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Original Article

Factors Improving the Propagation of *Simkania negevensis* Strain Z in Cell Culture

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SUMMARY: The purpose of the present study was to develop an optimal method for culturing *Simkania negevensis*. Centrifugation was effective for the propagation of *S. negevensis*, but sonication was not effective. The addition of cycloheximide to the culture medium significantly decreased the number of inclusions. Pretreatment of host monolayers with diethylaminoethyl-dextran or polyethylene glycol was detrimental. The most optimal conditions were centrifugation of the inocula onto untreated Vero cells, and culture in RPMI 1640 medium containing 10% fetal calf serum without cycloheximide or antimicrobial agents.

INTRODUCTION

Simkania negevensis, which was first described as "the chlamydia-like microorganism Z" by Kahane et al. in 1993, is an obligate intracellular Gram-negative bacterium (1). This newly characterized microorganism has been associated with bronchiolitis in infants (2) and community-acquired pneumonia in adults (3), and is widespread in Israel, North America, and Western Europe (4-6).

Recent studies of the characteristics of *S. negevensis* growth, including the details of the replication cycle and drug sensitivities, have been reported. Kahane et al. described a culture method for *S. negevensis* (1,7,8), which included a culture medium with a high concentration of fetal calf serum (FCS) (15%), a low concentration of glucose (1%), and 1 μg/ml of cycloheximide. However, they have not considered centrifugation, sonication, or pretreatment of host cells with chemicals.

A number of methods have been demonstrated to facilitate *Chlamydiaceae* propagation in cell culture. Ten percent FCS is appropriate for chlamydial culture in terms of sensitivity and cost effectiveness. Some investigators have reported that pretreatment of the host cell monolayer with polyethylene glycol (PEG) increased the yields of *Chlamydia pneumoniae* and *Chlamydia trachomatis* (9-12). Pretreatment with diethylaminoethyl (DEAE)-dextran enhanced chlamydial infectivity depending on the strains or cell lines used (11,13-15). The addition of cycloheximide to the inoculation medium has been proven to increase the yields of *C. pneumoniae* and *C. trachomatis* (16,17). Several reports have demonstrated that sonication or centrifugation enhanced cell culture infectivity (11,17-20). Although this information is useful for chlamydial cell culture, no optimal culture method for *S. negevensis* has been fully defined to date.

In the present study, we developed an optimal culture method for *S. negevensis* by applying factors known to improve the growth of *Chlamydiae*.

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MATERIALS AND METHODS

***S. negevensis* and cell line**: We obtained *S. negevensis* Z (VR-1471) from the American Type Culture Collection. Inocula were diluted with sucrose-phosphate-glutamate (SPG) medium (sucrose, 75 g; KH_2PO_4, 0.52 g; $NaHPO_4$, 1.22 g; glutamic acid, 0.72 g; H_2O to 1 liter; pH 7.4 to 7.6). The titers of the inocula were adjusted to 4.0×10^4 IFU (inclusion forming unit) per ml. After adjustment, the inocula were stocked at −80°C until use. Vero cells used as host cells were kindly provided by Dr. S. Saika (Chiba Prefectural Institute of Public Health, Japan).

Culture of *S. negevensis*: The culturing of *S. negevensis* was carried out according to modifications of the method described by Kahane et al. (7). Vero cells were seeded in 24-well tissue culture plates containing round cover slips (14 mm in diameter). RPMI 1640 medium (GIBCO Invitrogen, Tokyo, Japan) supplemented with 10% FCS (JRH BIOSCIENCE, Lenexa, Kans., USA), and 0.2% sodium bicarbonate was used as a control culture medium. Two day-old monolayers were examined for confluency and the medium was removed prior to inoculation. A 0.25 ml of inocula containing 1.0×10^4 IFU was added to each well, and the plate was incubated for 5 more days after fresh culture medium was replaced to the wells.

Preparation of anti-*S. negevensis* sera of rabbits: Hyperimmune sera against *S. negevensis* were prepared in two rabbits by a single intracutaneous injection with a mixture of *S. negevensis* antigen and Freund's complete adjuvant, and seven intracutaneous injections with a mixture of *S. negevensis* antigen and Freund's incomplete adjuvant. The intervals between immunizations were 7 to 14 days.

Inclusion stain: After incubation for 5 days, the cover slips

Table 1. Effects of sonication and centrifugation on the growth of *S. negevensis*

treatment	experiment no. (inclusions per 30 fields)			mean	P[3]
	1	2	3		
none	152	125	154	144	
sonication[1]	141	151	138	143	0.982
centrifugation[2]	628	511	491	543	0.0102
both	651	564	517	577	0.00813

[1]: Inocula were sonicated at 20 kHz for 20 s with an ultrasonic liquid processor before being applied to host cells.
[2]: After inoculation with *S. negevensis*, the culture plate was centrifuged at 1,500 × g for 60 min at 35°C.
[3]: Statistical analysis was performed using Student's *t* test for paired samples, with $P < 0.05$ considered as significant.

Table 2. Comparison of different culture media used for the growth of *S. negevensis*

medium no.	additional components of culture media			experiment no. (inclusions per 30 fields)			mean	P[3]
	FCS concentration (%)	antimicrobial agents[1]	cycloheximide[2]	1	2	3		
control	10	No	No	651	564	517	577	
1	10	No	Yes	534	494	415	481	0.0201
2	10	Yes	Yes	482	509	373	455	0.0712
3	15	No	No	595	662	547	601	0.644
4	15	Yes	Yes	521	526	384	477	0.0845

[1]: The following antimicrobial agents added to the samples: 100 μg/ml of streptomycin and 100 μg/ml of vancomycin.
[2]: Supplemented cycloheximide was 1.0 μg/ml.
[3]: Statistical analysis was performed using Student's *t* test for paired samples, with $P < 0.05$ considered as significant.

were fixed with methanol. They were then incubated with rabbit polyclonal hyper-immune sera raised against *S. negevensis* for 1 h at 37°C, followed by washing with PBS and staining with mouse anti-rabbit fluorescein isothiocyanate-conjugated monoclonal immunoglobulin G (IgG) (F-4151; Sigma-Aldrich Japan KK, Tokyo, Japan). Inclusions were counted using a fluorescence microscope (OPTIPHOT-2; Nikon, Tokyo, Japan) at ×200 magnification and inclusions in 30 fields were counted. Each method was tested in triplicate. The results showed the average count from three cover slips.

Sonication and centrifugation: The inocula were sonicated at 20 kHz for 20 s with an ultrasonic liquid processor (Sonicator XL2020; Misonix Inc., Farmingdale, N.Y., USA) before application to the host cells. Centrifugation of *S. negevensis* onto the culture plate was performed at 1,500 × g for 60 min at 35°C (Centrifuge RB-18 IV; Tomy Seiki Co., Ltd., Tokyo, Japan). The cell culture was incubated for another 60 min at 37°C with 5% CO_2 in a humidified cabinet. After removal of the inocula, the cell culture was incubated with RPMI 1640 supplemented with 10% FCS and 0.2% sodium bicarbonate, which was free from antimicrobial agents and cycloheximide. When the monolayers of inoculated Vero cells were not subjected to centrifugation, they were incubated for 120 min at 37°C with 5% CO_2 in a humidified cabinet.

Concentration of FCS: RPMI 1640 medium containing 10 or 15% FCS was used to evaluate the effects on the propagation of *S. negevensis*.

Additional components of the culture medium: The antimicrobial agents, streptomycin and vancomycin, and cycloheximide (C-6255; Sigma) were dissolved in distilled deionized water, and were added to the "control culture medium" at final concentrations of 100 μg/ml of both streptomycin and vancomycin, and 1.0 μg/ml of cycloheximide.

Pretreatment of Vero cells: PEG and DEAE-dextran were used for the pretreatment of Vero cells. PEG (P-3640, Sigma; average molecular weight: 3,350) was dissolved in distilled deionized water at a concentration of 7% (wt/vol). DEAE-dextran (D-9885, Sigma; molecular weight: 5×10^5) was dissolved in Hanks' balanced salt solution at a concentration of 30 μg/ml. Prior to inoculation, confluent monolayers of Vero cells were incubated with PEG for 60 min at 37°C or DEAE-dextran for 15 min at room temperature, respectively. After treatment, each of the chemicals was removed prior to inoculation.

Statistical analysis: Student's *t* test was used for the comparison of inclusion numbers.

RESULTS

Sonication and centrifugation: The results of the four different methods employed for the propagation of *S. negevensis* in combination with sonication and centrifugation are summarized in Table 1. The number of inclusions observed after centrifugation was significantly greater than when centrifugation was omitted. Sonication was not found to be more effective than the control.

Comparison of culture media: To evaluate the optimal culture medium for *S. negevensis*, various culture media with different components were used. Each culture method included a sonication and centrifugation procedure. The number of inclusions decreased significantly when cycloheximide was added to the control culture medium. There was no significant difference between groups in terms of growth with FCS concentrations of 10 or 15% (Table 2).

Pretreatment of Vero cells: Pretreatment with either PEG or DEAE-dextran significantly decreased the number of *S. negevensis* inclusions (Table 3). In these experiments, PEG was found to damage the monolayers.

Table 3. Effects of the pretreatment of Vero cells on the growth of *S. negevensis*

pretreatment	experiment no. (inclusions per 30 fields)			mean	P[3)]
	1	2	3		
none	651	564	517	577	
PEG[1)]	372	305	255	311	0.000545
DEAE-dextran[2)]	406	366	388	387	0.0298

[1)]: Prior to inoculation, host cells were incubated with PEG for 60 min at 37°C.
[2)]: Prior to inoculation, host cells were incubated with DEAE-dextran for 15 min at room temperature.
[3)]: Statistical analysis was performed using Student's *t* test for paired samples, with $P < 0.05$ considered as significant.

DISCUSSION

S. negevensis is known to have a characteristic replication cycle and drug sensitivity profile (7,8). However, this organism is in the early stages of investigation, and only a few clinical isolates have been obtained. The development of an appropriate method to obtain live strains of *S. negevensis* from clinical specimens is important for the success of future study of this organism.

Sonication of the inocula did not have a significant effect on growth of the cell culture. Sonication has been reported to enhance the infectivity of both clinical specimens and adapted strains of *Chlamydiaceae* (19,20). It is assumed that sonication destroys chlamydial inclusions containing infectious particles and disperses chlamydial particles that aggregate, thereby enhancing the adsorption of infectious particles onto host cells. However, in the present *S. negevensis* cultures, the sonication step appears to have been unnecessary. The reason for this unexpected result remains unclear. We speculated that since infectious particles of *S. negevensis* are fragile, some of them might have lost their infectivity during sonication treatment. On the other hand, centrifugation of inocula onto host cells significantly enhanced the infectivity of *S. negevensis*, in agreement with observations using chlamydiae (11,17,18). Centrifugation is expected to bring *S. negevensis* particles in contact with host cells. However, Kahane et al. reported that the centrifugation step was not necessary for culturing *S. negevensis* (1). This difference is thought to be dependent on the centrifugation speed.

Our results demonstrated that the addition of cycloheximide significantly reduced the number of *S. negevensis* inclusions. These results are in contrast with earlier reported results from studies of chlamydiae (17,21). Cycloheximide is known to inhibit the metabolism of host cells (22), but not that of chlamydiae (23). Therefore, this effect might be unfavorable for the growth of *S. negevensis* in cell culture. However, in another chlamydial report, the addition of cycloheximide had no significant effect on the number of infected cells, but it greatly enhanced the yield of infectious progeny per infected cell (24). Although we assessed the number of inclusions in the present study, it was still necessary to analyze the infectious progeny in the inclusions. Concentrations of FCS between 10 and 15% have no effect on the number of inclusions, thus demonstrating that nutrient-rich media were not required for the propagation of *S. negevensis*.

Pretreatment with either PEG or DEAE-dextran improves the growth of chlamydiae. Although one report has indicated that the optimal concentration of PEG was 35% (10), other reports have indicated that this concentration was toxic to host cells (11,12). Therefore, we chose a concentration of 7% PEG, as it has also been reported to be the optimal concentration (12). However, our results did not indicate that pretreatment with PEG improved the growth of *S. negevensis*. In contrast, PEG treatment markedly decreased the number of inclusions. It was already known that high-molecular-weight molecule PEG reduced the dielectric constant and therefore increased the hydrophobicity of the cell membrane. This characteristic facilitates the fusion of infectious particles to host cell membranes (25). Our results suggest that PEG has a cytotoxic effect on Vero cells at a 7% concentration during the growth of *S. negevensis*.

Several investigations have shown that the optimal concentration of DEAE-dextran was 30 μg/ml (13). Our results using this concentration revealed a significant decrease in the number of inclusions. Kuo et al. reported that because the surfaces of both host cells and trachoma-inclusion conjunctivitis organisms are negatively charged, the pretreatment of cell monolayers with a polycation such as DEAE-dextran would enhance the adsorption of the organism onto host cells. Our results of pretreatment with DEAE-dextran did not agree with earlier observations of chlamydiae (13). The reasons for this difference remain unclear; however, we speculate that this difference in the effect of DEAE-dextran depends on differences in the surface structures among strains of *Chlamydiale*, including *S. negevensis*. Pretreatment with DEAE-dextran, used for the propagation of *S. negevensis*, might inhibit the adsorption of this microorganism. Further investigation is needed to clarify the mechanism of this phenomenon. The results of the present study have provided evidence that pretreatment with either DEAE-dextran or PEG should be avoided during the propagation of *S. negevensis*.

In conclusion, we provide a method to obtain the most optimal conditions for the propagation of *S. negevensis*. This method includes the centrifugation of inocula onto Vero cells and the use of RPMI 1640 medium supplemented with 10% FCS. The addition of cycloheximide to the medium and pretreatment of Vero cells should be omitted when using the present method. The addition of antimicrobial agents, such as 100 μg/ml of streptomycin and vancomycin, to the culture medium may be needed to isolate *S. negevensis* from clinical specimens. Further studies using clinical specimens will be required to further evaluate the usefulness of the culture method proposed in the present study.

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