APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2589–2593 Vol. 59, No. 8
0099-2240/93/082589-05$02.00/0
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Influence of Temperature and Relative Humidity on the Survival of *Chlamydia pneumoniae* in Aerosols

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Received 3 February 1993/Accepted 24 May 1993

The survival of *Chlamydia pneumoniae* in aerosols was investigated by using a chamber with a capacity of 114.5 liters. We injected 5×10^7 inclusion-forming units (IFU) of *C. pneumoniae* in aerosols with a droplet size of 3 to 5 μm. Samples were taken after 30 s and every 1 min thereafter. The survival of *C. pneumoniae* was measured at four temperatures (8.5, 15, 25, and 35°C) and at three different relative humidities (RH) of 5, 50, and 95% for each temperature. The survival rates of *Streptococcus pneumoniae*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Chlamydia trachomatis* LGV_2, and cytomegalovirus were also determined at 25°C and 95% RH and compared with that of *C. pneumoniae*. At the mentioned temperatures and RH, a rapid decrease of *C. pneumoniae* IFU was observed in the first 30 s. After this the decrease in the number of IFU was more gradual. The survival of *C. pneumoniae* in aerosols was optimal at 15 to 25°C and 95% RH; it was good compared with those of other microorganisms. A lower death rate was observed only in *S. faecalis*. In *C. trachomatis*, the death rate during the first 30 s was higher than that in *C. pneumoniae* (85 and 53.3%, respectively). After the first 30 s, the death rates in the two organisms were identical. It was concluded that transmission of *C. pneumoniae* via aerosols was possible. There is probably a direct transmission from person to person, taking into account the relatively short survival period of *C. pneumoniae* in aerosols.

Transmission of microorganisms which cause respiratory infections probably occurs via an airborne route. In contrast to transport over long distances, host-to-host transmission is a relatively rapid process which occurs via sneezing and coughing and which is accompanied by transmission of microorganisms in small droplets of 3 to 10 μm in diameter, the so-called aerosols.

During aerial passage, the survival of microorganisms in aerosols depends on five different factors: relative humidity (RH), temperature, level of oxygen, presence of UV radiation, and constituents of the aerosol and of air (4, 16). The degree to which these factors influence the survival of microorganisms in aerosols depends strongly on the type of microorganism and the time it has to spend in the aerosol. In general, the following rules apply. Gram-negative bacteria survive the best at low temperatures and RH (5, 10). Gram-positive bacteria survive better at low temperatures and high RH (5, 6). In almost all cases, gram-positive bacteria survive better and longer than gram-negative bacteria in aerosols. The sensitivity of gram-negative bacteria to oxygen depends on the species. Viruses with membrane lipids are more stable in aerosols than are viruses without membrane lipids (5). Viruses which possess lipids survive the best at RH below 50%, whereas viruses which do not possess lipids are most stable at RH above 50% (6).

C. pneumoniae is a recently reported microorganism, which can cause respiratory infections varying in seriousness from bronchitis to atypical pneumonia (10). Although no proof has yet been provided, it is assumed that *C. pneumoniae* is transmitted via aerosols. A possible indication for such a mode of transmission was provided by the accidental infection of laboratory personnel with *C. pneumoniae* (14). Transmission must progress particularly efficiently because 50 to 60% of the European and North American population experience infection with *C. pneumoniae* at the age of 5 to 15 years (10).

In the present study the survival of *C. pneumoniae* elementary bodies (EBs) in air was determined at different temperatures and RH in an aerosol chamber to obtain further insight into its mode of transmission. A possible relationship between climate/seasons and the efficiency of transmission could thus be investigated. To make the acceptable assumption that infection with *C. pneumoniae* occurs via the airborne route, survival of other respiratory microorganisms in the same aerosol chamber was also determined.

Transmission of the urogenital pathogen *Chlamydia trachomatis* LGV_2 requires contact between mucous membranes. If transmission of *C. pneumoniae* occurs via an airborne route, an adaptation of *C. pneumoniae* to this mode of transmission is likely and differences in the survival rate in aerosols between *C. trachomatis* LGV_2 and *C. pneumoniae* can be expected. Differences in the survival of *C. trachomatis* LGV_2 and *C. pneumoniae* were determined by comparing the infectivity decrease of both microorganisms in aerosols.

MATERIALS AND METHODS

Microorganisms and antigen purification. *C. pneumoniae* TW-183 was propagated in HL cells as described by Cles and Stamm (3). HL cells were seeded in 75-cm² 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 supplemented with 15 μg of DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.) per ml. The monolayer was subsequently inoculated with *C. pneumoniae*. After centrifugation at $1{,}200 \times g$ for 1 h, the medium was replaced with Eagle's modification of minimal essential medium (Flow Laboratories, Irvine, Scotland) con-

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taining 10% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah), 2 mM glutamine (Flow), 1% vitamins (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). Following incubation at 37°C for 72 h in an atmosphere containing 5% CO_2, *C. pneumoniae*-containing cells were sonicated and partially purified by differential centrifugation at 500 × *g* for 10 min and at 30,000 × *g* for 30 min. The 30,000 × *g* pellet was homogenized in SPG (20 mM phosphate buffer containing 0.2 M sucrose [Merck, Darmstadt, Germany], 49 mM glutamine [Sigma], 10% FCS, 18 μg of gentamicin per ml, 23 μg of vancomycin per ml, and 2.5 μg of amphotericin B per ml) and centrifuged at 53,000 × *g* for 1 h through a 30% Urografin solution (3.9 g of sodium amidotrizoate per 100 ml, 26 g of meglumine amidotrizoate per 100 ml, 146 mg of iodine per 100 ml). Chlamydiae in the resulting pellet were washed once with phosphate-buffered saline (PBS; pH 7.4) and stored in SPG at −80°C until use.

C. trachomatis LGV_2 (strain 343/Bu) was propagated in McCoy cells. Culture and purification of *C. trachomatis* LGV_2 were identical to those described for *C. pneumoniae*. We chose *C. trachomatis* LGV_2 so that we could compare two organisms that belong to the same genus but cause different infections.

Streptococcus faecalis, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae* were isolated from throats of patients with respiratory disorders. Pure cultures of the bacteria were established in Todd-Hewitt bouillon (Oxoid, Basingstoke, United Kingdom) by culturing for 16 h and were then stored in Todd-Hewitt bouillon supplemented with 10% glycerol (Merck) at −80°C until use. *S. faecalis* was included because its survival in aerosols is known to be good (13). *K. pneumoniae* and *S. pneumoniae* were included as examples of gram-negative and gram-positive microorganisms, respectively, which may cause infections in the respiratory tract.

Cytomegalovirus (CMV) Kerr was cultured in MRC-5 cells (Flow). The cells were seeded in 75-cm^2 tissue culture flasks (Costar) and grown to a confluent monolayer. The medium was then removed, and the cells were inoculated with 10^5 to 10^6 PFU of CMV in Dulbecco's modification of minimal essential medium supplemented with 2 mM glutamine, 2.5% FCS, 1% amino acids, and 18 μg of gentamicin per ml (DMEMS) and incubated at 37°C for 2 h under an atmosphere containing 5% CO_2. After incubation, the free virus was rinsed away and the cells were incubated in DMEMS at 37°C for 5 days under an atmosphere containing 5% CO_2. Virus-containing cells were subsequently sonicated, and the virus was partially purified by centrifugation at 1,500 × *g* for 10 min. The CMV-containing supernatant was stored at −80°C until use. CMV was included as an example of a virus which may cause infections in the respiratory tract.

Culture and staining. Tissue culture plates with 24 wells (Greiner, Solingen, Germany) containing HL cells for *C. pneumoniae* culture or McCoy cells for *C. trachomatis* LGV_2 culture were treated with Hanks' balanced salt solution containing DEAE as described above. Monolayers were inoculated with chlamydiae, centrifuged at 1,200 × *g* for 1 h, and incubated in EMEMS for 72 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the infected monolayer was fixed with 96% ethanol (Merck). *C. trachomatis* LGV_2 inclusions were stained with anti-*C. trachomatis* monoclonal antibody (Microtrak; Syva Co., Palo Alto, Calif.). *C. pneumoniae* was 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 with a Leitz Ortholux fluorescence microscope.

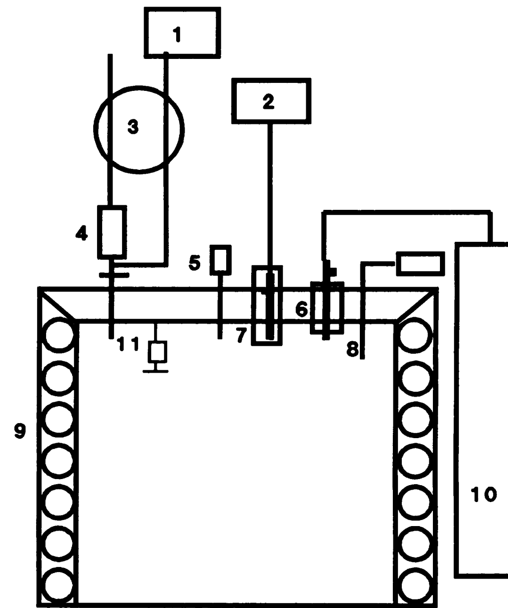

FIG. 1. Schematic diagram of the aerosol chamber. Numbers: 1, water vapor generator; 2, temperature and RH meter; 3, pump; 4, silica column; 5, filter (0.22 or 0.45 μm); 6, aerosol gun; 7, temperature and RH probes; 8, capillary impinger; 9, water jacket; 10, nitrogen cylinder; 11, ventilator.

S. faecalis and *K. pneumoniae* were cultured in Todd-Hewitt agar (Oxoid) at 37°C for 16 h. *S. pneumoniae* was cultured on blood agar (Oxoid) at 37°C for 16 h in an atmosphere containing 5% CO_2.

Tissue culture plates with 24 wells (Greiner) containing MRC-5 cells were inoculated with CMV and incubated at 37°C in an atmosphere containing 5% CO_2. After 1 h the medium was substituted with 1 ml of twofold strength basal medium (Eagle) without phenol red (Difco Laboratories, Detroit, Mich.), diluted twofold with 1.2% SeaKem-ME agarose (FMC Bioproducts, Rockland, Maine) in distilled water, yielding full strength agar containing 0.6% agarose. After incubation at 37°C for 10 to 14 days under an atmosphere containing 5% CO_2, the cells were fixed by incubation with 10% formalin in PBS for 24 h. After fixing, the agar layer was removed and the cells were stained for 1 min with 1% methylene blue in distilled water. After destaining with distilled water, the plaques were counted at a magnification of ×100.

Aerosol chamber. The aerosol chamber comprised a stainless-steel cylindrical container with a capacity of 114.5 liters (Fig. 1). The temperature in the chamber could be varied from 8.5 to 70°C by altering the temperature of the surrounding water jacket, which was connected to a water bath with a temperature regulator. The RH could be varied either by using water vapor (increase in RH) or by pumping air which had been passed through a silica column (decrease in RH) into the chamber. Eventual over- or underpressure was compensated for via a hydrophobic, autoclavable 0.45- or

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0.22-μm-pore-size filter (Millipore Intertech, Bedford, Mass.). The RH in the chamber was measured with an MC-2 humidity probe (Panametrics, Waltham, Mass.), and the temperature was measured with a PT-100 electrode. Both probes were autoclavable. The temperature and RH could be read on the homemade digital display. An FK-8 aerosol gun was used to generate aerosols (7). The pressure (5 kg/cm^2) that was required for injection was obtained from a nitrogen gas cylinder. At this pressure of injection, a 1-ml sample was dispersed in 4 s into aerosols with a droplet size of 3 to 5 μm. Samples were obtained with a stainless-steel capillary impinger with a diameter of 1 mm. The rate of sampling was 10 liters/min, and the size of the sample was 5 liters. The content of the aerosol chamber was kept homogeneous by using a ventilator with four fins with a diameter of 5.5 cm and a speed of 3,000 rpm. The infected compartment of the aerosol chamber was a closed system, which could be autoclaved.

In each experiment, 1 ml of PBS supplemented with 10% FCS containing either 5×10^7 inclusion-forming units (IFU) of chlamydia EBs or 1×10^8 to 5×10^8 CFU of *S. faecalis*, *S. pneumoniae*, or *K. pneumoniae* obtained from a 16-h culture was injected. For CMV, 1.4×10^7 PFU contained in 1 ml of DMEMS was injected. The sampling medium was the same as that used for injection and consisted of PBS supplemented with 10% FCS. The protein concentration of this medium (0.35 g/100 ml) is similar to the protein concentration found in saliva.

In separate experiments, the physical fallout was measured over 5.5 min for every temperature and RH by spraying 5×10^7 cpm ^{67}Ga-labeled gallium citrate in 1 ml of PBS containing 10% FCS. A 5-liter sample was taken after 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 min. The total number of counts per minute in the samples obtained at different times was subsequently set at 100%. In performing the control experiments with ^{67}Ga, it was observed that the maximum recovery at all RH tested was 2% of the total that was converted to aerosol. The physical fallout measured over the 5.5-min period appeared to be negligible. Theoretically, the physical fallout is the highest at high RH because droplets can form on the wall of the aerosol chamber. Several experiments for determining this fallout were repeated with ^{125}I-labeled *C. pneumoniae* EBs (9). ^{125}I-labeled *C. pneumoniae* EBs (5×10^7 IFU) were injected at 8.5, 15, 25, and 35°C and 95% RH. Subsequently, the physical fallout (decrease in the number of counts per minute) and the survival of *C. pneumoniae* under the tested conditions were determined. The physical fallout measured over 5.5 min was negligible, whereas the decrease in the number of infectious EBs was comparable to that of unlabeled *C. pneumoniae* EBs under the same conditions. On the basis of this information, we decided to express the decrease in the number of infectious organisms in aerosols as a ratio of viable microorganisms/^{67}Ga.

Prior to spraying the microorganisms in the aerosol chamber, we tested whether they could withstand the injection pressure of 5 kg/cm^2 and the high air speeds that are reached during sampling. For this purpose, prior to spraying, the microorganisms were cultured as described above. They were then injected directly into the sampling medium with the aerosol gun and cultured again. To check their survival during sampling, we suspended the microorganisms in sample medium and cultured them as described above. Subsequently, 5 liters of air was sucked via the capillary impinger through the sampling medium containing the microorganisms, and the microorganisms were cultured again. All the microorganisms used in this study appeared to withstand the injection pressure as well as the air speeds during sampling.

All experiments and checks were performed at least in duplicate.

Statistical analysis. Differences between the survival of *C. pneumoniae* at different temperatures and RH and those of *C. pneumoniae* and other microorganisms tested were analyzed by using multiple linear regression analysis after log transformation. The starting point of analysis is from 0.5 min after spraying the organisms.

RESULTS

The survival of *C. pneumoniae* over 5.5 min at different temperatures and RH is depicted in Fig. 2. An initial rapid inactivation of infectious *C. pneumoniae* EBs during the first 0.5 min was followed by a more gradual decrease in death rate over the next 5 min. It was also observed that the loss of infectious EBs was lowest at high RH. The survival at 95% RH was significantly higher than that observed at 5 or 50% RH ($P < 0.0001$). The best temperature for the survival of *C. pneumoniae* EBs in aerosols was between 15 and 25°C (Fig. 2B and C). However, the survival at 5°C did not differ significantly from that at 15 or 25°C. At 35°C only, the survival was lower than the survival at other temperatures ($P < 0.001$). The rate of survival at 50% RH was observed to be dependent on the temperature. The survival of *C. pneumoniae* EBs at 8.5°C and 50% RH (Fig. 2A) was almost identical to that at 95% RH. The survival at 15 and 25°C and 50% RH was identical to that at 5% RH but lower than the survival at 95% RH (Fig. 2B and C). Hardly any infectious EBs were observed at 35°C after 0.5 min when measured at 50% RH. The percent survival was then lower than that observed at 5 and 95% RH (Fig. 2D).

The survival of *C. pneumoniae* in aerosols was compared with that of other microorganisms at 25°C and 95% RH. A comparison of the results is depicted in Fig. 3. It can be seen that under the tested conditions, only *S. faecalis* in aerosols survived better than *C. pneumoniae*, but the difference was not statistically significant. The survival of the respiratory tract pathogen *K. pneumoniae* was slightly lower ($P = 0.062$), whereas the survival of the pathogens *S. pneumoniae* ($P < 0.0001$) and CMV ($P < 0.0001$) was clearly lower than that of *C. pneumoniae*.

The high inactivation of *S. pneumoniae* during the first 0.5 min is noteworthy. Thereafter, for the rest of the measurement period, there was no further decrease in the number of *S. pneumoniae* CFU.

The decrease in the number of infectious *C. trachomatis* LGV_2 IFU in aerosols during the first 0.5 min was much higher than that of *C. pneumoniae* IFU (85.0 and 53.4%, respectively). After the first 0.5 min, death rates in the two microorganisms were identical (12%/min). Overall, there was a significant difference in survival between the organisms ($P < 0.001$).

DISCUSSION

The percent survival of *C. pneumoniae* in aerosols was highest at high RH and at temperatures between 15 and 25°C. Dehydration appeared to be an important factor in the inactivation of *C. pneumoniae*, whereas extreme temperatures had a more limited influence on survival. Inactivation was highest immediately after injection of the aerosols. This phenomenon was also observed after spraying of other microorganisms (17, 20). The vapor pressure of the aerosol

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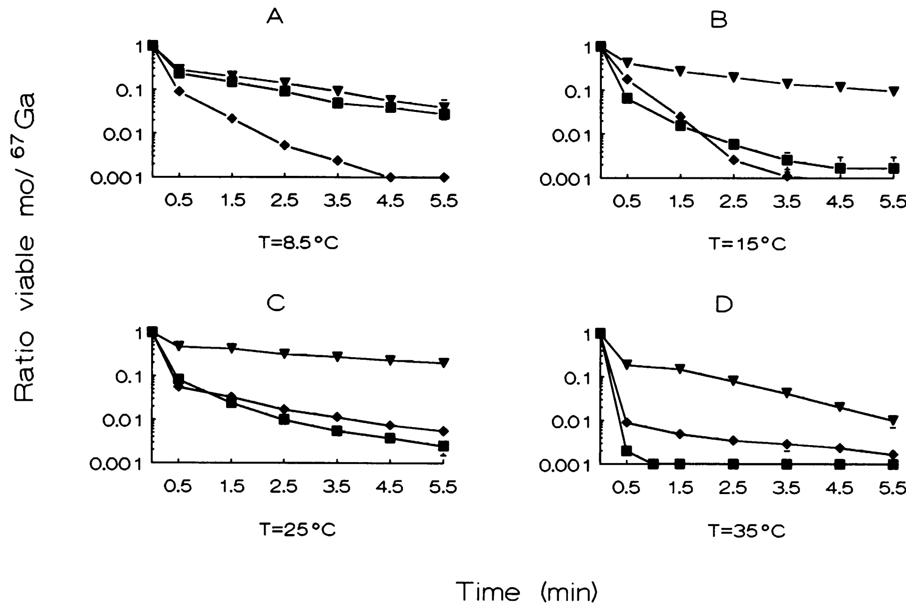

FIG. 2. Survival of *C. pneumoniae* at different temperatures and RH. The survival at 8.5°C (A), 15°C (B), 25°C (C), and 35°C (D) is expressed as the ratio of viable microorganisms (mo)/^{67}Ga. Survival of *C. pneumoniae* was measured over 5.5 min at 5% RH (◆), 50% RH (■), and 95% RH (▼). Error bars indicate the variation in the measurements. In cases when the error bars are not visible, the variation was so small that the bars are beyond the range of scale that was used.

was in equilibrium with that of the surroundings. At low RH, the water in the aerosol immediately evaporated into the surroundings. Increases and decreases in temperature caused higher and lower evaporation rates, respectively. Several membrane reactions may occur depending on the composition of the membrane and the presence or absence of a cell wall. Transition of lipids from the liquid-crystalline state to the gel state is a possible inactivation mechanism. The probability of such a phase transition increases with decreasing temperature and RH (8). Since a considerable proportion of the membrane lipids of chlamydiae consists of unsaturated fatty acids (15), it is possible that the temperature at which the transition to gel state occurs lies between 0 and 10°C. This may explain the lower survival of *C. pneumoniae* in aerosols at 8.5°C and 95% RH than at 15 to 25°C and 95% RH.

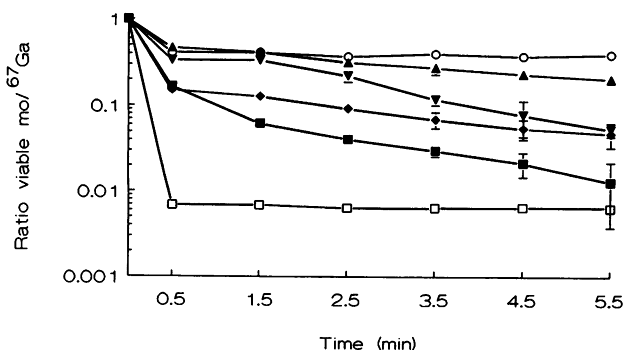

FIG. 3. Survival of different microorganisms at 25°C and 95% RH. Survival is expressed as the ratio of viable microorganisms (mo)/^{67}Ga. Survival over 5.5 min was measured for *S. faecalis* (○), *C. pneumoniae* (▲), *K. pneumoniae* (▼), *C. trachomatis* LGV$_2$ (◆), CMV (■), and *S. pneumoniae* (□). Error bars indicate the variation in the measurements.

Another inactivation mechanism is the occurrence of Maillard reactions at low to moderate (0 to 50%) RH. The probability of these reactions, which require low activation energy, increases with increasing temperature (5). Such reactions occur either between lipids and proteins or between proteins and proteins and result in removal of water. Inactivation of the proteins involved in anchoring and invagination of *C. pneumoniae* to the host cell may then occur, resulting in a decreased infectivity of *C. pneumoniae* in aerosols. Inactivation of proteins in the Maillard reaction may be an explanation of the low survival of *C. pneumoniae* EBs at 35°C, especially at 5 and 50% RH.

Damage to the outer membrane of gram-negative bacteria is another mechanism by which microorganisms can be inactivated during spraying in aerosols. As such, after spraying at 75% RH, 80% of *Escherichia coli* MRE 160 cells were susceptible to lysozyme action, indicating membrane damage (11). It was also reported that in bacteria and viruses, inactivation at high RH was the result of surface inactivation due to the (partial) removal of proteins from the outer membrane (2, 19). In contrast with bacteria, *C. pneumoniae* EBs, because they are metabolically inactive, possess no repair mechanisms, so that any eventual damage to the outer membrane cannot be repaired. Because the survival of *C. pneumoniae* was highest at high RH and because the membrane integrity of *C. pneumoniae* EBs depends on disulfide bridges between proteins in the outer membrane (1), in contrast to that of other gram-negative bacteria, it seems likely that the integrity of outer membrane proteins of *C. pneumoniae* is higher than that of other gram-negative bacteria. This limits the loss of outer membrane proteins during spraying, which has a beneficial effect on survival at high RH.

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At low RH, two other mechanisms, namely, the toxic effect of salts or amino acids in the aerosol from which the water has evaporated (19) and inactivation due to withdrawal of structural water molecules, play a role. Since *C. pneumoniae* EBs are resistant to high salt concentrations in liquid media (18), it is possible that, at low RH, inactivation occurs via the withdrawal of structural water molecules.

The results in Fig. 3 show that survival of *C. pneumoniae* in aerosols, under the conditions tested, is better than that of most other pathogens. Only *S. faecalis*, a bacterium known to survive well in aerosols at 95% RH (13), had a higher percent survival. Compared with pathogens that are likely to be transmitted by aerosols, i.e., *K. pneumoniae*, *S. pneumoniae*, and CMV, *C. pneumoniae* showed better survival. Although it cannot be excluded that another order of survival percentages between the organisms would be observed under different test conditions, transmission of infection with *C. pneumoniae* via the airborne route seems probable. The difference between the uropathogenic serovar *C. trachomatis* LGV_2 and the respiratory pathogen *C. pneumoniae* was noteworthy. *C. pneumoniae* was more resistant to the stress which occurred in the aerosol during the first 0.5 min after spraying. It is possible that the tertiary structure of one or more proteins in the outer membrane of *C. pneumoniae* is more resistant to dehydration and/or mechanical damage during the first 0.5 min.

When only the survival of *C. pneumoniae* in aerosols is taken into account, transmission of this pathogen occurs most efficiently when the temperature is between 15 and 25°C and the RH is high. However, a number of other variables such as UV radiation, host resistance, and minimal infectious dose should also be taken into consideration before the results are extrapolated to the real-world situation.

ACKNOWLEDGMENTS

We thank C. C. Kuo for kindly providing *C. pneumoniae* TW-183, C. A. Bruggeman for kindly providing cytomegalovirus Kerr, B. Tank for correcting the English text, and P. G. H. Mulder for providing the statistical model and performing the statistical analysis.

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