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# Transport and Storage Conditions for Cultural Recovery of *Chlamydia pneumoniae*

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***Chlamydia pneumoniae*** **is characterized by rapidly decreasing viability outside the host cell, and efficient preservation of its infectivity is a prerequisite for subsequent cell culture recovery. Extracellular survival of three *C. pneumoniae* stock strains and three wild-type strains subjected to simulated conditions of transport was therefore examined in order to establish recommendations for transport and storage of clinical specimens. The presence of fetal calf serum in transport media as well as refrigeration distinctly improved chlamydial retrieval during prolonged transport. Loss of infectivity was kept to a minimum in Eagle's minimal essential medium or sucrose-phosphate-glutamine medium. Storage at 22°C permitted a stock strain recovery of ≥81% after 12 h. When refrigeration to 4°C was provided, recovery rates of ≥74% could be achieved after 48 h. Though the strains were from different geographic regions, requirements for good survival were comparable and should therefore apply worldwide. The results indicate that the laboratory strains are not extremely labile. However, comparative examination of the wild-type strains showed less stability: primary isolates were not satisfactorily retrievable beyond 4 h at 22°C or beyond 24 h at 4°C. Further extension of storage times resulted in rapidly decreasing recovery, indicating a requirement to freeze samples at −75°C to preserve viability. Adherence to the shorter storage periods suggested by the data obtained with primary isolates is recommended to ensure successful transport until more extensive testing with clinical materials is available.**

*Chlamydia pneumoniae* is the recently identified third species of the obligate intracellular chlamydiae (8, 9). The pathogen is now recognized as a frequent cause of mild human respiratory tract infection but is also associated with approximately 10% of cases of pneumonia and possibly with other infections (7). Rapid loss of chlamydial viability leads to the unsatisfactory sensitivity of cell culture. The extraordinarily high prevalence of *C. pneumoniae* infections—nearly everybody appears to be infected by *C. pneumoniae* at least once during his or her lifetime—has therefore been established mainly by retrospective seroepidemiological studies based on the technically demanding microimmunofluorescence test (1). Serodiagnosis of acute infection is possible but often yields ambiguous results (4, 7, 15, 25). PCR is being evaluated as a reasonable alternative for direct detection (3, 6, 12) but is still in need of verification and comparison with culture results. Therefore, several efforts have been made to provide improved isolation schemes (13, 14, 18, 26) that can be employed in cell culture laboratories.

Preserving chlamydial infectivity in a clinical specimen is the obvious prerequisite for successful cultural diagnosis. Regarding the difficulty in recovering *C. pneumoniae*, extreme care in providing favorable environmental conditions during sample transport is mandatory. The problem of decreasing viability during transport is well known for *Chlamydia trachomatis* (17), but data on the requirements for extracellular survival of *C. pneumoniae* are scanty as a result of the limited amounts of clinical material available for comparative evaluation, rely on single-stock strains, and are in part contradictory (10, 24). In order to establish widely applicable sample transport recommendations, the infectivity of *C. pneumoniae* after storage was evaluated in this study by comparative use of three stock strains. Common transport media, storage time, and storage temperature were examined, as those parameters were expected to have the most prominent effect on chlamydial survival. To verify results obtained with easily grown stock strains, survival of three primary isolates was also investigated under simulated conditions of transport.

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## MATERIALS AND METHODS

**Culture of *C. pneumoniae* stock strains.** HEp-2 cells served as host cells for three representative *C. pneumoniae* strains: TW-183 (Washington Research Foundation, Seattle, Wash.), the type strain from Taiwan (9); CWL-029 (ATCC VR-1310), a respiratory isolate from the United States; and MUL-1, a regional respiratory isolate from our laboratory (11) which had been continuously propagated for about one year and which thus was considered to be adapted to laboratory conditions. Host cells were grown in Eagle's minimal essential medium with nonessential amino acids and 2 mM glutamine (EMEM) (GIBCO/BRL GmbH, Eggenstein, Germany), and 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). Chlamydiae were continuously cultured in an adaptation of recent protocols (13, 14, 18, 26): briefly, infectious inocula of the stock strains were centrifuged at 2,000 × *g* at 35°C for 45 min onto HEp-2 monolayers, and then supernatants were replaced by chlamydial isolation medium consisting of EMEM with 1 μg of cycloheximide per ml (Sigma Chemical Co., St. Louis, Mo.). After 3 days of incubation at 35°C in 5% $CO_2$, chlamydial growth was demonstrated by staining inclusions with a *C. pneumoniae*-specific mouse monoclonal antibody and fluorescein isothiocyanate-coupled secondary antibody according to the manufacturer's instructions (Cellabs Diagnostics Pty Ltd., Sydney, Australia). Infected monolayers were homogenized, the released elementary bodies were partially purified by differential centrifugation, and stock suspensions in EMEM were prepared for each strain that contained approximately $5 \times 10^4$ inclusion-forming units (IFU) per ml when cultured as described above.

**Simulated conditions of transport.** The partially purified chlamydiae were subjected to different conditions commonly employed for sample transport. Five transport media of different compositions were examined: sucrose-phosphate-glutamine medium (SPG) (75 g of sucrose, 0.52 g of $KH_2PO_4$, 1.22 g of $Na_2HPO_4$, 0.72 g of glutamic acid, distilled water to 1 liter, pH 7.4), SPG with 10% FCS, EMEM, EMEM with 10% FCS, and EMEM with 20% FCS. From each strain suspension approximately $5 \times 10^3$ IFU (100 μl) was added to 9.9 ml of each of the different transport media. Suspended chlamydiae then were immediately processed by cell culture or were held in aliquots for 4, 12, 24, 48, or 96 h at 4°C or at 22°C (room temperature) before retrieval by culture from

TABLE 1. Comparative cell culture recovery of *C. pneumoniae* stock strains after various conditions of transport

| Transport medium and temp (°C) | % Survival of *C. pneumoniae* at various storage times (h)[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strain CWL-029 | | | | | Strain MUL-1 | | | | | Strain TW-183 | | | | |
| | 4 | 12 | 24 | 48 | 96 | 4 | 12 | 24 | 48 | 96 | 4 | 12 | 24 | 48 | 96 |
| EMEM | | | | | | | | | | | | | | | |
| 4 | 90 | 68 | 34 | 18 | 11 | 93 | 63 | 40 | 19 | 11 | 79 | 72 | 32 | 12 | 1 |
| 22 | 85 | 57 | 18 | 9 | 5 | 81 | 41 | 10 | 9 | 6 | 80 | 42 | 10 | 1 | 6 |
| −75 | | | | | 52 | | | | | 45 | | | | | 38 |
| EMEM, 10% FCS | | | | | | | | | | | | | | | |
| 4 | 104 | 98 | 92 | 86 | 45 | 115 | 102 | 91 | 74 | 32 | 112 | 99 | 88 | 83 | 40 |
| 22 | 98 | 90 | 50 | 40 | 24 | 109 | 94 | 43 | 46 | 20 | 95 | 81 | 49 | 32 | 14 |
| −75 | | | | | 70 | | | | | 84 | | | | | 76 |
| EMEM, 20% FCS | | | | | | | | | | | | | | | |
| 4 | 97 | 104 | 91 | 85 | 40 | 107 | 96 | 90 | 80 | 44 | 90 | 88 | 91 | 85 | 40 |
| 22 | 102 | 95 | 53 | 33 | 19 | 100 | 83 | 58 | 30 | 11 | 102 | 98 | 56 | 37 | 9 |
| −75 | | | | | 65 | | | | | 73 | | | | | 82 |
| SPG | | | | | | | | | | | | | | | |
| 4 | 95 | 81 | 45 | 28 | 15 | 97 | 89 | 44 | 22 | 19 | 96 | 79 | 39 | 33 | 17 |
| 22 | 89 | 63 | 22 | 12 | 10 | 91 | 61 | 29 | 10 | 12 | 95 | 50 | 28 | 2 | 0 |
| −75 | | | | | 61 | | | | | 70 | | | | | 75 |
| SPG, 10% FCS | | | | | | | | | | | | | | | |
| 4 | 100 | 98 | 90 | 78 | 40 | 107 | 99 | 90 | 78 | 54 | 101 | 99 | 94 | 75 | 41 |
| 22 | 91 | 85 | 51 | 34 | 19 | 90 | 81 | 61 | 25 | 17 | 92 | 86 | 62 | 50 | 19 |
| −75 | | | | | 72 | | | | | 81 | | | | | 75 |

[a] Means from triplicate experiments; IFU counts obtained immediately after suspension of elementary bodies at 22°C were set at 100%.

0.2-ml portions (containing approximately 100 IFU) was attempted. Further aliquots of each strain suspension were cooled to 4°C for 30 min and then frozen and stored at −75°C for 96 h.

**Survival of stock strains.** Chlamydial recovery subsequent to the various storage conditions was assessed by centrifuging 0.2-ml aliquots from the transport media onto fresh HEp-2 cell monolayers seeded on 13-mm coverslips in 24-well tissue culture plates followed by the culture procedure described above. IFU counts per 0.2 ml then were determined by immunofluorescence microscopy. Frozen samples were rapidly thawed at 35°C and then cultured. For each condition of transport, a mean IFU count from triplicate experiments and the pertinent standard error of the mean were determined. IFU counts found immediately after suspension of chlamydiae in each transport medium were set as 100% recovery, and counts obtained after the different holding times and temperatures were expressed as percentages of this value, in order to represent mean survival percentages.

**Survival of wild-type strains.** Transport conditions were examined for three primary isolates not yet fully adapted to laboratory growth. The clinical isolates MUL-1 (this strain was also included among the stock strains after adaptation to laboratory conditions by one year of continuous growth), MUL-2, and MUL-3 had been recovered from bronchoalveolar lavage material. Since the amount of original lavage material was too limited for extensive comparative testing, pooled material from the first eight cell culture passages that had yielded the strains (stored at −75°C until use) was considered to contain not yet fully culture-adapted organisms. These materials containing each isolate were homogenized and then centrifuged for 1 h at 20,000 × *g* at 4°C, and the pellets were suspended in 4 ml of EMEM with 10% FCS. Aliquots were processed immediately or stored for 4, 12, 24, or 48 h at 4°C or at 22°C. Recovery by culture was then determined essentially as described above in replicate experiments, and the mean IFU counts obtained were compared.

## RESULTS

**Stock strains.** Survival rates of the stock strains under various conditions of transport are shown as percentages in Table 1. Recovery rates were consistent for the strains when equal conditions were applied. Addition of FCS to EMEM or SPG resulted in distinctly improved survival rates for holding periods of ≥12 h. Refrigeration to 4°C gave clearly better survival rates after 12 to 24 h of storage. In EMEM and SPG without FCS supplementation, survival ranged from 10 to 29% after 24 h at 22°C and from 0 to 12% after 96 h; at 4°C it ranged from 32 to 45% after 24 h and from 1 to 19% after 96 h. In EMEM and SPG with 10% FCS, survival was substantially improved and ranged at 22°C from 43 to 62% after 24 h and from 14 to 24% after 96 h; with additional protection by refrigeration to 4°C it ranged from 88 to 94% after 24 h and from 32 to 54% after 96 h. No distinct difference was noted between EMEM and SPG when both were supplemented with 10% FCS. Use of 20% FCS in EMEM did not result in a further improvement of chlamydial recovery. Stock strains did not appear to be particularly labile: recovery in FCS-supplemented media at 22°C was ≥90% after 4 h and ≥81% after 12 h, but further prolongation of storage resulted in a rapid loss of infectivity. Refrigeration to 4°C maintained infectivity for a longer period, and recovery rates of ≥88% after 24 h and ≥74% after 48 h were achieved. The standard error of the mean within single experiments was ≤15% after 4 h of storage, ≤25% after 96 h, and ≤20% in the remaining experiments (data not shown).

The effects of freezing at −75°C were also examined (Table 1). Again, survival rates were quite consistent for the strains but differed among the storage media. Survival rates varied from 38 to 52% in serum-free EMEM and from 61 to 75% in serum-free SPG. Supplementation of EMEM with 10% FCS had a distinct cryoprotective effect, improving survival rates to 70 to 84%. Serum-supplemented SPG yielded recovery rates similar to those seen in serum-free SPG and in EMEM with 10% FCS. Supplementation of EMEM with 20% FCS did not further improve survival. In summary, survival rates after a freeze-thaw cycle in serum-supplemented media were similar to those seen after 48 h of storage at 4°C in the 10% FCS-supplemented transport media. For the freezing experiments the standard error of the mean was ≤25% (data not shown).

**Wild-type strains.** Because EMEM with 10% FCS yielded good recovery of stock strains, this medium was also used in

TABLE 2. Comparative cell culture recovery of *C. pneumoniae* wild-type strains[a] in relation to transport time and temperature

| *C. pneumoniae* wild-type strain and transport temp (°C) | IFU/0.2 ml[b] after storage time (h) of: | | | | |
|---|---|---|---|---|---|
| | 0 | 4 | 12 | 24 | 48 |
| MUL-1 | | | | | |
| 4 | | 21 | 17 | 14 | 4 |
| 22 | 23 | 19 | 8 | 4 | 0 |
| MUL-2 | | | | | |
| 4 | | 7 | 12 | 8 | 2 |
| 22 | 12 | 9 | 2 | 0 | 0 |
| MUL-3 | | | | | |
| 4 | | 9 | 7 | 6 | 0 |
| 22 | 8 | 6 | 1 | 0 | 1 |

[a] Organisms recovered from pooled material of the first eight cell culture passages after primary isolation were considered to represent wild-type strains.
[b] Means from replicate testing.

the assessment of wild-type strains for transport at 4°C and at 22°C. Infectious inocula, like clinical specimens, contained highly diluted organisms (on average, 8 to 23 IFU/0.2 ml). The IFU counts found after various storage times and temperatures are given in Table 2. There appeared to be a more rapid and pronounced loss of viability than occurred with the stock strains. A sharp decline in viability was noted between 4 and 12 h of storage at 22°C. Refrigeration to 4°C shifted this decline beyond a 24-h holding period. Thus, all isolates could be recovered at high levels after 4 h at 22°C and after 24 h at 4°C, but prolongation of transport times invariably resulted in the rapid decline or total loss of chlamydial viability. An additional freeze-thaw cycle was not examined, as the isolates had already been stored at −75°C for up to one year and recovery was apparently not problematic.

## DISCUSSION

Successful recovery of *C. pneumoniae* from clinical samples obviously depends on the provision of environmental conditions that maximize the survival of the pathogen. In this study, an attempt was made to determine which conditions can be relied upon to prevent unacceptable loss of chlamydial viability. Stock strain survival was evaluated under simulated conditions of transport, and the results were verified with those from primary isolates. Limits to this approach are set by the complexity of the culture and detection system and the scarcity of positive clinical materials dispensable for comparative testing. The best survival rates for stock strains were obtained in refrigerated and FCS-supplemented transport media. SPG, a transport medium originally developed for preservation of rickettsiae (2) and later used for chlamydiae, and EMEM, the medium employed in the cell culture procedure, were equally effective in protecting chlamydial viability. In those media, recovery of stock strains was ≥81% after 12 h at room temperature and ≥74% after 48 h at 4°C, indicating that the laboratory-adapted strains were not particularly labile. However, the results from primary isolates indicated a greater lability: recovery was not satisfactory after more than 4 h at 22°C or more than 24 h at 4°C. Though the physiological basis of this finding remains unknown, the lability may be caused by not yet fully developed adaptive mechanisms for growth in the artificial culture environment. Adherence to the shorter storage periods implied by the data from the primary isolates appears advisable to ensure adequate transport. Considering the very small numbers of chlamydiae present in the clinical specimens, there might otherwise exist a high risk for false-negative results. Though the reference strains were from different geographic regions, survival requirements were consistent and should therefore apply worldwide.

Few data on extracellular survival of *C. pneumoniae* have been published. For laboratory strain AR-39 suspended in SPG without FCS, 39% viability at 22°C after 4 h and 1% viability after 1 day was reported (10). In the same study, infectivity was more stable at 4°C, then yielding 88% viability after 4 h and 70% viability after 1 day. Those values indicate markedly lower survival rates than were achieved in our study by the use of FCS-supplemented transport media. FCS apparently must not be omitted from transport media, although its use in the chlamydial isolation medium for cell culture is not obligatory (13). As in our study, a stabilizing effect of FCS during prolonged storage and a decreasing viability with elevated temperatures and extended holding periods have been noted by Theunissen et al. (24), but some results in their investigation, such as the 100% retrieval reported to have occurred in SPG with 10% FCS after 48 h at 5 to 20°C, differ from those of our study. We could not achieve a 100% retrieval beyond 12 h of storage in any of the media. Maybe the considerably larger inocula used in that study add to the difference. Sucrose-based collection media can be somewhat toxic to the host cell monolayer (19), especially when material from swabs is extracted in sucrose buffers and directly centrifuged onto monolayers. As no clear difference between EMEM and SPG was noted, we routinely use the cell culture medium EMEM with 10% FCS for collection and transport. Lyophilization of *C. pneumoniae* in glucose-supplemented FCS has previously been examined but resulted in only approximately 5% retrievable infectivity (23). Conditions for collection and transport of *C. trachomatis* have been examined more thoroughly than those for *C. pneumoniae*, but methods varied and no single superior transport medium has yet been determined. Use of sucrose-based media and of tissue culture media is common (5, 19, 21).

A firm recommendation of the storage time beyond which freezing will be superior to refrigeration in preserving chlamydial viability is difficult to establish. In a previous study, 3 days of storage at −75°C appeared distinctly better than storage for 4 h at 22°C or for 1 day at 4°C (10), but our study indicates that freezing may be further postponed by use of FCS-supplemented storage media. We found chlamydial stock strain retrieval after one freeze-thaw cycle to be approximately in the range of the survival rates seen after 48 h at 4°C in the FCS-supplemented transport media. For transport periods substantially exceeding 48 h for stock strains or 24 h for the wild-type strains, transport at −75°C should be considered. A short period of refrigeration prior to freezing *C. pneumoniae* has been shown to significantly enhance survival of the pathogen (10) and was therefore also used in our study. For *C. trachomatis*, various reports on the reduction of chlamydial infectivity after a freeze-thaw cycle exist, and, depending on the investigator, freezing of samples not to be processed immediately is preferred to storage at 4°C after 24 h (21), 48 h (20), or 72 h (5). A cryoprotective effect of sucrose and FCS has already been outlined for *C. trachomatis* (16). In our study, the cryoprotective values of sucrose and FCS appeared similar. Addition of FCS to SPG improved the cryoprotection only slightly, and a combination of the cryoprotective agents did not result in better recovery. Increasing the FCS supplementation of EMEM to 20% neither resulted in better cryoprotection nor had a toxic effect, as described for *C. trachomatis* (16).

The viability of strain TW-183 in aerosols has been demon-

strated to be influenced by temperature, time, and relative humidity, with a rapid decrease of infectivity occurring within 30 s (22). Naturally occurring transport of *C. pneumoniae* from host to host nevertheless permits very effective transmission, as was most impressively demonstrated by the extraordinarily high seroprevalence of *C. pneumoniae* (7). In general, maximizing infectivity by keeping samples refrigerated and minimizing time between collection and processing will be good practice. However, this investigation shows that in-house transportation of clinical specimens is practicable and should permit subsequent chlamydial recovery after about 4 h of storage at room temperature without prominent loss of infectivity. Additional refrigeration at 4°C prolongs the acceptable transport period to at least 24 h. Though *C. pneumoniae* certainly is an inconvenient organism to work with, it does not necessarily need to be considered extremely labile, and the establishment of transport conditions shown here to preserve infectivity should not pose major problems. In association with recently improved culture techniques, strict adherence to requirements for safe sample transport should render successful recovery of this frequent and fastidious pathogen more likely in the clinical laboratory.

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