Influence of Temperature and Relative Humidity on the Survival of Chlamydia pneumoniae in Aerosols

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The survival of Chlamydia pneumoniae in aerosols was investigated by using a chamber with a capacity of 114.5 liters. We injected 5 × 107 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 LGV2, 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 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 LGV2 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 LGV2 and C. pneumoniae can be expected. Differences in the survival of C. trachomatis LGV2 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 × 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% CO₂, 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 glucose [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 LGV₂ (strain 343/Bu) was propagated in McCoy cells. Culture and purification of C. trachomatis LGV₂ were identical to those described for C. pneumoniae. We chose C. trachomatis LGV₂ 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-cm² tissue culture flasks (Costar) and grown to a confluent monolayer. The medium was then removed, and the cells were inoculated with 10⁷ to 10⁸ 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% CO₂. 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% CO₂. 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 LGV₂ 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% CO₂. After incubation, the infected monolayer was fixed with 96% ethanol (Merck). C. trachomatis LGV₂ 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.

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% CO₂.

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% CO₂. 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% CO₂, 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 600.

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

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.
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/cm²) 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 × 10⁷ inclusion-forming units (IFU) of chlamydia EBs or 1 × 10⁸ to 5 × 10⁸ CFU of S. faecalis, S. pneumoniae, or K. pneumoniae obtained from a 16-h culture was injected. For CMV, 1.4 × 10⁹ PFU contained in 1 ml of 0.2% FCS 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 × 10⁷ cpm ⁶⁷Ga-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 ⁶⁷Ga, 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 ¹²⁵I-labeled C. pneumoniae EBs (9). ¹²⁵I-labeled C. pneumoniae EBs (5 × 10⁴ 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/⁶⁷Ga.

Prior to spraying the microorganisms in the aerosol chamber, we tested whether they could withstand the injection pressure of 5 kg/cm² 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 LGV2 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...
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. pneumo-

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)/67Ga. 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.

moniae in aerosols at 8.5°C and 95% RH than at 15 to 25°C and 95% RH.

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.

FIG. 3. Survival of different microorganisms at 25°C and 95% RH. Survival is expressed as the ratio of viable microorganisms (mo)/67Ga. Survival over 5.5 min was measured for S. faecalis (○), C. pneumoniae (▲), K. pneumoniae (▼), C. trachomatis LGV2 (●), CMV (■), and S. pneumoniae (□). Error bars indicate the variation in the measurements.
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, 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.

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