Molecular diagnosis and epidemiology of Mycoplasma Pneumoniae
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Molecular detection of *Mycoplasma pneumoniae* among general practitioner patients with acute respiratory infection and their household contacts reveals children as human reservoir

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ABSTRACT

During a 30 months prospective study in the Netherlands, the distribution of *Mycoplasma pneumoniae* and respiratory viruses among 1172 patients with acute respiratory infection (ARI) was studied in the outpatient general practitioner setting. Nose/throat samples from household contacts of *M. pneumoniae* positive patients were collected to study the effect of antibiotic treatment of index cases on *M. pneumoniae* transmission. Among the 1172 ARI patients *M. pneumoniae*, as detected by PCR, was present in 39 (3.3%) patients. The infection rate of *M. pneumoniae* was similar in all age groups. Of 79 household contacts, 12 (15%) had *M. pneumoniae*. The frequency of *M. pneumoniae* among household contacts of index cases treated with appropriate antibiotics and non-treated cases was similar. Nine of the 12 *M. pneumoniae* positive contacts were younger than 16 years (*P*=0.02), and 4 (44%) of them did not develop ARI. Therefore, children are apparently a relevant reservoir for *M. pneumoniae*. 
INTRODUCTION

Mycoplasma pneumoniae, a common respiratory pathogen, causes usually mild upper respiratory tract infections such as sore throat, pharyngitis, and tracheitis. In 5-10% of patients, M. pneumoniae infection results in tracheobronchitis or pneumonia. An estimated 2-4% of patients with M. pneumoniae infection requires hospitalisation (1). Data on the incidence and transmission of M. pneumoniae are derived mainly from large scale population studies performed in the 1960s and 1970s, making use of classical methods like culture and serology to diagnose M. pneumoniae (2,3,4,5). Modern techniques like PCR on easily obtainable specimens such as throat- and nasal swabs can be used to provide new insight into the epidemiology of M. pneumoniae infection in outpatient- and community settings (1,6,7,8).

In the Netherlands, respiratory infections, including those due to M. pneumoniae, are being studied in an outpatient general practitioner (GP) setting within the framework for monitoring influenza virus outbreaks, as recommended by the World Health Organization (9). This monitoring is performed in a national surveillance network by GPs evenly distributed over the country. GPs identify patients with acute respiratory infection (ARI), and register those ARI patients who present with influenza-like illness (ILI). GPs collect nose/throat swabs from randomly selected ARI patients. These samples are analysed for the presence of respiratory viruses and M. pneumoniae.

We aimed to assess (i) the frequency of M. pneumoniae infection among ARI patients in the outpatient GP setting, (ii) the distribution of M. pneumoniae patients by patient age and season as compared to patients with ARI due to respiratory viruses, (iii) the frequency of M. pneumoniae infection by age among household contacts of M. pneumoniae positive index cases, and (iv) the influence of antibiotic treatment of M. pneumoniae positive patients on transmission of M. pneumoniae in the household setting.

PATIENTS, MATERIALS AND METHODS

Design of the surveillance

GPs from 45 practices, evenly distributed over the Netherlands, participate in a nationwide sentinel surveillance network co-ordinated by the Netherlands Institute of Primary Health Care (NIVEL). The surveillance covers about 1% of the population in the Netherlands (15.7 million inhabitants). This sample of 150,000 people is representative for the national population in terms of age, sex and degree of urbanisation (10). Since 1970, GPs of the network register patients presenting with ILI to enable the calculation of the weekly incidence of ILI. Since 1992 GPs collect nose/throat samples from patients presenting with ILI and other ARI (hereafter referred to as ARI non-ILI) in order to assess the aetiology of such
infections. ILI is defined as a respiratory infection with acute onset, fever (a rectal temperature of at least 38 °C) and at least one of the following symptoms: coryza, sore throat, cough, frontal headache, retrosternal pain, myalgia. ARI-non ILI is defined as a respiratory infection with acute onset and at least one of the above mentioned symptoms. Patients presenting with ARI-non ILI are not registered by the GPs participating in the surveillance.

*M. pneumoniae* infection among patients with ARI

During a 30 months prospective study, from January 1, 1997 through June 30, 1999, GPs in each practice were requested to collect a nose/throat sample from randomly selected patients with ARI. Each week GPs sampled a maximum of two patients. One swab taken from the nose and one swab taken from the throat were placed together in 4 ml of Hanks’ balanced salt solution containing gelatine, lactalbumin, yeast and antibiotics, and were sent to the Virology Department of the Laboratory of Infectious Diseases and Perinatal Screening (LIS) at the National Institute of Public Health and the Environment by routine mail. The sample, referred to as nose/throat sample, was processed at the day of arrival. For each patient, GPs completed a questionnaire including patient characteristics, time of onset of illness, clinical signs and symptoms, and the presence of respiratory infections among direct contacts of the patients.

Detection of *M. pneumoniae* by PCR

Upon receipt of the samples at the laboratory, the nose and throat swabs were twirled in the transport medium and removed. Four hundred µl of this nose/throat sample was processed for *M. pneumoniae* PCR, as described previously (11). In short, the suspension was centrifuged at 12,000 x g for 30 min. DNA was isolated from the pellets using a proteinase K lysis protocol, and analysed for the presence of *M. pneumoniae* DNA using a nested PCR protocol with the P1 cytadhesin gene as the target. The samples were checked for the presence of PCR inhibitors, using an amplification control (11). In case of inhibition, the lysate was diluted 1:10 and retested in the PCR. Reaction products were analyzed by 2% agarose gel electrophoresis.

Detection of respiratory viruses

Specimens were routinely processed for isolation and detection by PCR of various respiratory viruses. Virus isolation was performed according to standard methods (12,13). Tertiary cynomolgus monkey cells (tMK) and human diploid lung fibroblasts (local cell line GaBi) in tubes were each inoculated with about 500 µl of the nose/throat sample. Cell cultures were inoculated in roller drums at 33°C. Another aliquot of 500 µl of the sample was used to inoculate tMK cells in flat-bottom tubes, which were subsequently centrifuged at 5,000 x g at RT for 75 min, and incubated stationary at 33°C. Influenza viruses were (sub)typed in
Table 1. Distribution of *M. pneumoniae* and various respiratory viruses in nose/throat samples from 1172 patients presenting with ARI to their GP, in 32 general practices in the Netherlands, January 1, 1997 to July 1, 1999

<table>
<thead>
<tr>
<th>Age category</th>
<th>No. of patients sampled (of total samples)</th>
<th>No. of patients with ≥ one respiratory pathogen (% of total per age category)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of patients (% of total per age category with a sample positive for</td>
<td>M. pneumoniae&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>0-4</td>
<td>6</td>
<td>114 (10)</td>
</tr>
<tr>
<td>5-15</td>
<td>12</td>
<td>140 (12)</td>
</tr>
<tr>
<td>16-25</td>
<td>13</td>
<td>170 (14)</td>
</tr>
<tr>
<td>26-40</td>
<td>25</td>
<td>348 (30)</td>
</tr>
<tr>
<td>41-60</td>
<td>26</td>
<td>291 (25)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>18</td>
<td>98 (9)</td>
</tr>
<tr>
<td>unknown</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>total</td>
<td>15.7 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1172</td>
</tr>
</tbody>
</table>

<sup>†</sup> percentages derived from figures per 01-1999 (18)
<sup>‡</sup> no significant difference between *M. pneumoniae* infection in different age categories was observed (χ² test, P>0.05)
<sup>‡‡</sup> the total number of patients with ≥ one respiratory pathogen is lower than the summarised totals of patients having specific respiratory pathogens, since 56 (9.9%) patients had more than one respiratory pathogen
hemagglutination inhibition assays (14). Other viruses were identified by standard methods (12,13). PCR was performed for RSV (15), rhinovirus and enterovirus (16) and coronaviruses OC43 and 229E (17).

Transmission of *M. pneumoniae* among household contacts of the patients

Household contacts of patients with a nose/throat sample positive for *M. pneumoniae* were asked to participate in the transmission study. From the household contacts a nose/throat sample was collected by the GP within 4 weeks after sampling of the index case. A questionnaire with the characteristics of the sampled persons, the presence of signs and symptoms of a respiratory infection and antibiotic usage was completed. Samples were processed for *M. pneumoniae* PCR.

Statistical analysis

The $\chi^2$ test was used to compare the frequency of *M. pneumoniae* infection in the different age categories and the frequency of *M. pneumoniae* among household contacts of index cases treated with appropriate antibiotics and of non-treated cases. For comparison of the frequency of *M. pneumoniae* in the winter period and the summer period the $\chi^2$ test with Yates correction was used. The $\chi^2$ test with Mantel-Haenszel trend analysis was used to compare the frequency of *M. pneumoniae* in the age categories of the household contacts.

RESULTS

Distribution of *M. pneumoniae* and respiratory viruses among GP patients with ARI

During the 30 months study period GPs in 32 (70%) of the 45 general practices participating in the surveillance collected nose/throat samples from 1172 ARI patients. The 1172 patients were a representative sample of the national population with respect to geographic and sex distribution (18). There was a slight overrepresentation of the youngest age category and an underrepresentation of people over 60 years of age (Table 1).

In total, 631 respiratory pathogens were detected in nose/throat samples from 565 (48%) patients. Mixed infections occurred in 56 ARI patients, predominantly in the 0 to 4 years age category.

Of the 1172 ARI patients, 772 (65%) presented with ILI and 400 (35%) with ARI non-ILI. *M. pneumoniae* DNA was present in samples from 39 (3.3%) of the 1172 patients, being 24 (3.1%) ILI patients and 15 (3.7%) patients with ARI non-ILI. Of the 39 *M. pneumoniae* positive patients, 22 (56%) were males and 17 (44%) were females. The detection rate of *M. pneumoniae* was similar in the different age groups ($P>0.05$) (Table 1). Mixed infections due
to *M. pneumoniae* and a viral agent were detected in 7 patients: in 4 with rhinovirus, in 2 with enterovirus, and in 1 with respiratory syncytial virus.

The vast majority of the samples (86%; 1012/1172) was obtained from patients presenting with ARI in the winter period, October through March (Fig 1). *M. pneumoniae* infection occurred during the whole year. Although the frequency (5.6%; 9/160) was higher in the summer period than in the winter period (2.9%; 30/1012) (Fig 1), this difference was not significant.

![Figure 1](image)

**Figure 1**
Total number of samples and of *M. pneumoniae* positive samples from patients consulting their GP for ARI by month in the Netherlands, from January 1, 1997 until July 1, 1999. Frequency of *M. pneumoniae* infections during summer periods was not significantly different from that during winter periods ($\chi^2$ test with Yates correction, $P>0.05$)

**Frequency of *M. pneumoniae* among household contacts**

The 39 *M. pneumoniae* positive index cases represented 39 households. In four households sampling of the household contacts was not possible within 4 weeks after samples from the index had been collected, and three patients and two GPs refused to participate in this part of the study. In total, nose/throat samples from all the 79 household contacts of 30 index cases were obtained. Samples were collected at a mean of 22 days (range 14 to 30 days) after initial sampling of the index case. *M. pneumoniae* was present in samples from 12 (15%) of the 79 household contacts (Fig. 2). Among these 12 *M. pneumoniae* positive contacts 9 were children (0-15 yrs) and 3 persons were older than 15 years ($P=0.02$). Of the 12 *M. pneumoniae* positive contacts 8 had an ARI, of whom 3 had consulted their GP because of
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ARI. Four of the children contacts were asymptomatic 4 weeks before and at least 4 weeks after sampling (Fig. 2).

*M. pneumoniae* infection among household contacts of antibiotic treated and non-treated patients.

Eight of the 30 index cases had been treated with doxycycline or macrolide antibiotics, whereas 3 had received β-lactam antibiotics and 19 had not used antibiotics. The frequency of *M. pneumoniae* positive throat samples among the 23 household contacts of the 8 doxycycline or macrolide treated cases was not significantly different from that among the 9 household contacts of the 3 β-lactam antibiotic treated cases and the 47 household contacts of the 19 cases who did not receive antibiotics ($P>0.05$) (Table 2). The 3 *M. pneumoniae* positive household contacts of the 8 index cases treated with doxycycline or macrolides had ARI, whereas among the 9 *M. pneumoniae* positive household contacts of the β-lactam antibiotic treated or non-treated cases 5 had ARI (Table 2).

<table>
<thead>
<tr>
<th>Treatment of <em>M. pneumoniae</em> positive index cases</th>
<th>No. of index cases</th>
<th>No. of household contacts</th>
<th><em>M. pneumoniae</em> positive contacts</th>
<th>ARI present</th>
<th>ARI absent</th>
<th>total (% of all contacts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline/macrolides</td>
<td>8</td>
<td>23</td>
<td>3</td>
<td>0</td>
<td>3 (13%)*</td>
<td></td>
</tr>
<tr>
<td>β-lactam antibiotics</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No antibiotics</td>
<td>19</td>
<td>47</td>
<td>5</td>
<td>4</td>
<td>9 (19%)*</td>
<td></td>
</tr>
</tbody>
</table>

*no statistically significant differences ($\chi^2$ test, $P>0.05$)

**DISCUSSION**

During a 30 months prospective study the distribution of *M. pneumoniae* and various respiratory viruses among GP patients with ARI in the Netherlands was studied. *M. pneumoniae* was sought for by PCR in nose/throat samples from 1172 such patients. Among pneumonia patients the incidence of *M. pneumoniae* infection diagnosed by serology and culture has been estimated at 200 per 100,000 population per year in a 12 years population study in the USA (2). The incidence of *M. pneumoniae* infection as diagnosed by PCR among outpatients with ARI and assessed in 5 successive winter periods in France ranged from 190
Figure 2
Number and age distribution of *M. pneumoniae* positive index cases participating in the transmission study (●), their *M. pneumoniae* negative household contacts (○) and *M. pneumoniae* positive symptomatic (▲) and asymptomatic (△) household contacts. Age categories are indicated by the dotted lines

*More M. pneumoniae* positive household contacts were detected in the youngest age categories (0-4 and 5-15 years) than in the other age categories (16 years and older) (Mantel-Haenszel $\chi^2$ trend analysis, $P=0.02$)

to 1,234 per 100,000 population (8). In our study we detected *M. pneumoniae* by PCR in samples from 39 (3.3%) of 1172 ARI patients, of whom 772 presented with ILI and 400 with ARI non-ILI. The calculated incidence of *M. pneumoniae* among the ARI patients presenting to the GP was 587 per 100,000 population per year in the Netherlands. The incidence of ILI among ARI patients was 2,410 per 100,000 population per year (19). The frequency of *M.
pneumoniae infection among these ILI patients was 3.1%. Therefore, the incidence of *M. pneumoniae* infection in GP patients with ILI was calculated to be 75 per 100,000 population per year. Although patients presenting with ARI non-ILI were not registered during this study, various sources provide data on the frequency of ARI among GP patients in the Netherlands (20). From these data and the ILI incidence data, the calculated incidence of ARI non-ILI was 13,840/100,000 population per year. Since we found *M. pneumoniae* present in 3.7% of the samples from ARI non-ILI patients, the incidence of *M. pneumoniae* infection among such patients was calculated to be 512/100,000 population per year. Thus, the incidence of *M. pneumoniae* among ARI patients presenting to the GP was 587 per 100,000 population per year. In the community, the incidence of *M. pneumoniae* infection is expected to be much higher as follows from our finding that among *M. pneumoniae* positive household contacts of *M. pneumoniae* positive patients, only 25% consulted their GP. The incidence of 587 *M. pneumoniae* infections in ARI GP patients per 100,000 population found in our study, reflects the incidence in a *M. pneumoniae* endemic 30 months period in the Netherlands (21), whereas the incidence of 1,234 per 100,000 population as reported in the French study was estimated in a *M. pneumoniae* epidemic winter period (8).

ARI due to *M. pneumoniae* occurred during all seasons. The number of *M. pneumoniae* positive patients was highest during winter months. The proportion of *M. pneumoniae* positive patients among patients with ARI was highest during summer months because of the low frequency of respiratory virus infections during these months (not shown).

In the GP patient population, *M. pneumoniae* infection occurred at all ages and no significant difference between the different age groups was present, similarly to the results as recently reported by Layani et al. for a French outpatient population (8). Their and our findings are not in agreement with data from other studies (2,22,23,24) in which school-age children (5 to 15 yr) and adults (30 to 45 yr) were identified as preference age groups for *M. pneumoniae* infection. A possible explanation could be that in these studies mainly hospitalised patients with *M. pneumoniae* infection were surveyed, whilst our study was performed in a GP setting.

Among the 79 household contacts of 30 *M. pneumoniae* positive patients, significantly more *M. pneumoniae* positive contacts were in the 0 to 4 and 5 to 15 years age groups than in the other age groups. The *M. pneumoniae* positive children had either no signs and symptoms of ARI or ARI was so mild that these children did not visit their GP. This strongly suggests that young children are a relevant reservoir for *M. pneumoniae*, and play an important role in transmission as has been reported for respiratory viruses (25,26,27,28). The finding also supports the assumption that clinical signs and symptoms of a first infection due to *M. pneumoniae* are absent or mild but that sensitisation occurs. A subsequent *M. pneumoniae* infection at older age may then induce more severe clinical manifestations (29).
The frequency of *M. pneumoniae* positive contacts in the households of the 8 doxycycline or macrolide treated index cases, of the 3 β-lactam antibiotic treated index cases and of the 19 non-treated index cases was similar. As doxycycline and macrolides are antibiotics by which *M. pneumoniae* infection is effectively treated (30), our finding suggests that *M. pneumoniae* transmission from such effectively treated cases still occurs in the household setting. It may be that transmission can be prevented only by early treatment of the index case. Similarly, spread of *Bordetella pertussis* among household contacts was prevented only if effective treatment of the index cases by erythromycin was initiated early in the disease (31,32).

In conclusion, our study shows a frequency of *M. pneumoniae* infection of 3.3% among GP patients presenting with ARI, and of 15% among their household contacts as detected by PCR. In the latter group, *M. pneumoniae* was mainly present in children aged 0 to 15 years in whom the infection was either mild or asymptomatic. The rather high infection rate among these household contacts of *M. pneumoniae* positive patients justifies the conclusion that children may be an as yet unrecognised reservoir for *M. pneumoniae*.

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