Role of Relative Humidity in the Survival of Airborne Mycoplasma pneumoniae

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Aerosols of Mycoplasma pneumoniae were studied at several relative humidities at a controlled temperature of 27 C. Production of an experimentally reproducible aerosol required pretreatment of the organism in its suspending fluid and was dependent on the type of fluid used in atomization as well as on the procedures used to produce an aerosol. The airborne particles studied were within the range of epidemiological significance, with most being 2 μm or less in diameter. Survival of the airborne mycoplasma in these particles was found to be best at very low and at very high humidities. The most lethal relative humidity levels were 60 and 80%, at which levels fewer than 1% of the organisms survived over a 4-hr observation period. However, survival of the organism at most relative humidity levels was such that long-term infectivity could be expected from aerosols of M. pneumoniae. Because of the extreme sensitivity of M. pneumoniae at critical humidity levels, control of the airborne transmission of these organisms may be possible in selected places.

In 1944, Eaton and his associates recovered a filterable agent associated with primary atypical pneumonia (11). This agent was thought to be a virus until 1962, when Chanock et al. (4) succeeded in growing the FH strain of the "virus" on a cell-free medium and determined that it was a pleuropneumonia-like agent which has subsequently been designated as Mycoplasma pneumoniae.

Although M. pneumoniae has not been reported as being an important cause of pneumonia in civilian adults (15, 20), it has been shown to be a major cause of pneumonia in children, young adults (9, 13, 22), and in military recruits (3, 5, 12). During a 7-year study (3), M. pneumoniae was associated with 38% of all pneumonias in Marine recruits, and during a 10-month period of this study, it was associated with 50% of all pneumonias.

Because M. pneumoniae is an important respiratory pathogen in selected populations and because airborne spread of pathogenic microorganisms is probably limited to indoor spaces (23), a critical study of the ability of M. pneumoniae to survive in aerosols over extended periods of time at various humidity levels was undertaken. With these data, it should be possible to determine the potential epidemiological significance of airborne M. pneumoniae as the etiological agent in primary atypical pneumonia. Such information is also desirable in determining whether atmospheric control of closed (barracks) environments can provide an effective means of preventing epidemic spread of this organism.

MATERIALS AND METHODS

Organism and growth conditions. M. pneumoniae strain M52 was obtained from York Crawford, U.S. Naval Medical Research Unit #4, Great Lakes, Ill. The organism was grown in the broth medium of Chanock et al. (4), modified to contain 20% inactivated horse serum (Hyland), 2% yeast autolysate (Albini), penicillin G (50 units/ml), and amphotericin B (0.05 mg/ml). When a solid medium was desired, 1.2% agar was added to the above medium. The aerosol impingers contained 20 ml of PPLO broth (Difco) without enrichments plus 0.2 ml antifoam B (Dow-Corning).

Aerosolization. Cultures in the stationary phase of growth, 168 hr, were aerosolized with a modified Wells reflux atomizer into two 500-liter stainless steel rotating drums (14) installed in a constant-temperature room held at 27 C ± 0.2 C. In all experiments, the drums were filled after a 10-min preaerosolization period, and the number of organisms in the atomizer after aerosolization was regarded as reflecting the true number of existing colony-forming units (CFU). The relative humidity inside the drums was controlled by mixing measured amounts of wet and dry air in the secondary air stream and was measured by a wet and dry bulb thermometer. Physical decay of the aerosol was determined by measuring the relative light scatter of the aerosol with a forward-angle light scatter photometer. Samples were taken from the drums at intervals over a 4-hr period with all-glass impingers (AGI-30) and decay rates were determined by plating serial dilutions of the impinger fluids.
Enumeration of *M. pneumoniae* by this assay procedure had a standard error of the mean of 8% at *P* = 0.05. Particle sizes were determined with the Andersen sampler (1) by using the agar medium described above.

**RESULTS**

**Effect of reflux action on viability.** The shearing forces produced during aerosolization with reflux-type atomizers frequently produce mechanical trauma and possibly physiological injury to some, but not all, bacterial species (21). To minimize such mechanical damage to *M. pneumoniae*, the effect of reflux action on the viability of *M. pneumoniae* suspended in several spray fluid slurries was determined. *M. pneumoniae*, suspended in the following spray fluids, was refluxed in an atomizer for 1 hr: spent medium, fresh complete medium, PPLO broth (Difco) without enrichments, 0.85% NaCl, and distilled water. Samples were taken periodically from each suspension (slurry) and the number of surviving organisms was determined by serial dilutions and plating of the samples.

In all fluids, there was an increase in the number of CFU during the first 5 min of reflux atomization (Fig. 1). This initial increase in the number of recoverable CFU is similar to that noted when mycoplasma species were subjected to sonic vibration (18) and probably reflects a disaggregation of the dividing cells into discrete CFU. The greatest increase (1.5 logs) in CFU was observed in the spent medium, which also provided the greatest protection for the organism during atomization in that 75% of the maximal titer was recovered after 1 hr of refluxing. About 40% was recovered after the same period from fresh medium, PPLO broth, or distilled water, whereas only 10% was recovered from 0.85% NaCl. To minimize these atomizer effects, all subsequent studies were conducted only on aerosols which had been prepared from slurries that had been refluxed for at least 10 min prior to filling the aerosol chambers.

**Impinger fluid effects.** Because the nature of impinger fluids is known to affect the recoverability of airborne bacteria (21), the above listed liquids (except spent medium) were tested as impinger fluids. Samples were taken for 1 min from a test aerosol with impingers containing each of the fluids. The total recoverable CFU per liter of aerosol was then determined for each fluid. The data presented in Table 1 show that PPLO broth and complete growth medium were equally effective in recovering viable mycoplasma from aerosols, whereas the number of CFU found in impinger fluids of either normal saline or distilled water was slightly lower. Consequently, PPLO broth was used as an impinger fluid throughout subsequent studies.

The effect of storage on the survival of organisms recovered from an aerosol of *M. pneumoniae* in an impinger containing PPLO broth was determined. *M. pneumoniae* aerosols were sampled and stored in the impinger fluid at room temperature, at −20 C, and −70 C, and the number of viable organisms was determined at irregular intervals for up to 36 weeks. The results (Table 2) showed that it was best to titrate impinger fluids immediately after sampling, because survival was poor upon storage under each of the conditions tested.

The size of airborne particles created by aerosolization is the result of many different influencing factors. However, under the proper conditions (15 psi of air pressure), the Wells reflux atomizer

![Graph showing effect of suspending fluid on the survival of Mycoplasma pneumoniae during reflux atomization.](https://example.com/graph.png)

**FIG. 1. Effect of suspending fluid on the survival of Mycoplasma pneumoniae during reflux atomization.**

<table>
<thead>
<tr>
<th>Impinger fluid</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPLO broth</td>
<td>1.4 × 10^4</td>
</tr>
<tr>
<td>Complete medium</td>
<td>1.2 × 10^4</td>
</tr>
<tr>
<td>0.85% NaCl</td>
<td>0.84 × 10^4</td>
</tr>
<tr>
<td>0.85% NaCl</td>
<td>0.84 × 10^4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.75 × 10^4</td>
</tr>
</tbody>
</table>

* Impinger samples taken for 1 min from a test aerosol at 10% relative humidity.
is designed to produce particles of 5 μm or less in diameter. An examination of the airborne particle size showed that, with the medium used in these experiments, 98% of the resultant particles were 2 μm or less, whereas the remaining 2% were less than 3.5 μm in diameter when the drum humidity was 80%.

Survival in aerosol. The ability of *M. pneumoniae* to survive in aerosols with relative humidities between 10 and 90% was determined. Samples were taken at 5, 30, 60, 120, 180, and 240 min after filling the drums.

Figure 2 summarizes the results of these experiments. The values are given as per cent survival of the initial samples (taken 5 min after the drums were filled) at each relative humidity, and are averages of six experiments which have been corrected for physical decay. The number of organisms in the initial sample was 10⁴ to 10⁸ CFU per liter of aerosol. *M. pneumoniae* survived best in aerosols at drier humidity levels. Thus, 50% of the organisms survived for 4 hr at 10% relative humidity, and 35% were recoverable after 4 hr at 25% relative humidity. At 90% relative humidity, only 20% survived the 4-hr test period, and at all other relative humidity levels examined, fewer than 10% of the cells were viable after 4 hr. *M. pneumoniae* displayed extreme sensitivity to two relative humidity levels, 60 and 80%. Figure 3 shows the effects of humidity on survival of *M. pneumoniae* as a function of aerosol age. Survival at 30 and 120 min are given at the humidity levels examined. In addition to the wide, midrange humidity zone, there was a narrow lethal zone at 80% relative humidity.

**DISCUSSION**

Our data indicate that *M. pneumoniae* is less fragile than is generally assumed. Even without a rigid cell wall, these bacteria were found capable of withstanding the stresses normally occurring throughout the procedures necessary for dissemination into air and recovery from these experi-

mental aerosols. They are able to survive reasonably well as stored suspensions or as aerosols at 27 C and dry humidity ranges; however, they do not survive well during prolonged storage after recovery from aerosols nor in aerosols at some humidity ranges, notably at 60 and 80% relative humidity.

Dunklin and Puck (10) first showed that the survival of airborne bacteria is relative humidity-dependent. As with other mycoplasmas which have been studied (24), aerosol survival of *M. pneumoniae* was found to depend on relative humidity, and the midrange humidities were found to be the most lethal. *M. pneumoniae*, however, showed an additional lethal zone at 80% relative humidity, which has not been seen with other mycoplasmas. This lethal zone at the 80% relative humidity level was quite narrow and, although narrow zones of lethality are rare (2), Cox reported such a narrow lethal zone in aerosol experiments with *Escherichia coli* (6). The significance of two such relative humidity-sensitive zones for an organism is unclear and is a phenomenon which has heretofore been reported only for bacteria (7, 8). However, the failure to find other lethal zones may be the result of study-

**TABLE 2. Recovery of Mycoplasma pneumoniae from impinger samples stored for various time periods at different temperatures**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (hr)</th>
<th>No. (10⁴)</th>
<th>Time (hr)</th>
<th>No. (10⁴)</th>
<th>Time (weeks)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 C</td>
<td>0</td>
<td>1.2 × 10⁴</td>
<td>0</td>
<td>1.2 × 10⁴</td>
<td>0</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.7 × 10⁴</td>
<td>24</td>
<td>1.2 × 10⁴</td>
<td>4</td>
<td>3.8 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.3 × 10⁴</td>
<td>48</td>
<td>1.2 × 10⁴</td>
<td>12</td>
<td>3.5 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.1 × 10⁴</td>
<td>24</td>
<td>3.4 × 10⁴</td>
<td>36</td>
<td>3.4 × 10⁴</td>
</tr>
</tbody>
</table>

**FIG. 2. Survival of airborne Mycoplasma pneumoniae at 27 C and various relative humidities.** Figure was obtained by plotting the mean values of six separate experiments at each relative humidity. No single value deviated more than 15% from those shown.
The rotating drum since the first samples after aerosolization were taken 5 min after the drums were filled. However, from the decay rates shown (Fig. 2), it seems that *M. pneumoniae* can remain viable in aerosols for a much longer period of time than previously reported (19).

The effects of freezing and thawing on the viability of stored *M. pneumoniae* are very limited. Kim et al. (18) reported that, after a drop in the number of viable organisms during the first freeze-thaw cycle, subsequent freeze-thaw cycles were without noticeable effect on survival. This initial loss in viability at −70°C is reflected in our work (Table 2), although prolonged storage at this temperature does not appear to have an adverse effect on survival (16, 17). However, as the loss in number of organisms is not uniformly constant, titration of material taken from aerosols should be performed as soon as possible.

In addition to the rise in titer observed during aerosolization experiments, a 1- to 2-log increase was observed between slurry counts taken before and after refluxing in all experiments. This phenomenon makes it imperative that, in all aerosol studies done with *M. pneumoniae*, the experimenter allow an aerosolization period before the aerosol is allowed to enter the chamber.

The size of the airborne particles is in agreement with those reported by Kundsín (19). However, particle size is a result of complex interactions involving the relative humidity, slurry solute concentration, and function and type of atomizer. Also, the minimal size of a CFU-containing particle is determined by the size of the organism itself. Because of the complexity of the conditions determining the size of airborne particles, extrapolation of the laboratory findings to environmental circumstances surrounding epidemiological conditions in regard to particle size may not be valid.

The results of these studies are sufficient to show that *M. pneumoniae* can survive in the airborne state for long periods of time at several relative humidities. The effect of such airborne stress on the virulence and pathogenicity of the organism is not known and is currently being investigated. If virulence is maintained during such a long-term exposure to the atmospheric environment, these data would confirm the epidemiological significance of disease transmission by airborne *M. pneumoniae*, and it becomes apparent that some measure of contagion control should be possible through control of relative humidity within living spaces.

**ACKNOWLEDGMENTS**

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\textbf{LITERATURE CITED}


