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Journal of Microbiological Methods 61 (2005) 17–24
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

High-yield culture and purification of Chlamydiaceae bacteria

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Received 29 April 2004; received in revised form 20 October 2004; accepted 27 October 2004
Available online 8 December 2004

Abstract

Research on intracellular bacteria of the family Chlamydiaceae, and the diseases they cause, requires large amounts of infectious elementary bodies (EB). We describe an approach that maximizes the generation of *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydia abortus*, or *Chlamydia pecorum* EBs in several replication cycles over approximately 10 days or more in a saturated equilibrium monolayer cell culture system. Buffalo Green Monkey Kidney (BGMK) cells, Human Epidermoid Carcinoma-2 (HEp-2) cells, or mouse McCoy cells were tested. BGMK cells best supported *C. pneumoniae* replication when cultivated in Iscove's Modified Dulbecco's Medium. From day 1 to day 9 after inoculation, *C. pneumoniae* genomes per ml culture medium increased from $10^{5.1}$ to $10^{8.6}$ in BGMK, from $10^{5.6}$ to $10^{8.1}$ in HEp-2, and remained at $10^{5.2}$ in McCoy cell cultures. Three-month pre-inoculation maintenance of BGMK cells in different culture media did not influence *C. pneumoniae* yields. Inoculation at multiplicities of infection (MOI) of 10 or higher and supplementation of the cell culture medium on day 7 after inoculation with 0.1% glucose enhanced *C. pneumoniae* EB yields in harvested cell culture medium. For purification, EBs in medium were concentrated by sedimentation, followed by low-speed centrifugation for removal of host cell nuclei, and by step-gradient centrifugation of the supernatant in a 30% RenoCal-76–50% sucrose step-gradient. Extensive sonication increased yield and infectivity of chlamydial EB. The combined method typically produced from 1000 ml infected BGMK culture medium 10 ml homogeneous, single-cell, highly infectious EB stock containing approximately 5×10^{11} *C. pneumoniae* genomes equivalent to $4–5\times10^{11}$ inclusion forming units.
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Keywords: Chlamydia pneumoniae; Culture; Purification; High yield

1. Introduction

Research on pathogenetic mechanisms of diseases induced by bacteria of the family Chlamydiaceae and identification of immunoprotective antigens requires large amounts of infectious elementary bodies. Numer-

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doi:10.1016/j.mimet.2004.10.020

ous reports about culture modalities attest to the difficulties associated with the generation of large quantities of chlamydial EB of these obligate intracellular bacteria, particularly for *Chlamydia pneumoniae* (Cles and Stamm, 1990; Kuo and Grayston, 1990; Wong et al., 1992). This organism typically produces little progeny, and production of large quantities of EB has been a problem. Typically, methods for propagation of chlamydiae not only rely on reagents to enhance infectivity (e.g., DEAE–dextran) and intracellular replication (e.g., cycloheximide) of chlamydiae, but also limit the viability of host cells. Such approaches typically entail a single infection of host cells followed by disruption of the infected cells before the destruction of the host cells by chlamydial replication (Caldwell et al., 1981). These methods have two disadvantages that severely limit the capacity for generation of chlamydial EBs: (i) the host cell population supports only a single cycle of chlamydial replication, and (ii) the disruption of the host cells necessitates extensive purification of chlamydial EBs, with an unavoidable loss of EBs.

In this report, we describe an approach that uses monolayer BGMK cells combined with an effective cell culture medium and glucose supplementation for optimum support of chlamydial replication in a 10-day or more equilibrated culture system. Harvest of the EB-saturated cell culture medium, but not of disrupted host cells, results in chlamydial EBs with little host cell contamination. Extensive sonication and a simple step-gradient centrifugation procedure produce highly concentrated, pure, homogeneous single-cell stocks of highly infectious chlamydial EBs.

2. Materials and methods

2.1. Cell culture

Cell lines frequently used for propagation of chlamydiae, Buffalo Green Monkey Kidney cells (Arens and Weingarten, 1981; Barron et al., 1970; Johnston and Siegel, 1992; Wills et al., 1984), Human Epidermoid carcinoma-2 cells (Kenney, 1993; Krech et al., 1989; Roblin et al., 1992; Tjhie et al., 1997), and mouse McCoy cells (Gordon et al., 1972; Johnston and Siegel, 1992; Krech et al., 1989; Wills et al., 1984) were purchased from Diagnostic Hybrids (Athens, OH). BGMK cells were maintained in Minimum Essential Medium with Earle's salts (EMEM) supplemented with 5% fetal bovine serum (FBS), 25 μg gentamycin/ml (Sigma, St. Louis, MO), 2 μg amphotericin B/ml (Invitrogen, Carlsbad, CA), and 8 μg/ml of the macrolide antibiotic tylosin (Sigma), at 36 °C with 5% CO_2 under humidity. Hep-2 and McCoy cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 36 mM sodium bicarbonate, 25 mM HEPES buffer, 0.45% glucose, 10% FBS, and antimicrobial supplements identical to EMEM.

For all experiments, the cells were grown as monolayers in 25- or 83-cm^2 flasks with 7.5 or 30 ml cell culture medium, respectively, at 36 °C with 5% CO_2 and 100% humidity. Monolayer cells were mobilized by 5 min trypsinization, and cell numbers were determined by with a hemocytometer. After seeding for use in chlamydial culture, cells were cultivated in media without tylosin.

2.2. Propagation of chlamydiae

C. pneumoniae strain CDC/CWL-029 (ATCC VR-1310, Manassas, VA) was used in all experiments to define the final methods, *Chlamydia trachomatis* serovar D strain UW-3/Cx (ATCC VR-885), *Chlamydia pecorum* strain E58 (ATCC VR-628), and *Chlamydia abortus* strain B577 (ATCC VR-656) were used for confirmation.

Chlamydial stock suspended in sucrose–phosphate–glutamate (SPG) buffer (Bovarnick et al., 1950) was placed in an ice water bath near the sonicator horn, sonicated at 250 W for 60 min, and suspended in 3 ml or 7 ml warm (36 °C) tylosin-free IMDM (Herbrink et al., 1991) with 10% FBS for inoculation of cell monolayers in 25- or 83-cm^2 flasks, respectively. Depending on the experiment, the cells were infected at an MOI of 4–20 (equal to about 4–20 IFU/host cell for 1 h sonication of the inoculum) chlamydial genomes copies per cell. Cell culture medium was removed by aspiration from the confluent cell monolayer, cells were washed once with 10 ml warm Hank's balanced salt solution (HBSS), HBSS was removed, and the inoculum was added. Culture vessels were taped to microtiter plate centrifuge carriers and centrifuged at 1300×*g* and 36 °C

for 1 h (Donati et al., 1995; Maass and Dalhoff, 1995). After resuspension of sediments by careful tapping of the flasks, the inoculum was removed by aspiration, the cells washed twice with HBSS, 7 ml or 30 ml tylosin-free IMDM+ 10% FBS containing a total of 0.45% (w/v) glucose was added and the flasks were incubated. In specific experiments, 20% sterile-filtered glucose in PBS was added after 4–9 days to increase glucose concentration in IMDM by 0.1%, 0.17%, 0.32%, 0.5%, 0.67%, or 1% (w/v). Chlamydial inclusion formation with blue-purple reticulate bodies and bright red elementary bodies in cell scrapings or culture supernatants obtained from infected flasks was evaluated by Giménez staining of methanol-fixed slide preparations (Giménez, 1964). This rapid method conformed very well with immunofluorescent staining of chlamydiae and allowed easy identification of adequately positive cultures.

2.3. Purification of chlamydial elementary bodies

Infected cell culture supernatants were first concentrated by centrifugation at 12,700×*g* for 30 min at 4 °C. Sediments of 50-ml round-bottom centrifuge tubes were resuspended in 5 ml cold SPG buffer by vortexing and by extensive 1 h sonication (250 W) of the tube in an ice water bath. Subsequent purification steps followed previously published methods (Caldwell et al., 1981; Howard et al., 1974; Miyashita and Matsumoto, 1992). Briefly, host cell nuclei and debris were separated from chlamydial elementary bodies by sedimentation at 500×*g* for 10 min at 4 °C. The supernatant was sonicated again for 10 min, and 22 ml were layered onto 10 ml 30% RenoCal-76 (Squibb, New Brunswick, NJ) in 30 mM Tris–HCl, pH 7.3, and a bottom layer of 10 ml 50% sucrose in 30 mM Tris–HCl, pH 7.3, in 50-ml centrifuge tubes. Step-gradient purification of chlamydial EB was achieved by centrifugation at 22,600×*g* for 1 h at 4 °C. After removal of the supernatant, the sediment of chlamydial EB and the turbid bottom layer were resuspended in 5 ml SPG buffer, and sonicated for 10 min at 250 W in ice water. The chlamydial EBs were washed twice with SPG buffer by centrifugation at 22,600×*g* for 30 min, resuspended in SPG buffer by vortexing and 10 min sonication in a final volume of 1/100 of the original volume of chlamydial cell culture medium, and stored in small aliquots at −85 °C.

2.4. Determination of C. pneumoniae genomes

Growth medium of infected cultures was collected daily (100 μl) for determination of *C. pneumoniae* genomes by quantitative *Chlamydia* 23S rRNA fluorescence resonance energy transfer (FRET) PCR in a LightCycler® real-time thermal cycler (DeGraves et al., 2003; Huang et al., 2001). For PCR quantification of *C. pneumoniae* genomes, total nucleic acids from culture media or chlamydial stocks were extracted by use of the High Pure PCR Template Preparation Kit (Roche Applied Sciences, Indianapolis, IN) and quantified by PicoGreen fluorometry (Molecular Probes, Eugene, OR).

2.5. Determination of C. pneumoniae inclusion forming units

Chlamydial inclusion forming units (IFU) as a measure of the infectivity of *C. pneumoniae* stocks were determined by coverslip culture of BGMK cells in shell vials (Huang et al., 1999). Serial dilutions of stocks were inoculated by centrifugation at 1300×*g* and 36 °C for 60 min onto confluent monolayers, and 30 h later the cells were methanol-fixed and stained with FITC-conjugated monoclonal antibody against chlamydial LPS (BioRad, Hercules, CA). The mean number of inclusions per eyefield was determined by epifluorescence microscopy, and the IFU were calculated per ml of original chlamydial stock.

2.6. Host cell contamination of C. pneumoniae stock

The degree of host cell contamination of purified *C. pneumoniae* stock was evaluated by comparative hybridization of non-infected BGMK cell DNA or *C. pneumoniae* EB DNA dot-blotted on nitrocellulose membrane with non-infected BGMK DNA. Purified BGMK cell DNA was labeled with digoxigenin by use of a kit according to manufacturer's instructions (Roche Applied Sciences). Dot blots containing 1 μg, 100 ng, 10 ng, 1 ng, or 100 pg of BGMK cell or *C. pneumoniae* DNA were probed with the labeled DNA, and the disappearance of the signal from the *C. pneumoniae* DNA in relation to the BGMK DNA was used to estimate the relative BGMK DNA content of *C. pneumoniae* DNA.

2.7. Mouse infectivity of C. pneumoniae stock

The infectivity of the *C. pneumoniae* stock prepared in the final protocol was evaluated by determination of the 50% lethal dose (LD_{50}) in a mouse lung disease model (Huang et al., 1999). Four groups of five female, 10-week-old A/J mice were infected intranasally under light isofluorane anesthesia with 60 μl of undiluted stock (6.2×10^{10} *C. pneumoniae* genomes per ml), or of stock 1:2, 1:4, or 1:8 diluted in SPG buffer. Death of mice was recorded, and the surviving animals were sacrificed 10 days after inoculation. The LD_{50} was calculated by logistic regression analysis of mortality. All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

3. Results and discussion

3.1. Optimal cell line

In an initial experiment, we evaluated three cell lines commonly used for culture of chlamydiae for their effectiveness in supporting replication of *C. pneumoniae*. Confluent monolayers of BGMK, HEp-2, or McCoy cells in triplicate 25-cm^2 culture flasks were inoculated with *C. pneumoniae* at an MOI of 10 by centrifugation at $1300\times g$ and 36 °C for 1 h. After removal of the inoculum, 10 ml IMDM with 10% FBS was added and the cultures were incubated at 36 °C and 5% CO_2. One-hundred microliter samples of cell culture medium were collected daily, nucleic acids were extracted, and the copy number of *C. pneumoniae* genomes were determined by *C. pneumoniae* 23S rRNA FRET qPCR.

Results shown in Fig. 1 indicate that *C. pneumoniae* genomes per ml medium increased from $10^{5.1}$ to $10^{8.6}$ in BGMK, from $10^{5.6}$ to $10^{8.1}$ in HEp-2, and remained at $10^{5.2}$ in McCoy cell cultures. The growth curves for both BGMK and HEp-2 cells indicate saturation characteristics of chlamydial replication in these cell cultures, while no net replication was observed in McCoy cells. These data suggest that after a period of logarithmic increase in chlamydial organisms, these infected cell cultures reach a steady-state equilibrium between chlamydial replication and decline of the host cell population due to chlamydial

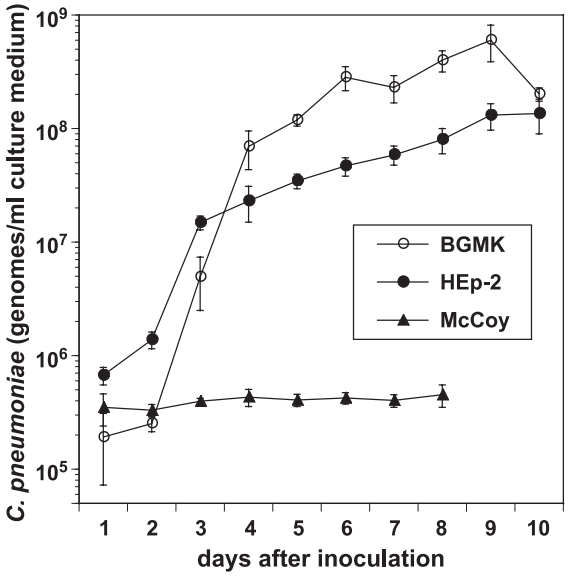

Fig. 1. Time dependence of *C. pneumoniae* concentrations in culture medium of three cell lines. The copy number of *C. pneumoniae* genomes was determined by *C. pneumoniae* 23S rRNA FRET qPCR. Concentrations of *C. pneumoniae* genomes per ml are shown as mean±S.D. Data for McCoy cell are shown only until day 8 because the cells detached from the flasks after that time. Differences in *C. pneumoniae* concentrations are significant at $p<0.05$ for all data points with non-overlapping standard deviation (Student's *t*-test).

replication. Typically, this equilibrium broke down at approximately day 12 when most of the cells died if the culture medium was not replaced. The inclusions in BGMK cells were typically larger than those of HEp-2, and particularly of McCoy cells, in which most inclusions contained only single or few chlamydial organisms. The percentage of BGMK cells with large productive inclusions densely packed with chlamydial EBs increased from less than 20% on day 4 to more than 80% on day 9. The percentage of large inclusions on day 9 was 10–20% lower in HEp2 cells than in BGMK cells. McCoy cells never showed large inclusions, even if most cells were infected. BGMK cells displayed contact inhibition without rounding and piling of cells that was strongly observed in McCoy and weakly in HEp-2 cell cultures. Therefore, they could be stored as confluent monolayer of cells, ready to use, for at least 10 days (Johnston and Siegel, 1992). Thus, BGMK cells clearly supported *C. pneumoniae* multiplication

optimally and were easiest to handle, and were selected for further experiments on optimum propagation of *C. pneumoniae*.

3.2. Maintenance of BGMK cells prior to *C. pneumoniae* inoculation

To investigate if pre-inoculation culture conditions selected for differential abilities to support chlamydial replication, we passaged BGMK cell for 3 months in rich (IMDM with 10% FBS) or standard medium (EMEM with 5% FBS). After inoculation, the concentrations of *C. pneumoniae* in culture medium of cells passaged in rich or standard medium did not differ. Therefore, we routinely passaged pre-inoculation BGMK cells in cost-efficient EMEM with 5% FBS.

3.3. Effect of multiplicity of infection on *C. pneumoniae* replication

The importance of the inoculum of infectious EBs per cell (MOI) on chlamydial replication and yields has been shown before (Meijer et al., 1996; Wyrick and Brownridge, 1978). We examined this question in our culture system by inoculating BGMK cell cultures after serial dilution of the inoculum with different MOIs of *C. pneumoniae* and determining the time course of the number of *C. pneumoniae* genomes in culture medium. MOIs of 10 and 20 resulted in significantly ($p<0.05$) higher production of *C. pneumoniae* organisms between days 2 and 9 than an MOI of 4 (Fig. 2). The parallel, but delayed chlamydial growth curve of 4 MOI inoculation suggests that at low MOI additional chlamydial replication cycles are required to achieve chlamydial concentrations similar to high MOI inoculation. Thus, low MOI inoculation suboptimally utilizes the host cell capacity to support chlamydial replication by resulting in delayed culture saturation equilibrium, with potentially lower chlamydial concentration. Therefore, we routinely infected BGMK cells with MOIs of 10 in subsequent experiments.

3.4. Sonication of chlamydial EBs

Earlier investigations had indicated that sonication of inocula increased numbers of chlamydial inclusions

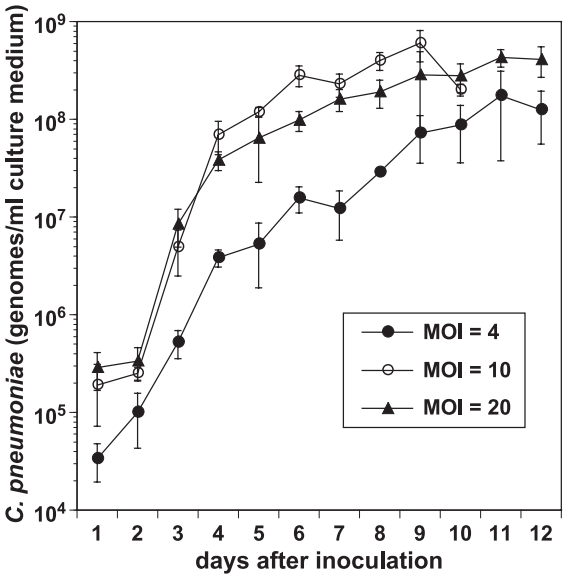

Fig. 2. Effect of the multiplicity of infection (MOI) on *C. pneumoniae* yields in BGMK cell culture. Triplicate confluent BGMK cell monolayers were inoculated with *C. pneumoniae* at MOIs of 4, 10, or 20. MOIs of 10 and 20 result in significantly higher production of *C. pneumoniae* organisms (±S.D.) between days 2 and 9 after inoculation ($p<0.05$, Student's *t*-test) than an MOI of 4. The culture at 20 MOI was terminated on day 10 because cells had died after day 9 sampling.

and chlamydial yields, but sonication at energy levels above approximately 1200 Ws/ml reduced the chlamydial yield significantly (Christoffersen et al., 1990; Warford et al., 1985). When we examined infected culture media, and particularly sediments after centrifugation at 12,700×*g* for concentration of the culture harvests, we noticed many clumps of chlamydial EBs, frequently attached to cell membranes. We assumed that more extensive sonication would break up these clumps, and extended sonication to 1 h. The tube with the resuspended sediments was placed near the horn of the sonicator (Branson sonifier, Model 102C) in an ice water bath, and sonication was performed at maximum power. We reasoned that the physical distance between sonicator horn and chlamydial EBs in this arrangement prevented damage of EBs by sonication that would result in reduced infectivity. This extended sonication approach resulted in increased yields during purification, as judged by quantitative *Chlamydia* 23S rRNA PCRs of culture supernatants and Giménez stains of chlamy-

dial preparations at each step, and homogeneous stocks of uniformly dispersed EBs.

In a separate experiment, we analyzed the influence of sonication on the infectivity of purified *C. pneumoniae* stocks containing 6.2×10^{10} and 2.92×10^{10} *C. pneumoniae* genomes per ml, respectively. Stocks were thawed and sonicated for 10 min or 1 h at 250 W in an ice water bath. The infectivity of the stocks was determined by centrifugal inoculation of triplicates of dilutions of the stocks onto BGMK cell monolayers in shell vials, followed by fluorescent staining of intracellular chlamydial inclusions after 30 h with monoclonal antibody against chlamydial LPS. IFU data in Fig. 3 indicate that intensive sonication for 1 h significantly ($p<0.002$) increased infectivity of both *C. pneumoniae* stocks by approximately 33% as compared to sonication for 10 min (6.2×10^{10} genomes stock: 4.26×10^{10} vs. 5.64×10^{10} IFU; 2.92×10^{10} genomes stock: 2.39×10^{10} vs. 3.18×10^{10} IFU). The data show also a high infectivity per chlamydial genome, approaching equality between

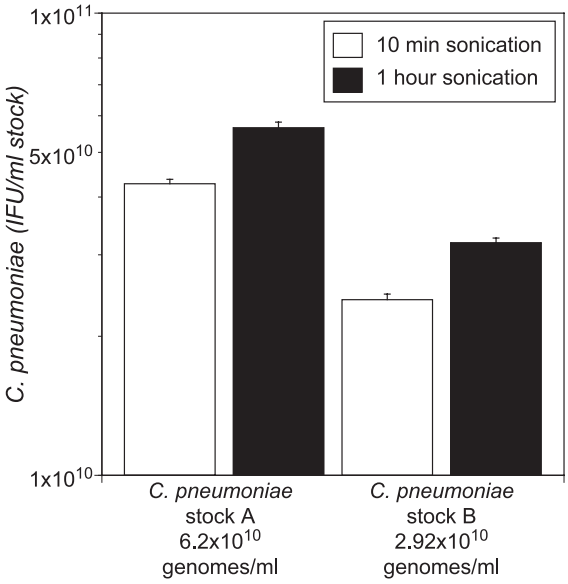

Fig. 3. Influence of sonication intensity on infectivity of *C. pneumoniae* stocks. The infectivity of the stocks (IFU±S.D.) was determined by centrifugal inoculation of triplicates of dilutions of the stocks onto BGMK cell monolayers in shell vials, followed by fluorescent staining of intracellular chlamydial inclusions after 30 h with monoclonal antibody against chlamydial LPS. Both stocks have a significantly ($p<0.002$, Student's *t*-test), approximately 33% higher infectivity after 1 h sonication than after 10 min sonication.

chlamydial genomes and infectious units. Based on the finding that long sonication described above increased chlamydial yields as well as infectivity, we incorporated extensive sonication at every step in the purification procedure as well after thawing of frozen stock.

3.5. Glucose supplementation

Addition of glucose to chlamydial culture media has been shown to enhance chlamydial replication and yields (Harper et al., 2000; Iliffe-Lee and McClarty, 2000). We also observed markedly enlarged inclusions and increased concentrations of *C. pneumoniae* after supplementation of culture medium at various time points before harvest of infected culture medium. In preliminary experiments, we found that addition of glucose 3 days before harvest, on day 7 after inoculation, appeared to maximize *C. pneumoniae* yields, without accelerating lysis of cells.

In a concluding experiment, we asked which amount of glucose added to the culture medium maximized the chlamydial yields in *C. pneumoniae* stocks propagated and purified in a final, optimal protocol. Confluent BGMK cell monolayers in triplicate 83-cm^2 cell culture flasks were centrifugally inoculated with 10 MOI *C. pneumoniae*, incubated with 30 ml IMDM containing 10% FBS, additional glucose was added after 7 days in different concentrations to flasks, and the cell culture medium was harvested on day 10. Preparation of purified *C. pneumoniae* stocks followed the optimal protocol as described in Section 2.3.

As evident in Fig. 4, concentrations of *C. pneumoniae* genomes per ml stock indicate that addition of approximately 0.1% glucose on day 7 is optimal and results in final *C. pneumoniae* stocks containing significantly ($p=0.037$), approximately 17-fold higher amounts of chlamydiae than stocks produced from cell culture harvest without glucose supplementation. Therefore glucose supplementation at 0.1% approximately 3 days prior to harvest of infected cell culture medium was incorporated in the standard procedure.

3.6. Confirmatory analyses

We estimated the amount of host cell contamination of *C. pneumoniae* stock by probing serially diluted

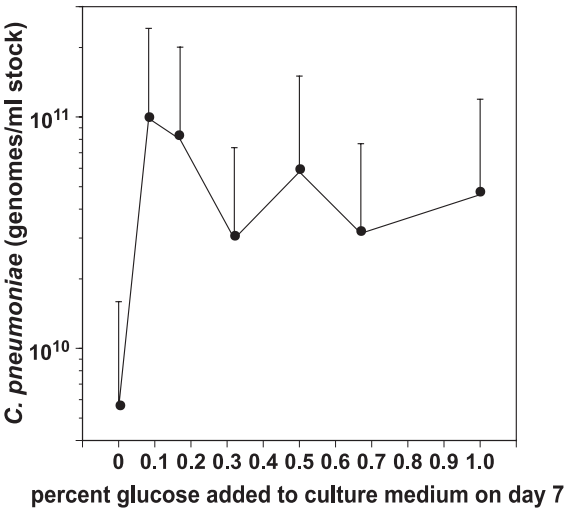

Fig. 4. Influence of glucose supplementation of the cell culture medium on the overall *C. pneumoniae* yield in concentrated stock preparations. Concentrations of *C. pneumoniae* genomes per ml stock (±S.D.) were consistently higher from glucose-supplemented cultures than from cell culture without glucose supplementation (0.1% glucose supplementation: $p<0.037$, Student's *t*-test).

BGMK cell and purified *C. pneumoniae* DNA with digoxigenin-labeled BGMK cell DNA. Dot blots of 1 μg, 100 ng, 10 ng, 1 ng, or 100 pg of BGMK cell showed a strong signal, while only dots with 1 μg, 100 ng, 10 ng, but not with 1 ng, or 100 pg of *C. pneumoniae* DNA showed a signal. This indicates a low contamination of *C. pneumoniae* DNA with less than 1% BGMK cell DNA.

To estimate the biological potency of the *C. pneumoniae* stock, we determined the mouse intranasal 50% lethal dose by inoculating four groups of five A/J mice. All mice of the highest inoculation group (inoculum per mouse 1.86×10^9 *C. pneumoniae* genomes), two mice of the next, and one mouse of the third dilution group died between days 3 and 7 after inoculation, all other animals survived. Logistic regression analysis indicated that intranasal challenge with 1.745×10^9 genomes of purified *C. pneumoniae* was the lethal dose for 50% of intranasally inoculated mice. This indicates a potent, highly concentrated inoculum since it is typically not possible to lethally infect mice with *C. pneumoniae.*

We confirmed the overall chlamydial culture-purification protocol by propagating other chlamydial strains in BGMK cells. For *C. abortus*, all parameters were identical to *C. pneumoniae*. Slower replication kinetics of *C. trachomatis* and cytotoxicity of *C. pecorum* required modifications of glucose supplementation and harvest time points. For *C. trachomatis*, glucose was added on day 10, and culture medium was harvested on day 14. *C. pecorum* was highly cytocidal and therefore most difficult to propagate to high concentrations in culture medium. A short-term culture of only 5 days after high MOI inoculation without centrifugation was required for harvests with little cell debris, and glucose supplementation was not beneficial under these conditions. Chlamydial concentrations in *C. abortus* and *C. trachomatis* stocks were similar to those of *C. pneumoniae* stocks, but concentrations of *C. pecorum* stocks were approximately 10 times lower.

In summary, we describe here an approach that maximizes the generation of chlamydial EBs in several replication cycles over 10 days or more in a steady-state equilibrium culture system. Monolayer BGMK cells serve as host cells with robust support of chlamydial replication, with higher yields than HEp2 cells as recently described by Mukhopadhyay et al. (2004). And directly sonicated cultivation cell line would not good to the operator's health. Supplementation of the cell culture medium with glucose 3 days before harvest of the medium strongly enhances chlamydial yields. Harvest of the cell culture medium prior to large-scale lysis of the host cells results in a population of chlamydial EBs with little host cell contamination that requires only a simple step-gradient purification procedure for production of highly pure, homogeneous single-cell stocks of highly infectious chlamydial EBs. We are aware that many protocols for purification of chlamydial EBs, including that of Mukhopadhyay et al. (2004), disrupt infected cells to increase chlamydial yields. In our experience, the potential gain is balanced by the need for more thorough purification with an associated loss of EBs. Extensive sonication ensures a single cell suspension of chlamydial EB with vastly improved infectivity over purification protocols with less sonication (Huang et al., 2001). The combined culture-purification procedure from 1000 ml infected BGMK culture medium typical results in yields of 10 ml EB stock containing a total of approximately 5×10^{11} C. pneumoniae genomes equivalent to $4–5\times10^{11}$ inclusion forming units.

Acknowledgements

This investigation was supported by NIH Grant AI47202 to B.K.

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