Molecular diagnosis and epidemiology of Mycoplasma Pneumoniae
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Citation for published version (APA):
CHAPTER 8

General Discussion
GENERAL DISCUSSION

*Mycoplasma pneumoniae* as cause of community-acquired pneumonia and other respiratory infections

The aetiology of infections of the respiratory tract has intrigued physicians for generations. In the late 1930ies the main bacterial causes of pneumonia had been recognised. Then, the occurrence of the so-called “atypical pneumonia” drew the attention of physicians. The term atypical referred to pneumonias for which no known bacterial cause by Gram stain and culture was found, and later on also to pneumonias not responding to antibiotic treatment with sulfonamides and penicillins. The development of better techniques to isolate fastidious bacteria and to characterise viruses revealed that the pathogens causing atypical pneumonia were respiratory viruses such as influenza virus, respiratory syncytial virus and adenovirus, and various bacteria such as *M. pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii* and probably many other micro-organisms.

The proportion of patients with community-acquired pneumonia (CAP) due to *M. pneumoniae* varies in different studies (1-3). Several factors influence the frequency of *M. pneumoniae* infection such as whether there is an epidemic situation of *M. pneumoniae* at the time a study is performed, the age group involved in the study but also the techniques used for laboratory diagnosis.

Only a minor portion of *M. pneumoniae* infections will result in the onset of pneumonia. It has been estimated that 5-10% of infected patients develop pneumonia, depending the patients’ age (4) and the presence of underlying diseases (5-7). Pneumonia due to *M. pneumoniae* is usually mild as indicated by its name ‘walking pneumonia’. In a 12 years population study, only 2% of all patients with *M. pneumoniae* pneumonia were hospitalised (8). On the other hand, of all patients hospitalised with CAP, *M. pneumoniae* has been identified as the etiologic bacterium in 4.5-5.6 % of the cases (8-11). In the Netherlands, the proportion of CAP caused by *M. pneumoniae* probably is similar to that reported from other industrialised countries. In a prospective study of CAP among adults requiring hospitalisation performed in the Netherlands in 1991 through 1993, *M. pneumoniae* was identified in 6 % of the cases (12).

In our study among 144 adults hospitalised with CAP *M. pneumoniae* infection was identified in 12% of the patients (Chapter 3). This high percentage was achieved by the combined use of PCR on multiple respiratory specimens and serology. During the initial phase of this study a *M. pneumoniae* epidemic situation existed in the Netherlands (13) which may have contributed to the high percentage of *M. pneumoniae* among the CAP patients. In the Netherlands, about 110.000 cases of CAP occur each year, of which about 12% are
hospitalised (14). If in an estimated 6 to 10% of these patients *M. pneumoniae* is involved in the aetiology, about 800 to 1300 hospitalised *M. pneumoniae* CAP cases occur annually.

Mortality due to *M. pneumoniae* infection has been reported to be rare. In a meta-analysis on the outcome of patients admitted with CAP, patient mortality among patients with CAP due to *M. pneumoniae* was 1.4% (15).

As mentioned before only a minor portion of *M. pneumoniae* infections result in pneumonia. Therefore, the number of patients admitted to the hospital with any *M. pneumoniae* infection is higher than the number of patients hospitalised with CAP due to *M. pneumoniae*. To gain insight into the proportion of patients with *M. pneumoniae* among patients hospitalised because of community-acquired respiratory infection, we conducted a 15 months prospective study among children (0.5-18 years) admitted to the outpatient departments (OPD) of an academic hospital (AMC) and a general hospital (Boven-IJ) (Chapter 2). Results obtained by *M. pneumoniae* PCR performed on throat swab specimens obtained from these children were compared to results obtained by serological methods. In 10% of the patients *M. pneumoniae* infection was diagnosed. The study was performed during an endemic period of *M. pneumoniae* in the Netherlands (13). Therefore, the frequency of community-acquired respiratory tract infections due to *M. pneumoniae* among children admitted to OPDs may even be higher in a *M. pneumoniae* epidemic period.

*M. pneumoniae* in the community and general practitioner setting

Only a limited number of studies have been performed on the aetiology of community-acquired respiratory infections in patients not requiring hospitalisation. Some of those studies focused on adults with CAP (16) or on childhood pneumonia (4), others on lower respiratory tract infection in adults (17) or on both upper and lower respiratory tract infection (RTI) in adults (18). The frequency of *M. pneumoniae* in these study populations varied from 22% in the CAP patients (4,16) to 0.5 and 11% in the patients with RTI (17,18). The significant difference in frequency of *M. pneumoniae* between the latter two studies can be explained by the difference in inclusion criteria and in laboratory techniques used.

In the Netherlands, general practitioners (GP) participating in a nation wide surveillance network, collect nose/throat samples from patients presenting with acute respiratory infections (ARI) in order to assess the aetiology of such infections. The aim of this surveillance is to monitor influenza virus outbreaks, but the approach also provides data on the frequency of other, mostly viral, respiratory pathogens in ARI in a GP setting. The frequency of *M. pneumoniae* among the ARI patients varied from 1.3% (1994-1995) to 5.4% (1997-1998) (19,20). During a 30 months period, we studied the *M. pneumoniae* infected patients identified in this surveillance (3.3%) and their household contacts (Chapter 5). From the data
obtained in this study and the incidence of ARI in GP practices in the Netherlands (21), we calculated an annual incidence of *M. pneumoniae* in GP patients presenting with ARI of 587 per 100,000 population. This means that each GP, providing care for an average of 2500 patients in the Netherlands, is consulted by 15 patients with ARI due to *M. pneumoniae* annually. In total, about 92,000 *M. pneumoniae* infections in ARI patients presenting to their GP occur each year in the Netherlands.

We found *M. pneumoniae* to be present equally distributed over the different age-categories. In most studies school-age children and adults (30 to 45 year) have been identified as preference age groups for *M. pneumoniae* infection. Also the data obtained by us from laboratory registration systems in England/Wales and the Netherlands (Chapter 1) showed that *M. pneumoniae* infection is most prevalent in these age groups. However, both the difference between study populations (hospitalised or primary care patients) and the difference between laboratory techniques applied (serological or molecular diagnosis) can be responsible for this discrepancy. Thus, also in patients older than 45 years with RTI or CAP, *M. pneumoniae* has to be considered as the etiologic agent. Indeed, in some recent studies the significance of *M. pneumoniae* in pneumonia leading to hospitalisation in elderly patients has been documented (10,22).

Another important finding from our study was the identification of asymptomatic children with *M. pneumoniae* present in their throats among the household contacts. From this finding and the finding that significantly more children than older household contacts were *M. pneumoniae* positive, we concluded that children are a relevant reservoir for *M. pneumoniae*. This may have implications for vaccination strategies in case vaccination against *M. pneumoniae* is reconsidered in the future.

### Molecular techniques for diagnosis

When the research described in this thesis was started, application of PCR in routine diagnostics of infectious diseases was relatively new. Therefore, it was difficult to estimate the impact of PCR on diagnosis of *M. pneumoniae* infection. Would such a sensitive technique lead to 'overdiagnosis' of *M. pneumoniae* infection partly due to detection in healthy carriers? How should the clinician anticipate on a positive PCR result from the laboratory?

Insight into how to interpret positive *M. pneumoniae* PCR results, provided all precautions have been taken to avoid laboratory contamination, has grown during the past years.

In our prospective study among 144 adults hospitalised with CAP, various sites of the respiratory tract of the patients were sampled. Specimens were used for *M. pneumoniae* PCR and the results were compared with those obtained by which antibody response to *M.
pneumoniae can be assessed. We observed that sputum was the respiratory specimen with the highest percentage of *M. pneumoniae* DNA in adults with CAP. The percentage of *M. pneumoniae* DNA-positive nasopharyngeal- and throatwash specimens was 50% less than that of sputum specimens. However, about 30% of the patients admitted with CAP cannot expectorate sputum (2; this thesis, chapter 3). Therefore, in a relevant proportion of cases detection of *M. pneumoniae* DNA has to be performed on less representative respiratory specimens, such as nasopharyngeal swabs or throat washes.

A major advantage of molecular diagnosis of *M. pneumoniae* in CAP patients is the availability of rapid results from the laboratory. With these results, appropriate antibiotic treatment can be started in an early phase of the infection. However, in both studies in which we applied PCR for diagnosis of *M. pneumoniae*, in about 20% of the cases *M. pneumoniae* diagnosis could only be established by antibody detection in paired sera (Chapter 2 and 3). In a recent study evaluating PCR for etiologic diagnosis in CAP patients, a low sensitivity of *M. pneumoniae* PCR compared to serological diagnosis was reported (23). However, only throat swab specimens were used for *M. pneumoniae* PCR in this study. Based on our own results, we conclude that application of *M. pneumoniae* PCR, in addition to serological methods, will increase the number of patients diagnosed with *M. pneumoniae* infection, especially in patients with impaired immune response.

As PCR is a sensitive technique for detection of *M. pneumoniae* DNA, and thus may identify possible “carriers”, we studied age-matched controls of the children with community-acquired respiratory infection (Chapter 2). No positive PCR results were found among these controls. However, in a follow-up study among patients residing in a home for mentally disabled, we detected *M. pneumoniae* DNA in the throat for several weeks after respiratory infection due to *M. pneumoniae* (24).

To enable discrimination between strong and weak positive PCR results, we developed a semiquantitative PCR for *M. pneumoniae*. Application of this technique on throat specimens obtained from hospitalised and non-hospitalised *M. pneumoniae* DNA positive subjects, revealed a higher *M. pneumoniae* load among the hospitalised patients (Chapter 4). A relation between *M. pneumoniae* load and severity of disease is thus very likely. Future developments in automatic PCR quantification (’real time PCR’) will allow easier application of this technique in *M. pneumoniae* disease (25) as it is already the case in infections caused by bloodborne viruses such as hepatitis B virus, hepatitis C virus and human immunodeficiency virus.
Tools for molecular epidemiology

Molecular typing of numerous bacterial and viral pathogens has allowed detailed studies of transmission and epidemiology. In the case of *M. pneumoniae* the genome appears to be highly conserved, which hampers the development of genotyping tools for this microorganism. By the end of the 1980ies, two groups among *M. pneumoniae* isolates could be discriminated, based on variation in their P1 genes (26,27). The P1 gene encodes a 170 kDa protein, which has been identified as major cytadhesin and therefore virulence factor of *M. pneumoniae* (28). Based on differences in the P1 gene, a PCR restriction fragment length polymorphism (RFLP) was designed (29). Application of this technique divided 215 Japanese *M. pneumoniae* isolates collected over a 20 years period into the two groups (30). Years in which one group is most prevalent are succeeded by years in which the other group prevails (30;31). During the past ten years, effort has been put in the search for more variation in the genome of *M. pneumoniae* clinical isolates. Techniques using the whole *M. pneumoniae* genome as target for typing, like random amplified polymorphic DNA (RAPD) analysis and amplified-fragment length polymorphism (AFLP) analysis also revealed the existence of only two groups among *M. pneumoniae* clinical isolates (32,33). Pulsed-field gel electrophoresis (PFGE) as applied by Cousin et al. showed only one subgroup among the P1 type 2 isolates (34).

We applied three genotyping methods to 2 *M. pneumoniae* reference strains and 21 clinical isolates (Chapter 6). As variation in the P1 gene may occur through recombination between repetitive sequences in the P1 gene itself and at other locations of the genome (35), and as antigenic variants of in the P1 gene may be selected under immune pressure, we focused on the P1 gene as genotyping target. An extended set of restriction enzymes was used in the PCR RFLP analysis of the P1 gene of the strains studied, and revealed 8 subtypes among the P1 type 1 and type 2 strains. Furthermore, we performed sequence analysis of the 16S-23S spacer region and part of the 23S rRNA gene, a method that has been successfully applied in strain differentiation of various bacterial species. As a third approach genome sequence data of *M. pneumoniae* reference strain M 129 (a P1 type 1 strain) (36) were used to develop primers for amplification of large interrepeat fragments by long PCR, which were subsequently analysed by RFLP. By the last two methods, the strains were divided into two genomic groups. An association between the P1 type, 23S rRNA gene 'type' and long PCR type for all 23 strains was found. These findings confirmed the existence of two genomic groups among *M. pneumoniae* clinical isolates, but expanded possibilities for strain differentiation based on variation in the P1 gene.
Unravelling the P1 cytadhesin gene

Although the P1 RFLP technique is a PCR based typing method for which, in contrast to PFGE, not a large amount of chromosomal DNA is required, still long PCR fragments of about 2.5 kb are necessary to perform the RFLP. Such long fragments are not easily amplified directly from clinical specimens (own observation). This means that isolation of *M. pneumoniae* is still required to perform this typing method. As we now identified clinical *M. pneumoniae* isolates with P1 gene variation within the P1 types 1 and 2, a set of strains with unique RFLP patterns were selected for sequence analysis of their P1 genes (Chapter 7). Indeed, as expected from the RFLP patterns, nucleotide sequence variations within P1 type 1 and P1 type 2 strains were detected, which enabled us to identify targets in the P1 gene for direct genotyping. In addition, stretches in the P1 gene containing variable sequences were identified. In the P1 gene sequence of one strain, part of the variable sequence was identical to that in a recently reported novel P1 gene sequence (37). Regions homologous with the variable stretches as detected in the P1 genes of our *M. pneumoniae* isolates, were found outside the P1 gene locus in the genome sequence of reference strain M129. Further studies to elucidate whether intrachromosomal recombination is a mechanism generating this P1 gene variability, are needed.

Most point mutations detected in the P1 gene sequences resulted in amino acid changes. Several of these amino acids were located in domains containing cytadherence mediating epitopes (38,39), and therefore can be relevant for function of the P1 protein and escape from the host immune response.

In the studies described in this thesis, we have shown that *M. pneumoniae* plays an important role in the aetiology of respiratory infections, both in hospitalised patients as in patients with milder respiratory infections. Application of molecular techniques to detect of *M. pneumoniae* decreased the number of patients hospitalised with a community-acquired pneumonia of ‘unknown origin’ and enabled estimation of the incidence of *M. pneumoniae* in a general practitioner patient population.

Furthermore, molecular techniques revealed more variation in the major cytadhesin P1 gene of clinical isolates of *M. pneumoniae* than thus far anticipated. The recent sequencing of the entire genome of *M. pneumoniae*, allowed speculations about possible mechanisms causing this P1 gene variability. However, the role of recombination events in generating P1 gene variation has not been proven.
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