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Factors Influencing the Infectivity of *Chlamydia pneumoniae* Elementary Bodies on HL Cells

J. J. H. THEUNISSEN,[1]* B. Y. M. VAN HEIJST,[2] J. H. T. WAGENVOORT,[2] E. STOLZ,[1] AND M. F. MICHEL[2]

Departments of Dermato-Venereology[1] and Clinical Microbiology,[2] Erasmus University, Rotterdam, The Netherlands

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The influence of variations in the pH, NaCl concentration, temperature, and concentrations of calcium and magnesium ions on the survival of *Chlamydia pneumoniae* elementary bodies (EBs) outside the host cells was investigated. The survival was determined after various incubation periods by counting the inclusion-forming units after *C. pneumoniae* was cultured for 72 h on monolayers of HL cells. The normal physiological conditions were restored prior to infecting the HL cells with *C. pneumoniae*. Declines in the infectivities of *C. pneumoniae* EBs were observed at pH values of lower than 5 and higher than 8 or at NaCl concentrations of less than 80 mM. The viability of *C. pneumoniae* EBs in SPG medium decreased as the temperature and/or incubation period increased. Incubation temperatures of up to 20°C and incubation periods of up to 48 h did not affect the viability of *C. pneumoniae*. One hundred percent of the *C. pneumoniae* EBs were infective after 1 h of incubation at 35°C, whereas 90, 50, and 40% survived after incubations of 8, 24, and 48 h, respectively. The viability of *C. pneumoniae* was unaffected within the investigated range of Ca^{2+} and Mg^{2+} ion concentrations in the medium. The presence of 10% fetal calf serum in the incubation medium had a stabilizing effect on the viability of *C. pneumoniae*. This effect became more pronounced as the incubation period increased.

Chlamydia pneumoniae, previously known as *Chlamydia psittaci* TWAR (5), is an important cause of respiratory complaints in adults (11). *C. pneumoniae* epidemics have been observed both in local communities and across entire nations (8, 13). Since the initial isolation of *C. pneumoniae* from patient material is tedious, the diagnosis is generally based on the presence of acute-phase antibodies (6). In contrast to *Chlamydia trachomatis*, which requires mucous membrane to mucous membrane contact for its transmission, *C. pneumoniae* is probably transmitted via aerosols. *C. pneumoniae* infections are not dependent on the season (15). The microbe must be able to withstand physical changes in its environment in order to survive outside its host.

Changes in pH, salt concentration, temperature, and concentrations of Ca^{2+} and Mg^{2+} ions were investigated in order to establish the limit(s) to which these parameters affected the infectivity of *C. pneumoniae* elementary bodies (EBs) on HL cells.

MATERIALS AND METHODS

***C. pneumoniae* strain and antigen purification.** *C. pneumoniae* TW-183 was propagated in HL cells as described by Cles and Stamm (3). Briefly, HL cells were seeded in 25-cm^2 tissue culture flasks (Costar, Cambridge, Mass.). After the cells had grown to a confluent monolayer, they were preincubated for 15 min in Hanks balanced salt solution (Flow, Irvine, Scotland) containing 15 μg of DEAE dextran (Sigma) per ml. The monolayer was subsequently inoculated with *C. pneumoniae*. After centrifugation for 1 h at 1,200 × *g*, the medium was replaced by Eagles modification of minimal essential medium (Flow) containing 10% fetal calf serum (FCS), 2 mM glutamine (Flow), 1% vitamin (Flow), 18 μg of gentamicin per ml, 23 μg of vancomycin per ml, 2.5 μg of amphotericin B per ml, and 1 μg of cycloheximide (Sigma) per ml (EMEMS). Cells containing *C. pneumoniae* were sonicated following a 72-h incubation at 37°C in an atmosphere containing 5% CO_2. *C. pneumoniae* EBs were partially purified by differential centrifugation at 500 × *g* for 10 min and at 30,000 × *g* for 30 min. After centrifugation, EBs were suspended in SPG, comprising 20 mM phosphate buffer containing 0.2 M sucrose (Merck, Darmstadt, Germany), 49 mM glutamine (Sigma), 10% FCS (HyClone Laboratories, Inc., Logan, Utah), 18 μg of gentamicin per ml, 23 μg of vancomycin per ml, and 2.5 μg of amphotericin B per ml, at a concentration of 2×10^8 inclusion-forming units (IFU) per ml and stored at −80°C.

Culturing and staining. Tissue culture plates with 24 wells (Greiner, Solingen, Germany) containing confluent monolayers of HL cells for *C. pneumoniae* culture were pretreated with Hanks balanced salt solution containing DEAE dextran as described above. The monolayer was then inoculated with *C. pneumoniae*, and the infected monolayer was centrifuged for 1 h at 1,200 × *g* and incubated in EMEMS at 37°C in an atmosphere containing 5% CO_2 for 72 h, after which the infected monolayer was fixed with 96% ethanol. *C. pneumoniae* inclusions were stained with anti-*C. pneumoniae* monoclonal antibody (Washington Research Foundation, Seattle, Wash.) and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) in 0.003% Evans blue (Sigma). Inclusions were counted at a magnification of ×320 by using a Leitz Ortholux fluorescent microscope.

Incubation with variations in pH. *C. pneumoniae* was diluted to a concentration of 2×10^5 IFU/ml with SPG from pH 4 to 10 at increasing steps of 0.5 pH units. After incubation for 1, 4, 8, 24, and 48 h at 22°C, a 0.2-ml sample was withdrawn from each tube and placed into 1.8 ml of EMEMS. Prior to infecting HL cells, any deviation from pH 7.5 was corrected by bubbling air or CO_2 into the solution. Tissue culture plates with 24 wells containing monolayers of

* Corresponding author.

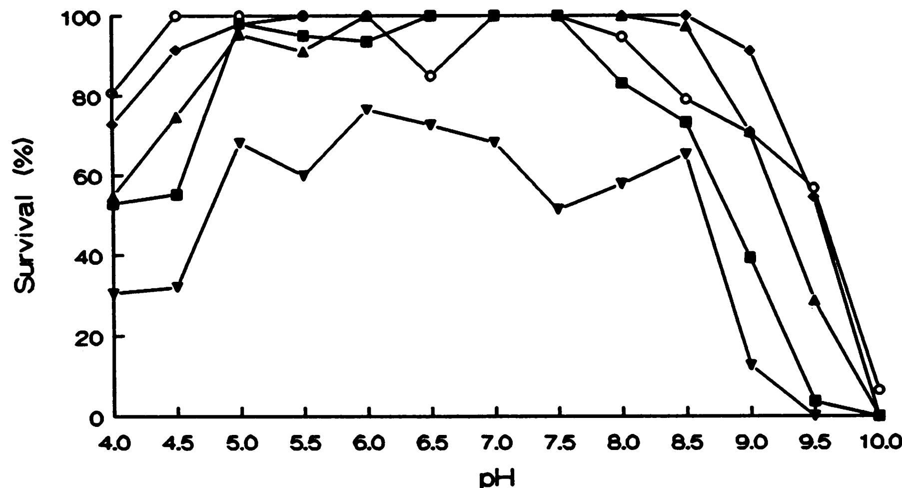

FIG. 1. Survival of *C. pneumoniae* in SPG with changing pH and incubation periods at 22°C. Percent survival was measured after incubations of 1 h (○), 4 h (◆), 8 h (▲), 24 h (■), and 48 h (▼) at pHs that ranged from 4.0 to 10.0 (increasing in steps of 0.5 pH units); n = 9; standard error of the mean, ≤15% [data not shown]).

HL cells were inoculated in triplicate with 0.4-ml samples. As positive controls, four wells were inoculated with a 0.4-ml suspension of *C. pneumoniae* in EMEMS. The concentration of *C. pneumoniae* EBs in the control tubes was identical to the end dilution in the tubes which were incubated at various pHs. The number of IFU per well in the positive control was set at 100%.

Incubation with variations in NaCl concentration. *C. pneumoniae* was diluted to a concentration of 2×10^4 IFU/ml with distilled water containing 10% FCS at NaCl concentrations ranging from 15.4 to 400 mM at increasing steps of 40 mM. After different incubation periods (as described above for variations in pH), 1.6 ml of distilled water containing 10% FCS and NaCl was added to 1 ml of the sample, such that the final concentration of NaCl in each tube was physiological (154 mM). The subsequent steps were the same as those described above for variations in pH.

Incubation with variations in temperature. *C. pneumoniae* was diluted with SPG to a concentration of 2×10^5 IFU/ml. Incubations were carried out at temperatures ranging from 0 to 45°C at increasing steps of 5°C. After different incubation periods (as described above variations in pH), a 0.2-ml sample from each tube was withdrawn and placed into 1.8 ml of EMEMS. The subsequent steps were the same as those described above for variations in pH.

Effect(s) of SPG and FCS. (i) SPG. *C. pneumoniae* was diluted with phosphate-buffered saline (PBS) to a concentration of 2×10^5 IFU/ml at pH 4.5, 7.0, and 9.0 instead of with SPG at similar pH values. Incubations were carried out at 5, 25, and 45°C. The subsequent steps were the same as those described above for variations in pH.

(ii) FCS. *C. pneumoniae* was diluted with distilled water to a concentration of 2×10^4 IFU/ml and at NaCl concentrations of 80, 154, 240, 320, and 400 mM instead of with 10% FCS in distilled water containing similar NaCl concentrations. Incubation was carried out at 22°C. The subsequent steps were the same as those described above for variations in NaCl concentration.

Incubation with variations in the concentrations of Ca^{2+} and Mg^{2+} ions. *C. pneumoniae* was diluted to a concentration of 2×10^4 IFU/ml with distilled water containing 10% FCS, 154 mM NaCl, and $CaCl_2$ at concentrations that ranged from 0.346 to 4 mM at increasing steps of 0.5 mM or $MgCl_2$ at concentrations that ranged from 0 to 2.5 mM at increasing steps of 0.5 mM. After different incubation periods (as described above for variations in pH), a 1-ml sample was adjusted to physiological Ca^{2+} and Mg^{2+} concentrations of 2.5 and 1 mM, respectively, with 0.46 ml of EMEMS containing Ca^{2+} and Mg^{2+}, after which HL cells were infected. The subsequent steps were the same as those described above for variations in pH.

Statistical methods. Variations within a single experiment were determined by calculating the standard error of the mean.

RESULTS

Variations in pH. One hundred percent of the *C. pneumoniae* EBs survived after incubation for 24 h at 22°C in SPG medium at pHs ranging from 5 to 8 (Fig. 1). After 48 h of incubation at pHs ranging from 5 to 8, the viability of *C. pneumoniae* decreased to about 65%. At pH lower than 5 or higher than 8, there was a strong decline in the number of infectious organisms (Fig. 1). The survival of *C. pneumoniae* incubated for 4 h at 22°C in PBS at different pHs was similar to its survival in SPG medium. Incubations for longer periods showed that SPG had a stabilizing effect on the viability of *C. pneumoniae*. After incubations for 8, 24, and 48 h in PBS at pH 7.0, survivals of 78.2, 29.1, and 0%, respectively, were observed.

Variations in NaCl concentration. The viability of *C. pneumoniae* in medium containing 10% FCS was affected only at NaCl concentrations of less than 80 mM (Fig. 2). After longer incubation periods (24 and 48 h), a gradual decline in the viability was observed over the whole range of NaCl concentrations tested. The infectivity of *C. pneumoniae* was stabilized by FCS. The effect of FCS on the survival of *C. pneumoniae* EBs increased as the incubation periods increased. After incubations of 8, 24, and 48 h in distilled water at physiological NaCl concentration without FCS, survivals of 54.7, 16.9 and 0%, respectively, were observed.

Variations in temperature. No adverse effect on the viability of *C. pneumoniae* in SPG medium was observed after incubation at temperatures of between 0 and 20°C (Fig. 3).

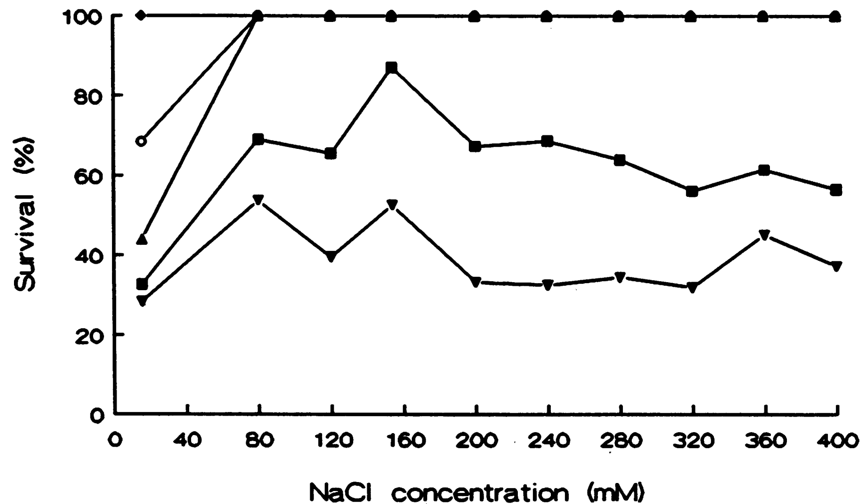

FIG. 2. Survival of *C. pneumoniae* in distilled water containing 10% FCS with changing NaCl concentrations and incubation periods at 22°C. Percent survival was measured after incubations of 1 h (○), 4 h (◆), 8 h (▲), 24 h (■), and 48 h (▼) at NaCl concentrations that ranged from 15.4 to 400 mM (increasing in steps of 40 mM; $n = 9$; standard error of the mean, ≤20% [data not shown]).

The viability of *C. pneumoniae* decreased at temperatures above 20°C, and this adverse effect of increasing temperature on the survival increased as the incubation periods increased. The stabilizing effect of SPG on the survival of *C. pneumoniae* increased as the temperature and/or the incubation period increased. Incubation in PBS at 25°C for 8, 24, and 48 h resulted in survivals of 78.2, 29.1, and 0%, respectively.

Variations in the concentrations of Ca^{2+} and Mg^{2+} ions. Variations in the Ca^{2+} ion concentration from 0.346 to 4 mM or the Mg^{2+} ion concentration from 0 to 2.5 mM at pH 7.5 and at the physiological NaCl concentration had no effect on the survival of *C. pneumoniae* (data not shown).

DISCUSSION

The results of this study showed that the survival of *C. pneumoniae* in SPG medium declines rapidly at pH lower than 5 or at pH higher than 8. Narita et al. (12) observed a decrease in the turbidity when *C. psittaci* EBs were exposed to an alkaline pH. Those investigators also demonstrated that the loss of turbidity was coupled with the loss of EB contents. It is likely that the loss of cell contents results in the death of the organism. However, those investigators (12) did not check the viability. It was demonstrated that the major outer membrane protein is acidic (1). Therefore, it is possible that exposure to an alkaline pH causes irreversible changes in the structure of the major outer membrane protein. A possible explanation for the loss of viability at pH lower than 5 may be that the reduction of disulfide bonds between cysteine-rich proteins in the outer membrane of chlamydiae results in the loss of rigidity of EBs.

C. pneumoniae EBs were well able to withstand the changes in the osmolarity of the medium. In medium containing FCS, the viability was observed to be affected only at NaCl concentrations of less than 80 mM in the concentration

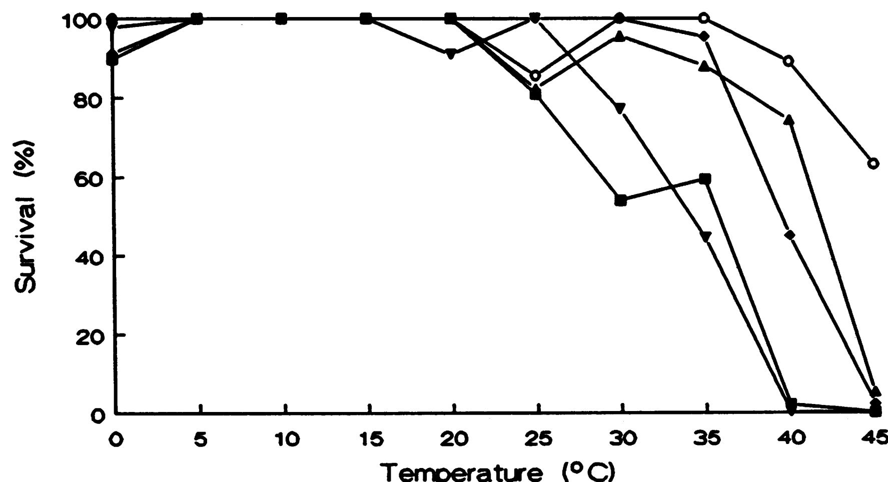

FIG. 3. Survival of *C. pneumoniae* in SPG with changing temperature and incubation periods. Percent survival was measured after incubations of 1 h (○), 4 h (◆), 8 h (▲), 24 h (■), and 48 h (▼) at temperatures that ranged from 0 to 45°C (increasing in steps of 5°C; $n = 9$; standard error of the mean, ≤25% [data not shown]).

range of 15.4 to 400 mM that was tested. *C. pneumoniae* EBs are probably resistant to hypertonic media since they are metabolically inactive. Loss of water to the hypertonic environment probably has no effect on the membrane proteins which are responsible for the adhesion of EBs to the host cells and invagination of EBs. In that case, the infectivity of EBs remains unimpaired. The rigidity of EBs may be lost because of water uptake in hypotonic media.

The survival of *C. pneumoniae* in SPG medium was observed to be 100% after incubation for 48 h at temperatures of between 5 and 20°C. The survival was observed to be 80% after incubation for 48 h at 25°C. Kuo and Grayston (9) observed a significant decline in the viability of *C. pneumoniae* AR-39 in SPG medium after incubation at 22°C. No living organisms were observed after 48 h. In comparison with the results of this study, in their study (9), survival also declined rapidly after incubation at 4°C. There are several possible explanations for these discrepancies. Kuo and Grayston (9) cultured *C. pneumoniae* on HeLa 229 cells, whereas it was later reported (3, 10) that HL cells are more suitable than HeLa 229 cells for culturing *C. pneumoniae*. Kuo and Grayston (9) used *C. pneumoniae* AR-39, whereas we used strain TW-183 in this study. Although it is true that both of these strains are identical with respect to DNA-DNA hybridization and restriction endonuclease analyses (6), it is possible that both strains may have background anomalies with respect to their adaptation in vitro and growth on cell lines. It is well known that the initial in vitro isolation of *C. pneumoniae* from patient material is tedious. However, subsequent propagation after the first few passages is relatively easier (13). A higher efficiency in the culturing of the TW-183 strain used in the present study could have been due to adaptation to laboratory conditions.

The orientation of the dipole moments of phospholipids in the membrane is influenced by the presence of divalent cations in the medium. Alterations in the dipole moments may indirectly cause conformational changes in the membrane lipids. The activity of the membrane-bound proteins may be regulated in this way (14). The infectivity of *C. pneumoniae* EBs was not affected by changes in the concentrations of either Ca^{2+} or Mg^{2+} ions. Up to three successive freeze-thawing cycles of *C. pneumoniae* did not result in any decline in the number of IFU. Other investigators (9) reported a decline of 11% in the number of IFU after freeze-thawing.

The survival of *C. pneumoniae* was considerably affected not only by SPG medium but also by the FCS contained in the SPG medium. The stabilizing effect of SPG medium containing FCS on the integrity of EBs became greater as the incubation period increased. The SPG medium was originally used since it showed a beneficial effect on the survival of rickettsiae outside its host (2). Since its introduction in 1969 for isolating *C. trachomatis* (4), SPG medium has generally been accepted as *Chlamydia* storage medium. The roles played by the various constituents of SPG medium on the stability of chlamydiae were never investigated further.

Survival of *C. pneumoniae* outside host cells is limited. Transmission of *C. pneumoniae* between various members of the same family (16) supports the concept of direct host-to-host transmission, although transmission via fomites cannot be totally excluded. A recent report also supports the possibility of spread by droplet aerosolization (7).

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