Molecular diagnosis and epidemiology of Mycoplasma Pneumoniae
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CHAPTER 1

General Introduction
GENERAL INTRODUCTION

1. Description of the organism

1.1 History

In 1944 Eaton and colleagues described an agent that passed through filters with pore sizes that retain bacteria and caused pneumonia when inoculated in rodents (1). The agent was believed to be a virus as it passed through bacteriologic filters and could be grown in chicken embryos only. The relation of the agent to the primary atypical pneumonia (PAP) syndrome was suggested by the fact that human serum from patients recovering from PAP neutralised the agent. In the early 1960ies it was established that the organism had many characteristics in common with the pleuro pneumonia-like organisms (PPLO) isolated in 1898 by Nocard and co-workers and causing contagious pleuropneumonia in cattle. Its mycoplasmal nature was established by growing the agent on cell free medium (2), and subsequently it was appropriately named *Mycoplasma pneumoniae*.

1.2 Taxonomy

*M. pneumoniae* is one of the currently recognised 102 Mycoplasma species in the order of Mycoplasmatales. Thirteen of these 102 Mycoplasma species have been isolated from humans. Mycoplasmas are classified in the Class Mollicutes (mollis=soft, cutis = skin) which name originates from the lack of a rigid cell wall, separating mycoplasmas from other bacteria. They are bounded by a cell membrane containing sterols. Because of their deformable membrane and small size (150-250 nm), mycoplasmas are able to pass through bacteriologic filters. As mycoplasmas lack a typical bacterial cell wall, they are not visible on Gram staining and resistant to cell wall-active antimicrobial agents such as penicillins and cephalosporins. Mycoplasmas are able to grow in cell free media and belong to the smallest self-replicating organisms with genome sizes of 580-1350 kb. Recently, sequence data from the entire genomes of *M. pneumoniae* (3) and *M. genitalium* (4) became available. This information will enhance the knowledge of the molecular biological basis of these microorganisms. The small genome is remarkable for such successful pathogens. The question is whether there is any relation between genome size and the ability of mycoplasmas to grow in vitro. Recently, Hutchison *et al.* sorted out the number of essential genes necessary for replication of *M. genitalium* (5). Their conclusion was that 140 of the 340 genes, at least under laboratory conditions, are not a prerequisite for the propagation of this smallest-known free-living micro-organism. In addition, obligate intracellular bacteria like *Chlamydia pneumoniae*, *Coxiella burnetii*, and *Rickettsia* species have genomes larger than many of the self-replicating mollicutes, making a direct relation between genome size and the ability to grow in vitro unlikely. The genome sequence projects provided the genetic explanation for the requirements
of serum, peptone, and yeast extract in nutritious growth media for the in vitro growth of mycoplasmas. Only a limited number of genes allows biosynthetic pathways in mycoplasmas and no genes are present for amino acid synthesis (3,4). Therefore, M. pneumoniae and M. genitalium are totally dependent on exogenous supply of amino acids.

M. pneumoniae is a filamentous organism, about 10×200 nm in size, with a genome of 816 kb. Mycoplasmal DNA has an exceptional low guanine and cytosine (G&C) content, with M. pneumoniae showing the highest G&C content of 40 mol% of the entire genome. Characteristic for mycoplasmas is the use of TGA as a tryptophan codon instead of a stop codon. The flask shaped cells of M. pneumoniae have a terminal tip structure and exhibit gliding motility on solid surfaces (6,7). The terminal tip structure is responsible for attachment of the organism to cell membranes. Of the surface proteins required for cytaadherence, the 169 kDa protein P1 has been most extensively studied (8-11).

1.3 Habitat

Human mycoplasmas colonise mucous membranes in the ororespiratory or genitourinary tract, where some species behave as commensals. Under immune suppression, for example after organ transplantation, in AIDS patients or in patients with hypogammaglobulinaemia, isolation of mycoplasmas from joints, pericardial tissue or from respiratory tract tissue different from their usual habitat has been described (12,13).

Human mycoplasmas have been shown to be taken up by polymorphonuclear leukocytes (PMNL) and macrophages (14), but whether mycoplasmas can enter epithelial cells is unclear. M. fermentans and M. penetrans have been found in nonphagocytic cells in AIDS patients (15,16). The mechanism of cell entry is not fully understood. The specialised tip structure of M. genitalium and M. penetrans seems to play a role in cell entry but also mycoplasma species lacking a tip structure like M. fermentans and M. hominis have been found intracellular by electronic microscopy (17).

Whether mycoplasmas replicate intracellularly remains to be resolved. There are indications for cell lysis of human lung fibroblasts after infection with M. genitalium (18) and cell disruption after invasion with M. penetrans (16). The intracellular localisation can protect mycoplasmas against the host immune system and antibiotics and may be responsible for latent or chronic infection states.

M. pneumoniae has been shown to parasitize cell surfaces of mammalian cells and to enter the intracellular environment where it is located throughout the cytoplasm and perinuclear regions. It can persist intracellularly for at least 7 days (19). Baseman isolated M. pneumoniae mutants, which are able to cytaadhere but not to invade cells, suggesting separate mechanisms for adherence and invasion (19).
2. Epidemiology

2.1 Epidemiology

*M. pneumoniae* causes usually a mild respiratory disease. Most cases will not lead to a doctor's visit and confirmed diagnosis is often not accomplished in daily practice. Available data on morbidity and mortality due to *M. pneumoniae* infection from regular sources (statistics of death due to pneumonia, statistics on hospitalisation due to pneumonia or laboratory data on *M. pneumoniae* infection) thus will reveal only a minor part of actually occurring *M. pneumoniae* infections.

The way diagnosis of a *M. pneumoniae* infection is established will influence the outcome of epidemiological studies. Most studies on *M. pneumoniae* epidemiology performed in the past, made use of data obtained by serological testing or by culture of the organism. A deficient knowledge in parameters influencing the antibody response exists. Persistent antibodies and antibody response after reinfection may differ and probably depend on the age and immune status of the people under investigation. Although isolation of *M. pneumoniae* is tedious and takes several weeks to complete, culture of *M. pneumoniae* has provided additional information for epidemiology of the organism only in settings in which special attention to culture was paid. Prolonged shedding of the micro-organism has been described in certain populations and is probably related to several factors like the immune status of the host, the age of the host, intervention with antibiotics, and may be, the virulence of the strain. However, the asymptomatic carrier state is believed to be rare, although contradictory results have been reported (20,21).

Many studies have been performed in outbreak investigations of respiratory disease due to *M. pneumoniae*, for example among military and in boarding schools (22-24). Long term studies, as performed in civilian populations in Seattle (1962-1975)(25) and Tecumseh (26), have provided data on community infection rates and epidemiological patterns.

*M. pneumoniae* infection occurs in temperate climates throughout the year, but the absolute number of infections is higher during the winter season. Because most respiratory infections due to other pathogens such as rhinovirus, respiratory syncytial virus and influenza virus occur in wintertime, the proportion of *M. pneumoniae* infection among respiratory infections is highest in summer.

Although *M. pneumoniae* is endemic in most civilian populations where the infection has been sought for, an epidemic pattern is evident from long-term population studies (27-32). Epidemics occur with intervals of 4-5 years. However, a change in this epidemic pattern has been observed in Denmark, possibly related to a change of protective immunity in children, which can be a result from increased use of day-care facilities (27). As can be concluded from long term population studies among patients with pneumonia (25) and data from laboratories registering *M. pneumoniae* infection (29)(this thesis), the more severe *M. pneumoniae*
infections, occur predominantly in school-age children, with another incidence peak in young adults. Although these data suggest that the overall incidence is lowest in the over 40 age group, it is likely that there is an underestimation of M. pneumoniae infection occurring in people older than 40 years. Antibody titers due to M. pneumoniae infection in older people are significantly lower compared to younger people (29; this thesis, Chapter 3), implying that M. pneumoniae infection in the older age groups is not diagnosed in case only serological assays are performed. In studies performed among outpatient populations with respiratory tract infection, in which diagnosis of M. pneumoniae infection was made by PCR, no difference in incidence between the different age groups is found (33; this thesis, Chapter 5).

2.2 Results of registration of M. pneumoniae from Laboratory Reporting Systems in Europe

To gain insight into the epidemiological pattern of M. pneumoniae in Europe, the data from national diagnostic laboratory reporting systems in different countries have been analysed. Only countries having data reported per quarter or per month for at least 10 years were included. These countries were: Finland (1972-1998)(34), England and Wales (1975-1998)(35), the Netherlands (1981-1998)(36) and Denmark (1972-1999)(37). Data from Iceland (1987-1996) reported earlier (29), were also included in the analysis. As M. pneumoniae infection is not a notifiable disease, the information available is based on volunteer reporting systems (Finland, England/Wales, the Netherlands) or on data from the national laboratory performing M. pneumoniae tests (Denmark, Iceland). The reported data obtained from laboratory diagnosed M. pneumoniae infection, most often by antibody detection in serum (CFT and/or ELISA), are presented as absolute numbers.

In those countries for which monthly data were available, most infections occurred during the winter period (November until March)(Fig 1). In epidemic episodes, however, the infection is diagnosed during the whole year, without preference for the winter period (Fig 2). The data showed that endemic cases occur on a regular basis with epidemic outbreaks every 4 to 5 years. This pattern is most regular in England/Wales and possibly in Iceland, for which country only 10 years data are available. Epidemic outbreaks in the different countries do not always coincide (Fig 2), most likely because of the protective immunity built up in a certain population.

The laboratory reporting systems from England/Wales and the Netherlands also register sex and age of the patients diagnosed with M. pneumoniae. M. pneumoniae infection occurred slightly more frequent in male patients with an exception for the 16 to 40 years age group in which more female patients were diagnosed with M. pneumoniae infection (Fig 3). In both countries there was a peak incidence of infections in young children (4 to 10 years) and a smaller, second peak in adults of 30 to 40 years old (Fig 4).
Figure 1
Seasonal distribution of *M. pneumoniae* in 4 Northern European Countries (data derived from (34-37))
Figure 2
Endemic and epidemic periods of *M. pneumoniae* as registered by clinical diagnostic laboratories in 5 Northern European countries (data derived from(29;34-37))
Figure 3
Age and sex distribution of patients diagnosed with *M. pneumoniae* infection in England/Wales and the Netherlands (data derived from (35,36))
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Figure 4
Age distribution of patients diagnosed with *M. pneumoniae* infection in England/Wales and the Netherlands (data derived from (35,36))
2.3 Transmission

Transmission of respiratory pathogens can occur by aerosols (e.g. influenza virus, *Bordetella* spp.), by personal contact (e.g. Epstein-Barr virus) or even via contaminated objects (respiratory syncytial virus). In the case of *M. pneumoniae* it is uncertain whether the spread is primarily by droplets, or by direct or indirect contact, or by all of these three routes. Infection with *M. pneumoniae* with small-particles (2-3um) in the hamster model resulted in both upper and lower respiratory tract infection, whereas infection after exposure to large-particle aerosols was limited to the upper respiratory tract (38).

The incubation period of *M. pneumoniae* is approximately 3 weeks. Spread of a *M. pneumoniae* infection in the community is slow. However, within communities, microepidemics can arise, in which spread probably occurs from person to person. Only a few common source outbreaks of *M. pneumoniae* have been reported (39-41).

3. Clinical disease

3.1 Respiratory infection

*M. pneumoniae* infection should be suspected in patients with a respiratory infection with clinical symptoms as seen with influenza-like illnesses such as fever, cough, headache, malaise, and myalgia although the onset of an infection with *M. pneumoniae* is usually less abrupt compared to an influenza virus infection. Also in people with milder respiratory infections *M. pneumoniae* may be the cause of such a disease. Non-productive cough is a characteristic sign in *M. pneumoniae* infection. In children cough can be paroxysmal. In an estimated 5-10% of patients *M. pneumoniae* infection progresses to tracheobronchitis or pneumonia. Sputum may be produced in such cases. Gram staining of sputum specimens reveals only inflammatory cells. Pneumonia is usually mild and self-limited, resolving even without antibiotic treatment (42). However, severe and fulminant cases have been reported (43,44) and may be underdiagnosed.

3.2 Complications

A variety of complications of *M. pneumoniae* infection have been described, but their frequencies are rarely known (45). Skin rashes, one of the commonest complications occurred in 10-20% of the recognised *M. pneumoniae* infections (46,47). The Stevens Johnson syndrome (erythema multiforme) although rare, can be a life threatening complication. Other non-pulmonary common complications comprise haemolytic anaemia, thrombocytopenia, thrombosis and disseminated intra-vascular coagulation. Furthermore, neurological complications like encephalitis, meningitis and the Guillain Barre syndrome, but also myocarditis and arthritis have been described and are believed to be due to circulating
immune complexes. Recovery of the organism from cerebrospinal fluid (48,49), synovial fluid (50-52) and the description of mycoplasmal bacteremia as detected by molecular methods (53,54) suggest systemic spread of the bacterium.

4. The bacterium and the host

4.1 Virulence and evasion of the host immune response

The clinical picture of mycoplasma infections is more suggestive of damage due to host immune and inflammatory responses rather than to direct effects by mycoplasma cell components. *M. pneumoniae* can be considered as a surface parasite of the respiratory epithelium. The adhesion is essential for colonisation and infection as the loss of adhesive capacity results in loss of infectivity. For *M. pneumoniae* and *M. genitalium* the mycoplasma adhesins, their genes (P1, P30, and MgPa) and encoded proteins have been studied most extensively. The topography of the adhesion molecules with epitope mapping using monoclonal antibodies against adhesion molecules in situ and against synthetic oligopeptides provided insight into the conformation of the adhesins in the membrane (55). However, in *M. pneumoniae* a number of accessory membrane proteins are involved in the process of gliding motility and concentration of the adhesin molecules at the attachment tip organelle. Some of the genes and encoded proteins of the *M. pneumoniae* cytoskeleton have been identified (30, 40 & 90 kDa, P65 and HMW1 and HMW3) and are shown to be proline rich and possess other characteristics of eukaryotic cytoskeletal proteins (56-59).

For persistence in the host, pathogenic bacteria develop mechanisms to deal with the host immune response. One of these mechanisms consists of variation in antigenic structure in order to avoid host immune recognition. For *M. hominis* deletions in a repeat-containing gene encoding a surface localised antigen resulted in antigenic variation (60). Also in the case of *M. genitalium* regions in the MgPa adhesin gene encoding adherence mediating epitopes have been described to be highly variable, thus leading to avoidance of the host immune response (61). For *M. pneumoniae*, deletion of repeated sequences in the gene encoding the 30 kDa protein of the *M. pneumoniae* attachment organelle, resulted in a hemadsorption negative mutant of *M. pneumoniae* (62). Regions of the P1 cytadherence gene which are recognised by cytadherence-inhibiting anti P1 monoclonal antibodies have been shown to vary, at least between P1 group I and II *M. pneumoniae* isolates (63,64).

4.2 Host susceptibility to *M. pneumoniae* infection

People with congenital or acquired immunodeficiencies are recognised to be more susceptible for a variety of mycoplasma infections. The number of recurrences of *M. pneumoniae* infection in such patients compared to people with normal immunity is also
higher. In hypogammaglobulinaemic patients and in patients undergoing organ transplantation and immunosuppressive therapy, dissemination of mycoplasmas such as *M. pneumoniae*, *M. hominis* and *Ureaplasma urealyticum* to joint and bone tissue has been reported (50,65-69).

Primary infection with *M. pneumoniae* does not protect against a subsequent infection with the bacterium as follows from clinical studies (70) and animal studies (71). Reinfection of animals showed more lymphocyte infiltration than primary infected animals, indicating that specific cell-mediated host response might play a role in the pathology of a reinfection. Experimental reinfections of mice showed elevated mRNA expressions for proinflammatory cytokines (72). In humans, the difference in clinical disease between a mild primary infection and more severe manifestations after a second infection with *M. pneumoniae* (73), might be due to enhanced immune response as found in the reinfection animals (74).

5. Diagnosis

Diagnosis of *M. pneumoniae* infection relies mainly on laboratory tests, as discrimination of *M. pneumoniae* from other (mostly viral) respiratory pathogens on clinical parameters only, is difficult (75). Specific laboratory diagnosis can be made directly, by culture or detection of antigen or DNA of the organism, or indirectly by detection of antibodies.

5.1 Culture

Culture of *M. pneumoniae* is labour intensive and requires specific media and experienced personnel to perform. *M. pneumoniae* is a relatively slow growing bacterium. After adaptation to artificial media the mean generation time is 6 hours (76). In clinical practice this means that incubation from 1 week to 4 weeks is necessary for propagation of the micro-organism, which makes culture inappropriate for the routine diagnosis in a clinical setting. If culture is attempted, the transport medium should contain peptide broth, serum albumin and antibiotics to retard overgrowth by other bacteria. Throat swabs, nasopharyngeal aspirates and sputum samples are all suitable for culturing of *M. pneumoniae*, provided they have been suspended in appropriate transport medium. Inoculation should be performed within 24 h or, if not possible, samples should be stored at -70 °C. Culture media to be used for *M. pneumoniae* have to contain beef heart infusion broth, supplemented with fresh yeast extract and horse serum providing sterols and nucleic acid precursors. Inoculation is performed using both solid and liquid medium, the latter containing glucose and phenol red. *M. pneumoniae* ferments glucose, which is detected by a colour change of the medium from red into yellow. On solid media *M. pneumoniae* appears in ‘mulberry’ colonies (Fig 5), which are able to hemadsorb chick and guinea pig erythrocytes. Specific antisera are required to provide definite identification, although close serologic relationship between *M. pneumoniae* and *M.*
genitalium may encounter difficulties in identification (77). Although the usefulness of culturing *M. pneumoniae* in daily practice is limited, culture remains important as a standard in studies in which non-cultural methods are evaluated. Also the development of antimicrobial drug resistance and antigenic variation can only be studied if clinical isolates of *M. pneumoniae* are available.

![Image of M. pneumoniae colonies on agar](image)

**Figure 5**

*M. pneumoniae* colonies on agar (40x magnification) (courtesy to AF Angulo, RIVM Bilthoven, The Netherlands)

5.2 Direct detection of *M. pneumoniae*

As an alternative for culture, direct antigen tests have been developed. A species specific probe test (Gene-Probe), an EIA and immunoblot assays making use of monoclonal antibodies against *M. pneumoniae* proteins have been applied in direct detection of (antigen of) the micro-organism in nasopharyngeal specimens (78,79).

In the last decade studies using amplification of species specific fragments of the *M. pneumoniae* genome by PCR have been published. Target sequences used for molecular detection of *M. pneumoniae* are mostly chosen in the P1 cytadhesin gene (80-85) or in the 16 SrRNA gene (86,87).

For preparation of clinical samples before amplification, various procedures have been described. Since mycoplasmas lack bacterial cell wall material, DNA can be made available by relative simple procedures. A boil-freeze method (thermal shock) has been applied to nasopharyngeal aspirates (85) and also lysis with proteinase K on aspirates (88), bronchoalveolar lavages (84), and throat swab specimens (75,89) has been applied. For preparation of tissue or blood specimens a more elaborate DNA extraction is required. The
use of an internal process control is necessary in order to avoid false negative results (82,85,90). Up to 20% of the throat swab samples after a proteinase K lysis showed inhibition of the amplification process (90) and up to 25% of nasopharyngeal aspirates after freeze-boiling showed inhibition of the PCR (85), as detected by the lack of the internal control band after gelelectrophoresis. Using β globine primers, a more roughly way to test for inhibition, up to 10% of the aspirates showed inhibition after proteinase K lysis (88). Identification of the PCR products has been performed by (microtiter well) hybridisation (89,91) as well as by a nested PCR (90). Detection limits of *M. pneumoniae* by PCR in clinical samples have been described to vary between 1.0-50 colony forming unit (CFU), 10-100 colour changing units (CCU)) and 5-50 fg of template DNA (53,82,87,90,92). Only a few studies compared reproducibility of PCR results obtained with different primersets in different laboratories (75,85).

5.3 Serology

In routine laboratories antibody detection in serum is the most commonly used technique in diagnosis of *M. pneumoniae* infection. The first serological assay performed was the cold hemagglutinin (CA) test. Cold agglutinins are non-specific IgM class antibodies against the I antigen of erythrocytes. The specificity of the test can be increased when a cut-off titre of >40 is used as diagnostic criterion (93). As more specific tests became available, the CA test is not commonly used anymore.

The most widely used serological assay is the complement fixation test (CFT) in which CF antibodies against *M. pneumoniae*, in conjunction with antibodies against other, mostly viral, respiratory pathogens are determined. The sensitivity of this assay depends on whether the first serum sample is collected early or late after onset of illness and on the availability of paired sera collected with an interval of 2 to 3 weeks. On interpretation of positive CF titers one has to bear in mind that the membrane glycolipids-based antigen from *M. pneumoniae* is not highly specific. Cross reactivity with e.g. vegetable lipids (94) and human brain tissue antigens has been demonstrated (95).

ELISA tests have been developed to detect specific immunoglobulins of different classes, most of them using a crude *M. pneumoniae* antigen, which includes the cross reacting glycolipid fraction. More specific test results can be obtained by using isolated P1 protein as antigen, which is one of the major virulence factors of *M. pneumoniae* (96). Proteins derived from parts of the P1 gene have also been applied as antigen in an ELISA test (97). Commercial availability of these tests sofar is limited. Immunoblotting, to confirm positive results from the less specific serological assays, have shown strong reactivity against the 169 kDa P1 protein of *M. pneumoniae* (98), but reactivity against this protein among clinically healthy persons interferes with conclusive interpretation. Recently, a commercially available
immunoblot making use of various recombinant proteins based on *M. pneumoniae* genome sequence data (3) has been developed.

6. Treatment and prevention

6.1 Treatment

Upper respiratory tract infection due to *M. pneumoniae* is mostly undiagnosed and is not treated with antibiotics. Also pneumonia is often a self-limiting disease, but appropriate antimicrobial treatment shortens the duration of the illness (99;100). Respiratory infection caused by *M. pneumoniae* is generally treated with tetracycline or erythromycin. As mycoplasmas lack a cell wall, *M. pneumoniae* is unaffected by β-lactam antibiotics such as penicillin and cephalosporins. Clinical improvement upon institution of appropriate antimicrobial therapy is not always accompanied by early eradication of the organism from the respiratory tract. The capacity of *M. pneumoniae* to reside intracellularly may be responsible for this. Also the newer macrolides (clarithromycin and azithromycin) and to lesser extent fluoroquinolones have been found to have in vitro and in vivo activity against *M. pneumoniae* (101).

6.2 Prevention

In terms of transmission, the respiratory route is the least subject to effective control of infectious diseases. In non-respiratory transmission such as vehicle-borne, gastro-intestinal infections, intervention is possible in the food chain or in drinking water systems (102;103). In case of vector borne infections the vector can be eliminated (104) and in case of genital infections change in behaviour will reduce transmission of the causative micro-organism (105). For some respiratory pathogens control is possible by intervention in the environment. This is the case for example with *Legionella pneumophila* (106) or *Chlamydia psittaci* (107). However, for many respiratory pathogens, including *M. pneumoniae*, environmental control is not feasible and the only way of controlling these infections is through immunisation of the host or providing chemoprophylaxis.

Prevention of *M. pneumoniae* infection through vaccination has been studied as early as the 1960ies, the time that *M. pneumoniae* was recognised as a respiratory pathogen. These early vaccine studies made use of an inactivated *M. pneumoniae* vaccine after administration of which volunteers were challenged with agar-grown mycoplasma. Those responding serologically to the vaccination showed protection to respiratory infection. In volunteers who failed to respond to the vaccine with antibody development, more severe disease developed after challenge compared with the controls (108). In larger vaccine trials among military personnel this sensitisation effect could not be confirmed and the protective efficacy of the
vaccine against development of *M. pneumoniae* pneumonia was about 66% in the first year following vaccination (109). However, the overall success of formalin inactivated vaccines has been disappointing and no acceptable live vaccine has been developed for human use.

The molecular characterisation of the major adhesin P1 of *M. pneumoniae*, prompted trials to use purified P1 as vaccinogen. The results of immunisation with P1 in animal studies were however disappointing (110), showing the same lymphocyte infiltration in lung tissue after challenge with *M. pneumoniae* in immunised animals compared to control animals. The conclusion is that until now, no useful vaccine (inactivated or live) for *M. pneumoniae* has been developed. DNA vaccines, having the advantage of inducing both humoral and cell mediated response, have been applied successfully in animals preventing mycoplasmal disease (*M. pulmonis, M. hyopneumoniae*) (111). This generation of vaccines may become useful in human mycoplasma disease in the future as well.

Only limited data is available on the effect of prophylactic antibiotic use. In a long-term care facility prophylactic antibiotic usage has shown to significantly reduce the secondary attack rate of *M. pneumoniae* infection (112).
Scope of the thesis

*Mycoplasma pneumoniae* is a common respiratory pathogen in humans. The usual mild clinical presentation of the infected patient does not encourage laboratory diagnosis to be performed. However, in case antibiotic treatment is instituted this should be different from the first choice recommended antibiotics for respiratory infections in general practitioner (GP) settings. As the organism is notorious for its difficult laboratory diagnosis, molecular techniques should enable fast diagnosis and subsequently appropriate antibiotic treatment. Current knowledge of the epidemiology of *M. pneumoniae* is based on data predominantly derived from studies using serology or culture for diagnosis.

In this thesis the value of molecular techniques in diagnosis of *M. pneumoniae* in both children and adult patients with a respiratory infection is investigated (Chapters 2 and 3). For reliable detection of *M. pneumoniae* DNA by PCR, control for inhibition of the amplification reaction is required. Therefore, we developed an amplification control (AC) to detect inhibition, which is described in chapter 4. This AC was applied to clinical specimens, submitted for *M. pneumoniae* PCR. Furthermore, we applied a semiquantitative PCR to enable discrimination of strong and weak positive PCR results in specimens obtained from hospitalised and non-hospitalised patients with a respiratory tract infection (Chapter 4).

To expand knowledge of epidemiological patterns of *M. pneumoniae*, the PCR was applied to nose/throat specimens obtained from patients with acute respiratory infection, identified by GPs in a routine surveillance for respiratory pathogens. *M. pneumoniae* transmission was studied in the households of the *M. pneumoniae* positive patients (Chapter 5).

Molecular epidemiology of *M. pneumoniae* is hampered, as variation in its genome allows the distinction of only two types. In order to refine molecular typing, clinical isolates of *M. pneumoniae* collected over time in Denmark and the Netherlands, were subjected to various molecular typing methods (Chapter 6). For identification of targets, which should enable direct genotyping of *M. pneumoniae* in clinical specimens, P1 genes were sequenced. In addition, these sequences were analysed to uncover possible mechanisms generating P1 gene sequence variation (Chapter 7).
REFERENCES


Introduction

Chapter 1


Introduction


Chapter 1


