group of shell vials was pretreated with 7% PEG for 1 h at 37°C as previously described (10).

Each spiked specimen was divided into three aliquots—one for each culture method (standard, PEG pretreatment, and trypsin pretreatment). One aliquot of each specimen was pretreated with 0.1% trypsin for 30 min at 37°C as described by Kazuyama et al. (5). The remaining aliquots were left untreated. The specimen aliquots were inoculated into the appropriate shell vials, centrifuged, and incubated for 3 days. The coverslips were then fixed and stained, and the IFU were counted as described above.

In order to test enough replicates for statistical analysis, 96-well trays were used for all other experiments. Six 96-well trays were seeded with HEp-2 cells. NP and blood specimens were collected and processed as described above. A 50- μ l volume of each spiked specimen was inoculated onto eight untreated and eight PEG-pretreated monolayers as previously described (10). The row between each specimen set was not inoculated and served as a negative control for carryover or contamination between sets of specimens. The trays were centrifuged and incubated as described above for either 3 days, 7 days, or 7 days with additional centrifugation on days 3, 4, and 5 and with medium refreshment on day 3.

At the appropriate time point, the monolayers were fixed and stained, and the inclusions were counted as described above. In order to determine the increase in IFU after extended culture time and multiple centrifugations, a serial dilution titration was performed on infected HEp-2 cells harvested from a well for each culture method.

All analyses were performed by nonparametric procedures. The Wilcoxon rank sum test was used when comparing two methods. The Kruskal-Wallis test was used when comparing three methods. A *P* value of 0.05 or less was considered significant.

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TABLE 1. Number of chlamydial inclusions recovered from spiked NP and blood specimens (eight wells) after 3 days of incubation

Specimen type and no.	Median (range) no. of IFU recovered		<i>P</i> ^a
	Standard method	PEG pretreatment	
Blood			
1	10 (8–15)	5.5 (2–10)	0.0014
2	7 (4–11)	6 (5–10)	0.3917
3	24 (17–28)	17 (10–23)	0.0084
4	19 (15–32)	16.5 (10–24)	0.2261
NP			
1	4 (0–45)	4.5 (1–13)	0.9578
2	14.5 (7–35)	11 (3–14)	0.1015
3	10.5 (5–12)	11.5 (8–23)	0.2223
4	8 (5–20)	8.5 (5–19)	0.6657

^a Statistical analysis was performed with the Wilcoxon rank sum test, with *P* ≤ 0.05 considered significant.

Preliminary experiments with the three culture methods showed no significant differences in recovery of *C. pneumoniae* for NP mock specimens (data not shown). For spiked blood specimens, the PEG pretreatment method may have improved recovery, although the small sample size precluded definitive conclusions (data not shown). No significant difference was seen in recovery of *C. pneumoniae* between the trypsin pretreatment and standard methods. Since the trypsin pretreatment method did not improve recovery from either specimen type, it was not evaluated further.

Repeating the 3-day culture comparison of the standard and PEG pretreatment methods failed to show a significant differ-

ence in recovery from either type of spiked mock specimen (Table 1).

Extending culture time to 7 days without additional centrifugations did not improve recovery by either culture method (Table 2). However, extending the culture time with additional centrifugation on days 3, 4, and 5 dramatically increased the number of inclusions seen for both culture methods (Table 2). Titration of the cells harvested from wells after 7 days of culture with additional centrifugation showed a 500- to 5,000-fold increase in inclusions for both PEG-pretreated and unpretreated wells (data not shown). No inclusions were detected in the uninoculated control wells.

Extension of culture time to 7 days with additional centrifugations increased recovery of *C. pneumoniae* from mock specimens for both the standard and the PEG pretreatment culture methods, in agreement with the observations reported by Tjhie et al. (10). Tjhie et al. suggested that the additional centrifugations allowed for elementary bodies from lysed host cells to initiate new rounds of infection (10). This is likely, since centrifugation upon initial inoculation is necessary for *C. pneumoniae* to efficiently infect host cells (6). Extension of culture time to 7 days without additional centrifugation did not significantly improve recovery of *C. pneumoniae* from the mock specimens. Comparable numbers of inclusions were seen on days 3 and 7, which is in agreement with data reported by others (7, 10).

In this study, PEG pretreatment did not improve recovery of the organism over the standard method for NP mock specimens and only improved recovery from mock blood specimens in the first experiment. Subsequent experiments using more replicates for each specimen showed that PEG pretreatment did not increase the number of IFU detected.

TABLE 2. Number of chlamydial inclusions recovered from spiked specimens (eight wells) after 7 days of incubation

Specimen type and no.	Median (range) no. of IFU recovered		<i>P</i> ^a
	Standard method	PEG pretreatment	
NP			
No additional centrifugations			
1	64.5 (24–93)	37.5 (24–59)	0.0513
2	148.5 (115–221)	141.5 (87–189)	0.8335
3	118 (96–150)	110 (30–197)	0.7525
4	87.5 (57–126)	74.5 (50–133)	0.9163
Additional centrifugations			
1	>500 (>500)	>500 (>500)	ND ^b
2	>500 (>500)	>500 (>500)	ND
3	>500 (>500)	>500 (>500)	ND
4	>500 (>500)	>500 (>500)	ND
Blood			
No additional centrifugations			
1	4.5 (0–6)	4.5 (1–8)	0.9571
2	4.5 (1–7)	6 (0–12)	0.2911
3	16 (4–23)	11 (5–16)	0.1242
4	7 (1–21)	7 (0–26)	0.4929
Additional centrifugations			
1	164 (80–>300)	235 (100–>300)	0.3270
2	>500 (>500)	>500 (>500)	ND
3	>500 (>500)	>500 (>500)	ND
4	>500 (>500)	>500 (>500)	ND

^a Statistical analysis was performed with the Wilcoxon rank sum test, with *P* ≤ 0.05 considered significant.

^b ND, not determined.

Trypsin pretreatment did not significantly change recovery from either type of mock specimens, contrary to data reported by Kazuyama et al. (5). The experiments described by Kazuyama et al. included a range of trypsin concentrations, and the recovery results varied with each concentration and strain used (5). It is possible that the trypsin concentration needs to be optimized in each laboratory for this method to function properly.

The mock specimens used in this study were spiked with *C. pneumoniae* CM1, a laboratory-adapted strain. Culture-positive patient specimens are difficult to obtain, making an evaluation such as this study with true patient specimens difficult to perform. In addition, *C. pneumoniae* strains isolated from patient specimens may require growth conditions different from those of laboratory-adapted strains (7). For these reasons, the findings of this study should be confirmed with true patient specimens.

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REFERENCES

1. Danesh, J., R. Collins, and R. Peto. 1997. Chronic infections and coronary heart disease: is there a link? *Lancet* **350**:378–379.
2. Grayston, J. T., C. C. Kuo, S. P. Wang, and J. Altman. 1986. A new *Chlamydia psittaci* strain, TWAR, is isolated in acute respiratory tract infections. *N. Engl. J. Med.* **315**:161–168.
3. Grayston, J. T. 1992. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin. Infect. Dis.* **15**:757–763.
4. Grayston, J. T., L. A. Campbell, C. C. Kuo, C. H. Mordhorst, P. S. Saikku, D. H. Thom, and S. P. Wang. 1990. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. *J. Infect. Dis.* **161**:618–625.
5. Kazuyama, Y., S. M. Lee, K. Amamiya, and F. Taguchi. 1997. A novel method for isolation of *Chlamydia pneumoniae* by treatment with trypsin or EDTA. *J. Clin. Microbiol.* **35**:1624–1626.
6. Kuo, C.-C., and J. T. Grayston. 1988. Factors affecting viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. *J. Clin. Microbiol.* **26**:812–815.
7. Maass, M., and U. Harig. 1995. Evaluation of culture conditions used for isolation of *C. pneumoniae*. *Am. J. Clin. Pathol.* **103**:141–148.
8. Saikku, P., K. Mattila, R. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* **2**:983–986.
9. Saikku, P., M. Leinonen, L. Tenkanen, E. Linnanmaki, M. R. Ekman, V. Manninen, M. Manttari, M. H. Frick, and J. K. Huttunen. 1992. *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki heart study. *Ann. Intern. Med.* **116**:273–278.
10. Tjhhie, J. H. T., R. Roosendaal, D. M. MacLaren, and C. M. J. E. Vandembroucke-Grauls. 1997. 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. *J. Clin. Microbiol.* **35**:1883–1884.
11. Wong, K. H., S. K. Skelton, and Y. K. Chan. 1992. Efficient culture of *Chlamydia pneumoniae* with cell lines derived from the human respiratory tract. *J. Clin. Microbiol.* **30**:1625–1630.
12. Wong, K. H. 1993. Origin of HEP-2 cells used for culture of chlamydiae. *J. Clin. Microbiol.* **31**:470–471. (Letter.)