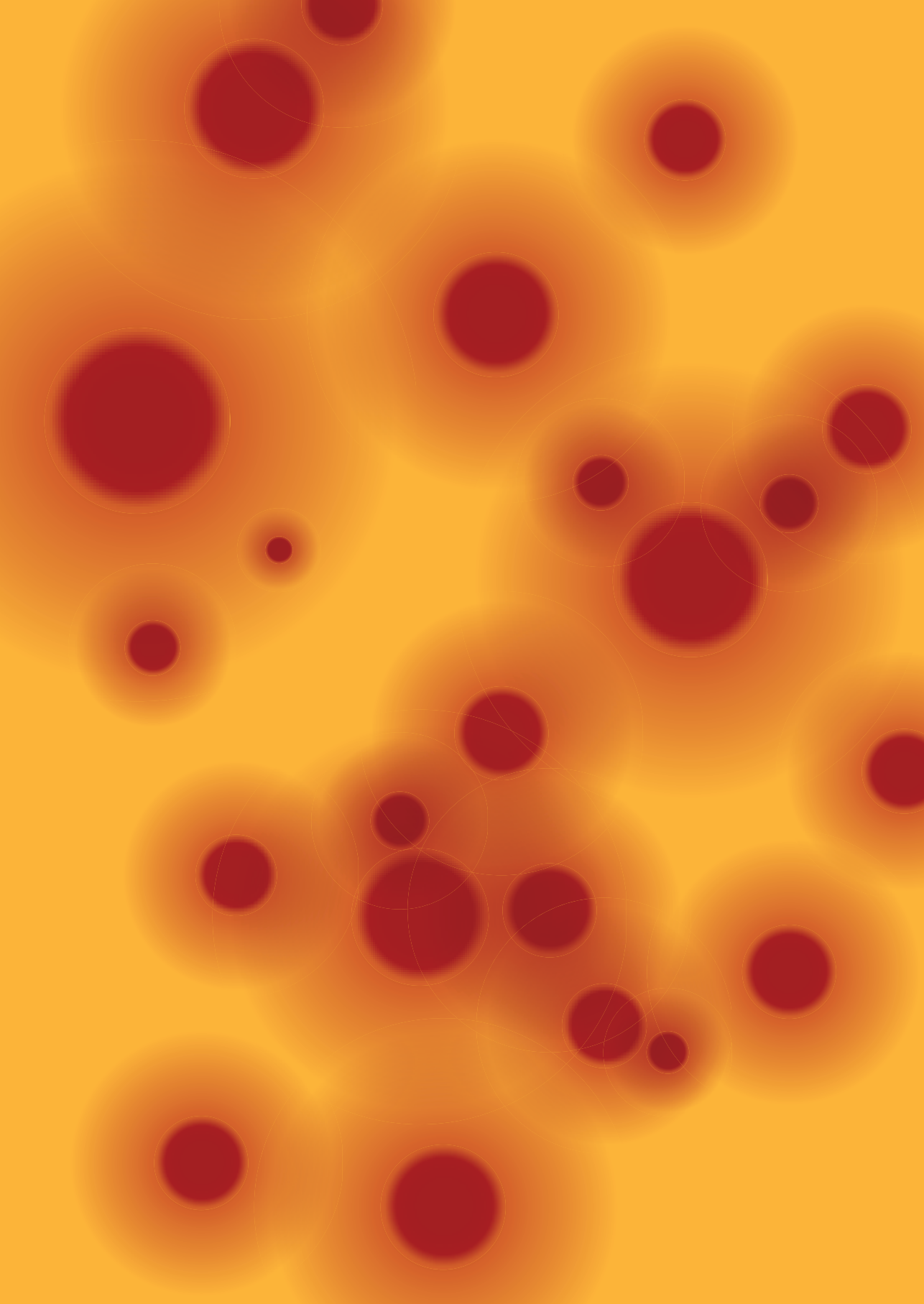
Emiel B.M. Spuesens

Mycoplasma pneumoniae

Bacterial genetic variation and colonization of the respiratory tract of children



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BACTERIAL GENETIC VARIATION AND COLONIZATION OF THE RESPIRATORY TRACT OF CHILDREN

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Chapter 1

General introduction

Respiratory tract infections (RTIs) form a major burden of disease in children worldwide. The World Health Organization estimates there are 150.7 million cases of pneumonia each year in children younger than 5 years, with as many as 20 million cases severe enough to require hospital admission[1]. Moreover, mortality (death rates) due to pneumonia in Europe varied from 0.10-1.76 per 100000 between 2000-2010[2].

A wide range of microorganisms is accepted as potential pathogen of the respiratory tract in children, including viruses and bacteria. *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* are the two most common bacterial causes of lower RTIs in hospitalized children (Table) [3, 4]. This thesis focuses on *M. pneumoniae*, the smallest free living micro-organism that is responsible for up to 40% of children hospitalized because of community acquired pneumonia (CAP) [5].

INTRODUCTION TO *M. PNEUMONIAE*

History and biological characteristics. *M. pneumoniae* was first isolated from the sputum of a patient with primary atypical pneumonia by Eaton et al. in 1944 [6]. The bacterium was thereafter known as the Eaton agent, and was considered a virus until it finally became clear that antibiotics could be effective against it. Chanock et al. succeeded in culturing the Eaton agent on cell-free medium and proposed the taxonomic designation *M. pneumoniae* in 1963 [7]. *M. pneumoniae* is currently classified as a member of the family *Mycoplasmataceae* and order *Mycoplasmatales* within the bacterial class of Mollicutes (lat. soft skin). The common features shared by all the bacteria in this class are a permanent lack of a rigid cell wall, small cellular dimensions, and a small circular genome with a low G+C content (20-40%) [5, 8, 9].

M. pneumoniae possesses a membrane containing sterols and has a size of approximately 0.2 µm by 2 µm. So far, the complete genome sequence of three *M. pneumoniae* strains has been determined. Analysis of genomic studies has resulted in the identification of two subtypes, i.e. subtype 1 and subtype 2, and variants thereof. The complete genome of *M. pneumoniae* strain M129 (a subtype 1 strain) has a length of 816,394 bp and contains only 689 open reading frames (ORFs). The genome length of *M. pneumoniae* strain FH and 309 (both subtype 2 strains) is 811,088 bp and 817,176 bp respectively [10, 11]. The limited size of the genomes of mycoplasmas is generally considered to be the result of a gradual loss of genome information from a common Gram-positive ancestor.

As a consequence of their small genome, *M. pneumoniae* strains have a limited metabolic capacity and activity, and are thus dependent on their host for the production of e.g. purines and pyrimidines [8]. It is therefore important that *M. pneumoniae* is in close contact with its host to scavenge for such nutrients. To establish close contact with the host respiratory epithelium, *M. pneumoniae* contains a specialized attachment organelle. This organelle consists of a number of adhesion proteins and accessory proteins that are essential for attachment [12-14]. The most important adhesion protein is the P1 protein. Together with the P40 and P90 proteins, it is primarily responsible for attachment to the host respiratory epithelium [12, 15-17] (Figure). Loss of (a part of) this protein or (a part of) one of its counterparts results in the inability to attach to respiratory epithelium and a non-virulent phenotype [15, 18].

Antigenic variation of the attachment organelle. The P1 and P40/P90 proteins are encoded by the MPN141 gene and the MPN142 gene, respectively [8, 19, 20]. Parts of the MPN141 and MPN142 genes exist in multiple versions in the genome [9, 21]. The different versions of these repetitive DNA elements, which are called RepMP elements, are similar but not identical in DNA sequence. It is speculated that in order to evade the host immune response, *M. pneumoniae* may change the MPN141 and MPN142 genes

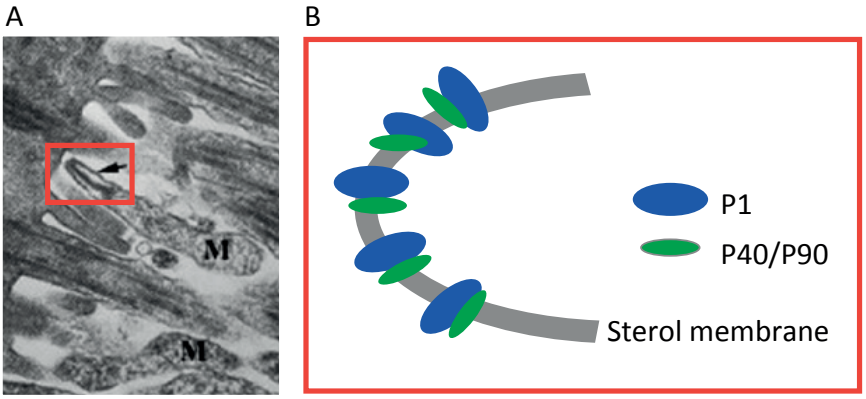

Fig. (Left) Transmission electron microscopy of *M. pneumoniae* (M) and its attachment organelle (in the red box indicated by the arrow). (Right) A simplified schematic representation of the attachment organelle and the location of the most important attachment proteins in the sterol membrane. Figures adapted from S. Rottem, Physiology Reviews [14].

and, consequently, the encoded proteins, by homologous DNA recombination between the RepMP elements in the genome. This process may result in antigenic variation. In addition to the basic subtype of an *M. pneumoniae* strain (i.e. subtype 1 or subtype 2), these recombination events result in additional genetic variation located in the MPN141 and MPN142 genes. Genetic variation is not only important for immune evasion, but might also play a role in pathogeneity of *M. pneumoniae*. However, the effect of genetic variation on pathogeneity has never been demonstrated for *M. pneumoniae*. One of the reasons for this lack in knowledge could be the lacking consensus about how to study and interpret genetic variation of *M. pneumoniae*. Therefore, first a thorough analysis of the current genetic variation of *M. pneumoniae* had to be performed. The analysis of this genetic variation is the main subject of part 1 of this thesis.

Epidemiology and clinical syndromes of *M. pneumoniae* infection. *M. pneumoniae* is an endemic cause of respiratory tract infections unbound to a specific season. Cyclic epidemics in developed countries occur every 3-7 years and are attributed to decreased herd immunity. During an epidemic, local outbreaks are common in schools, military institutions and nursing homes [22-25]. *M. pneumoniae* is found particularly in school-aged children, but may affect all age groups. *M. pneumoniae* infection may manifest itself as upper RTIs, lower RTIs or a combination of both. The most common upper RTIs due to *M. pneumoniae* are pharyngitis and acute bronchitis. In general, these infections are mild and self-limiting. However, up to 40% of patients with CAP are infected with *M. pneumoniae*, which makes CAP the primary burden of disease.

Symptomatic disease usually develops during a couple of days with unspecific symptoms of RTI indistinguishable from e.g. a viral illness. Symptoms may include cough, fever,

Table. Pathogens identified in hospitalized children with community-acquired pneumonia in developed countries (total exceeds 100% because co-infections are taken into account) Adapted from Michelow et al. [3].

Respiratory syncytial virus and other viruses	58%
Streptococcus pneumoniae	44%
Mycoplasma pneumoniae	14%
Chlamydophila pneumoniae	9%
Mycobacterium tuberculosis	1%
Staphylococcus aureus	1%
No pathogen identified	21%

sore throat, headaches, muscle pains, general malaise and fatigue [26]. Some symptoms (such as chest pain and lack of wheeze) seem to occur more in *M. pneumoniae*-infected children than in children infected with other pathogens [27]. Also, a clinical syndrome, i.e. 'walking pneumonia', has previously been described that was considered to be characteristic for *M. pneumoniae* RTI [5]. However, a recent Cochrane review showed that a reliable diagnosis of *M. pneumoniae* RTI cannot be made based on clinical symptoms and signs alone [27]. After the primary illness, patients may complain of persistent unspecific symptoms such as recurring headache, general malaise and fatigue, which may last for months. More importantly, also respiratory complaints may last for a longer period. It is believed that this is especially true for children with an underlying pulmonary illness such as asthma, which led to the notion that there is a possible causal relationship between *M. pneumoniae* infection and the onset of asthma, asthma progression or asthma exacerbation [28]. This is, however, still a matter of debate.

Besides respiratory tract infections, *M. pneumoniae* has been associated with a number of extra-pulmonary manifestations. It is believed that as many as 25% of persons colonized with *M. pneumoniae* may experience an extra-pulmonary sign or symptom at a variable time period after colonization. This is irrespective of the actual occurrence of symptoms related to an RTI. The etiology of many of these manifestations is not exactly known and they may be of variable severity. Both direct invasion of other sites than the respiratory tract and cross-reaction of antibodies or auto-immunity have been suggested as an explanation.

Central nervous system diseases have been recognized as the most frequent occurring extra-pulmonary manifestations of variable severity. In children, encephalitis is the most frequently observed neurological complication, which in theory may be attributed to the cross-reaction (molecular mimicry) of anti-*M. pneumoniae* antibodies and myelin components, such as gangliosides [29]. Examples of other severe complications, which have been described less frequently, are Guillain-Barre syndrome and acute disseminating encephalomyelitis [30, 31].

Extra-pulmonary manifestations may further include dermatologic manifestations (erythematous maculopapular rash and, rarely, Stevens-Johnson syndrome), hemolytic anemias and several forms of arthralgia or arthritis. Very sporadically, renal complications (e.g. glomerulonephritis, tubulointerstitial nephritis and IgA nephropathy) and cardiac complications (e.g. pericarditis, myocarditis and pericardial effusion) have been described [5].

Microbiological tools to diagnose *M. pneumoniae*. The diagnosis of an *M. pneumoniae* infection has been the subject of many studies since the first isolation of this pathogen from the sputum of a patient with primary atypical pneumonia in 1944 [5]. Although the media used for culturing *M. pneumoniae* have been optimized over the years, culturing remains an insensitive tool to detect *M. pneumoniae* in clinical samples, and is successful in only 60% of the time in experienced laboratories. Moreover, due to the lack of a rigid cell wall and limited metabolic capacity [5], *M. pneumoniae* is difficult to culture. The current diagnosis of *M. pneumoniae* RTIs therefore relies on the detection of either serum antibodies against *M. pneumoniae* or bacterial DNA in samples of the upper respiratory tract (URT), as recommended in the guidelines published by the British Thoracic Society and the Infectious Disease Society of America [32, 33].

Serology, i.e. the detection of antibodies against *M. pneumoniae* proteins in the serum of patients, is a widely available diagnostic procedure in microbiological laboratories. Although several serological assays are currently available, their outcomes are often not comparable. The assays with the highest sensitivity and specificity, however, are based on the ELISA technique. As these tests are commercially available, and culture is relatively insensitive and time-consuming, ELISA has become the cornerstone of the diagnosis of *M. pneumoniae* infections. Nevertheless, the use of serology also has a clear downside since it requires a serum sample taken in the acute phase of the disease and a convalescent serum sample taken 2-4 weeks later to provide a reliable result. The inherent retrospective diagnosis, after the collection and testing of a second serum sample, is not helpful for clinicians to make a therapeutic management decision in the acute phase of the infection.

In the past two decades, a solution to the drawbacks of culture and serology has seemingly been provided by PCR. Consequently, PCR-based methods are increasingly used in daily clinical practice, as well as in clinical studies for the detection of *M. pneumoniae* DNA, because they provide fast and sensitive results in the acute phase of an infection. However, if *M. pneumoniae* would be commonly carried asymptomatically in the URT of children, the detection (of DNA) of this bacterial species may not indicate a symptomatic infection. There have been some studies that report asymptomatic carriage with *M. pneumoniae*. However, all these studies suffer from inherent shortcomings in study design (e.g. lack of a control group or follow-up) or diagnostic methods (e.g. low sensitivity of culture methods and serology) [25, 26, 34-36]. Nevertheless, the existence of

asymptomatic carriage would have major implications for the interpretation of the results of currently used molecular detection methods for *M. pneumoniae*, and particularly for clinical management of suspected *M. pneumoniae* RTIs. To overcome the problems in previous studies on asymptomatic carriage of *M. pneumoniae* we conducted a large prospective study, using a clearly defined control group and several accepted microbiological tools to detect *M. pneumoniae*.

Treatment of *M. pneumoniae* respiratory tract infections. While *M. pneumoniae* lacks a rigid bacterial cell wall, the bacterium is protected by a sterol-containing membrane. As a direct result, *M. pneumoniae* has an innate resistance to any antibiotic that is directed to the destruction or disruption of a bacterial cell wall, such as beta-lactams and glycopeptides [37]. In general, antimicrobials that are directed at the DNA metabolism and protein synthesis have a good inhibitory activity against *M. pneumoniae*. Tetracyclines, doxycycline and fluoroquinolones therefore have a good in vitro inhibitory effect against *M. pneumoniae*, but have known side effects and affect the growing bones in children. As a consequence, macrolide (ML) antibiotics are the preferred choice of antibiotics in children. These antibiotics also have a better MIC value than most other antibiotics directed against *M. pneumoniae* [37].

The past decade has witnessed a growing number of reports on antimicrobial resistance of *M. pneumoniae*. This is particularly the case in Asia, but also in Europe and the USA, an increase in the prevalence of ML-resistant *M. pneumoniae* isolates has been observed [38-49]. ML resistance is caused by single-nucleotide polymorphisms (SNPs) in the bacterial ribosomal RNA gene. Due to these mutations, the attachment of the ML molecules to the peptidyl transferase loop of the bacterial ribosome is ineffective. It is believed that a high prevalence of ML-resistant strains in a certain area is the result of a high use of ML antibiotics. We studied in a large cohort MPML resistence in the Netherlands for the first time. In this thesis, we present a first report on *M. pneumoniae* ML-resistance in the Netherlands.

INTRODUCTION TO THE MAIN HYPOTHESES AND OBJECTIVES OF THIS THESIS

It is important to realize that humans are surrounded by millions of different bacteria. These bacteria live in our gut, our respiratory tract, or on our skin, and many of them are probably not harmful for humans and could even be beneficial under normal circumstances. In this theory, bacteria are 'carried' by humans without causing disease, which we call carriage or asymptomatic colonization. Throughout this thesis, we use both terms interchangeably to indicate the unharmful presence of potential pathogens in the respiratory tract. These potential pathogens include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*. Under certain circumstances, these bacteria can cause respiratory tract infections, which may even develop into life-threatening infections such as sepsis and meningitis. These circumstances needed for the progression from colonization to invasive disease can be divided in: (1) host factors involving a breach of the natural immunological barriers due to local mucosal changes or a underlying mucosal immunological disease or immaturity, and (2) pathogen factors such as differences in the composition and expression of surface-associated proteins and other virulence factors that are needed for acquisition, adherence and invasion [50-54].

Since *M. pneumoniae* is a bacterium that also causes respiratory tract infections, it is not inconceivable that this pathogen has a similar 'lifestyle' as the other bacteria mentioned above. Indeed, previous studies reported the presence of *M. pneumoniae* in seemingly healthy individuals. However, these studies suffered from drawbacks related to either the study design or limitations of the diagnostic assays that were employed [25, 34-36, 55-58]. We therefore initiated a study to confirm the hypothesis that *M. pneumoniae* is able to colonize the human respiratory tract in an asymptomatic fashion.

Due to the absence of specific clinical, biochemical or radiological findings, the diagnosis of an *M. pneumoniae* RTI has to be supported by microbiological findings. At the start of our studies and at present, different diagnostic techniques are available, but all of these have drawbacks that should be taken into consideration before defining an RTI as an *M. pneumoniae*-induced infection.

Assuming that our hypothesis would be confirmed, we also set out to study whether possible asymptomatic carriage could be distinguished from infection using the currently available microbiological diagnostic tools for *M. pneumoniae*. In addition, we sought to investigate the genetic composition of different *M. pneumoniae* strains to identify possible factors in the genome that predispose the bacterium to invasiveness in the respiratory tract of children. The overall aim of the studies is to find factors that could be important in the progression from asymptomatic colonization to invasive infection with *M. pneumoniae*.

Specific aims are:

- To identify asymptomatic colonization by *M. pneumoniae* using current available microbiological tools.
- To find host factors associated with asymptomatic carriage versus symptomatic disease.
- To classify genetic variation in *M. pneumoniae* in a concise understandable manner and to make a classification system that covers the description of current and future possible variants.
- To find specific genetic pathogen variation that is associated with disease and disease severity.
- To investigate the upcoming ML-resistance in the Netherlands.

THE OUTLINE OF THIS THESIS

This thesis contains two general parts, i.e. **Part 1** and **Part 2**. As a prerequisite for the clinical studies, **Part 1** contains studies on the genetic variation of *M. pneumoniae* and the classification of adhesion gene variants. **Chapter 1** contains a general introduction to *M. pneumoniae*. **Chapter 2** is a review in Dutch about the genetic variation of *M. pneumoniae* and of the current molecular methods used for genotyping. This review is in part a summary of the findings in chapters 3-6. In **Chapters 3-5**, genetic variation of *M. pneumoniae* due to homologous DNA recombination is described, including a novel classification scheme for genetic variants. In **Chapter 6,** the design of a convenient molecular typing assay for *M. pneumoniae* is described and optimized for use in clinical samples. This assay is partly based on the findings in chapter 3.

Part 2 of this thesis focuses on the clinical aspects of *M. pneumoniae* colonization, infection and epidemiology of various bacterial genotypes and the dissemination of macrolide resistance in the Netherlands. **Chapter 7** describes our primary study (*Mycoplasma pneumoniae* Infection and Colonization in Children, MymIC study), which was performed to investigate the existence of carriage and the difference between carriage and infection by *M. pneumoniae*. A continuation on the topic of asymptomatic carriage with *M. pneumoniae* is provided in **Chapter 8**, in which we describe our genetic search for virulence factors in *M. pneumoniae* strains using Next Generation Sequencing. **Chapter 9** contains a retrospective study of 12 years in the Netherlands to investigate the epidemiology of macrolide resistance. **Chapter 10** is a review on the treatment of respiratory tract infections caused by *M. pneumoniae*.

Finally, in **Chapter 11**, the overall findings and implications of the studies described in this thesis are discussed. **Chapter 12** provides a summary of the thesis.

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Part 1

Genetic variation of *Mycoplasma pneumoniae*

Chapter 2

Moleculaire typering van *Mycoplasma pneumoniae*

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SUMMARY

Mycoplasma pneumoniae is considered to be a uniform bacterium. In addition to the two known subtypes of *M. pneumoniae*, a number of important genetic variations have been described in the MPN141 gene, which encodes the major adhesin, the P1 protein, of the bacterium.

In this paper, the molecular typing techniques available for *M. pneumoniae* are described on the basis of the known genetic variation. These techniques can be divided in techniques that only determine the major subtype of *M. pneumoniae* (subtype 1 and subtype 2) and those that determine the 'MPN141 type', which requires sequencing of the entire gene. It is important to describe the variation in *M. pneumoniae* in a systematic fashion with the use of the recently published classification system. Future studies should be focused on the relation between the different molecular *M. pneumoniae* types and clinical featues, such as severity of disease.

SAMENVATTING

Mycoplasma pneumoniae is een luchtwegpathogeen die wordt beschouwd als een genetisch stabiele bacterie. Naast de twee bekende subtypes van *M. pneumoniae* (subtype 1 en subtype 2), kunnen echter een groot aantal genetische varianten van de bacterie worden onderscheiden. Deze varianten verschillen van elkaar in de sequentie van het MPN141 gen dat codeert voor een belangrijk oppervlakte eiwit (het P1 eiwit). Aan de hand van de bekende genetische variatie in *M. pneumoniae*, zullen in dit artikel de voor deze bacterie beschikbare moleculaire typeringstechnieken worden beschreven. Deze kunnen worden ingedeeld in technieken die zijn gericht op het bepalen van het subtype of op het bepalen van het 'MPN141 type'. Het is van belang de variatie tussen subtypes en tussen MPN141-types eenduidig te beschrijven met behulp van een recent gepubliceerd classificatie systeem. Onderzoeken in de toekomst dienen zicht te richten op de relatie tussen de gevonden variatie in *M. pneumoniae* en klinische parameters zoals bijvoorbeeld de ernst van een infectie.

INLEIDING

Mycoplasma pneumoniae is een humaan pathogeen en behoort tot de klasse van de *Mollicutes* (lat. zachte huid). De naam van deze klasse refereert aan het feit dat de bacteriën die tot deze klasse behoren geen celwand hebben. Een andere belangrijke eigenschap van deze bacteriën is dat ze een klein circulair genoom hebben dat een grootte heeft tussen de 0,58 en 2,2 megabaseparen (Mbp). Naast *Mycoplasma genitalium* (stam G37; 580.076 bp) is *M. pneumoniae* (stam M129; 816,394 bp) één van de kleinst zelfreplicerende micro-organismen van de klasse *Mollicutes*.[1]

M. pneumoniae veroorzaakt verscheidene luchtweginfecties, waaronder tracheobronchitis, faryngitis en pneumonie. Ongeveer 40% van alle gevallen van 'community-acquired pneumonia' wordt veroorzaakt door *M. pneumoniae* en verreweg de meeste van deze gevallen komen voor op de kinderleeftijd (80-85%).[1]

Tot nu toe is er nog geen associatie gevonden tussen de ernst van infecties en een bepaald moleculair type van *M. pneumoniae*. Kenri et al. beschreven echter dat ongeveer elke decade een nieuw moleculair type dominant wordt in de populatie.[2] Om meer te weten te komen over de variatie van *M. pneumoniae* in relatie tot epidemiologische gegevens en klinische gegevens, is moleculaire typering van groot belang. Alvorens de moleculaire typering en bijbehorende technieken van *M. pneumoniae* te bespreken, geven wij eerst een overzicht van de mogelijke variatie in *M. pneumoniae*.

GENETISCHE VARIATIE VAN *M. PNEUMONIAE*

Van twee *M. pneumoniae* stammen is tot nu toe de volledige genoom sequentie bepaald. Deze twee stammen vertegenwoordigen de twee subtypen van *M. pneumoniae*, namelijk subtype 1 en subtype 2 (zie onder subtype variatie). De stam M129 (subtype 1, ATCC 29342) heeft een genoomlengte van 816.394 bp en de stam FH (subtype 2, ATCC 15531) heeft een genoomlengte van 811.088 bp. Hoewel er vele verschillen zijn tussen de genoomsequenties van de M129 stam en de FH stam, wordt *M. pneumoniae* in het algemeen beschouwd als een genetisch stabiel micro-organisme. Er kan echter een grote variatie bestaan tussen stammen in het gen dat codeert voor het oppervlakte eiwit P1. Dit gen (MPN141) kan sequentie variatie (antigene variatie) ondergaan ten gevolge van homologe DNA recombinatie met sequenties die zich elders in het genoom van *M. pneumoniae* bevinden. Omdat bij de moleculaire typering in de literatuur regelmatig verwarring is ontstaan over het bepalen van het subtype van *M. pneumoniae* (subtype 1 en 2) en het bepalen van het 'MPN141 type', worden deze twee onderwerpen hieronder afzonderlijk behandeld.[3, 4]

Subtype variatie

Het belangrijkste onderscheid dat met bijna alle toegepaste moleculaire typeringstechnieken kan worden aangetoond is het onderscheid tussen subtype 1 en subtype 2 stammen. Dit genetisch onderscheid werd voor het eerst beschreven in het MPN141 gen en werd later ook ontdekt in andere genen zoals in het MPN142 gen, het MPN528a gen, het MPN535 gen en in het MPN340 gen.[5-9] Na analyse van de genoomsequenties van de M129 stam en de FH stam is gebleken dat subtype-specifieke sequenties op veel meer plaatsen in het genoom te vinden zijn. Veelal zijn dit base substituties en kleinere inserties of deleties. Het verschil tussen subtype 1 en subtype 2 stammen is echter zeer uitgebreid in het MPN141 gen en het MPN142 gen. In het MPN141 gen beslaat de variatie een lengte van 1246 baseparen en in het MPN142 gen 993 baseparen.

Variatie als gevolg van homologe DNA recombinatie

In 25 verschillende *M. pneumoniae* stammen zijn repeterende DNA sequenties beschreven. [10-12]In het algemeen wordt aangenomen dat deze repeterende sequenties in alle *M. pneumoniae* stammen te vinden zijn en elke stam eenzelfde set sequenties bevat met daarin kleine onderlinge verschillen. Deze sequenties worden aangeduid met 'RepMP' (repeated element in *M. pneumoniae*) gevolgd door één of meer getallen. In het MPN141 gen bevindt zich een RepMP2/3 element en een RepMP4 element en in het MPN142 gen bevindt zich een RepMP5 element (Figuur).

Van elk van deze elementen bestaan meerdere versies in het genoom van *M. pneumoniae*. Omdat deze versies grotendeels overeenkomen met het corresponderende element in het MPN141 of MPN142 gen, maar niet identiek zijn, kan uitwisseling tussen de elementen door middel van homologe DNA recombinatie optreden. Dit kan vervolgens leiden tot een verandering van sequentie in het MPN141 gen en/of het MPN142 en daarmee tot een verandering in aminozuurvolgorde van het coderende eiwit, namelijk het P1, P40 of P90 eiwit aan het oppervlakte van de bacterie. Aangezien deze drie eiwitten zeer immunogeen zijn, leidt een verandering in deze eiwitten mogelijk ook tot een verandering in virulentie en/of verspreiding van *M. pneumoniae*.

Tot nu toe zijn er een aantal varianten van het MPN141 gen gevonden als gevolg van homologe DNA recombinatie.[13-17] Er zijn echter slechts twee stammen gevonden met een variatie in het MPN142 gen.[11] Een aantal belangrijke kenmerken zijn overeenkomstig tussen elke variatie van het MPN141 gen of het MPN142 gen als gevolg van recombinatie.

Ten eerste is er tot nu toe bij ieder verandering sprake van eenrichtingsverkeer, waarbij de sequentie van een element de plaats inneemt van de homologe sequentie in het MPN141 of MPN142 gen, zonder dat de sequentie op de originele locatie wordt veranderd. De sequentie die oorspronkelijk aanwezig was in het MPN141 gen of MPN142 gen gaat daarbij verloren. Er is dus geen reciproke uitwisseling van sequenties,

maar altijd slechts één 'donor element' en één 'ontvangend element'. Dit mechanisme van 'unidirectionele' homologe DNA recombinatie wordt genconversie genoemd.[11, 12]

Ten tweede wordt altijd slechts een deel van het donor element gebruikt in de recombinatie en verandert ook slechts een deel van de MPN141 gen of MPN142 gen. Echter de lengte van de sequentie die wordt overgebracht kan zeer variabel zijn en varieert van enkele tientallen tot enkele honderden nucleotiden. Omdat er vanuit elk RepMP2/3, RepMP4 of RepMP5 element meerdere kleinere sequenties kunnen worden overgedragen naar het desbetreffende gen, is het aantal mogelijke recombinatie gebeurtenissen in theorie zeer groot en is het voorspellen van deze gebeurtenissen onmogelijk. Het lijkt er echter wel op dat bepaalde locaties in het MPN141 gen en het MPN142 'hotspots' zijn voor recombinatie. Behoudens de theoretische mogelijkheid tot overdracht van een bepaalde sequentie, is de functie en variatie van het uiteindelijke oppervlakteeiwit uiteraard van het grootste van belang. Het is daarom niet ondenkbaar dat ook de immunologische respons van een bepaald individu of de immunologische druk vanuit de populatie een belangrijke rol speelt in de gevonden variatie in het MPN141 gen en MPN142 gen van *M. pneumoniae* stammen en daarmee hun coderende eiwitten.[11, 12]

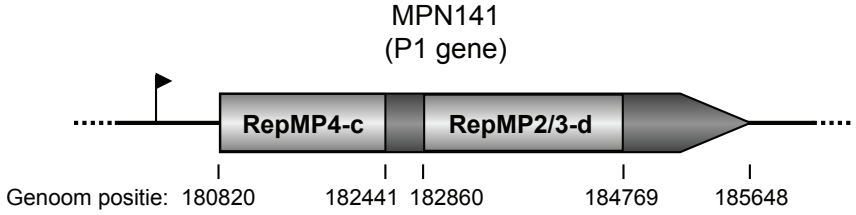

Fig. Het P1-gen (MPN141). Daarin bevinden zich twee RepMP elementen. Het MPN142 gen met daarin het RepMP5 element bevind zich direct na het MPN141 gen. Voor gedetailleerde informatie over de genoom posities en variaties in deze genen en de RepMP-elementen: zie hoofdstuk 2 & 3 van dit proefschrift.

MOLECULAIRE TYPERING VAN *M. PNEUMONIAE*

Op basis van de hierboven beschreven genetische variatie, kan de moleculaire typering van *M. pneumoniae* worden onderverdeeld in: het bepalen van het subtype en het bepalen van het MPN141 type of MPN142 type. In de afgelopen jaren zijn er verschillende moleculaire technieken gebruikt om *M. pneumoniae* te typeren (zie tabel), waaronder pyrosequencing, real-time PCR met 'high resolution melt analysis' (HRM), Multilocus Sequence Typing (MLST), Pulsed Field Gel Electrophoresis (PFGE), PCR-RFLP en sequencing.[2, 11-13, 15, 17-23] Een aantal van de belangrijkste technieken zal hieronder in detail worden behandeld.

Subtypering door middel van pyrosequencing

Aangezien de subtype-specifieke sequenties door het hele genoom voorkomen, is het niet noodzakelijk om de sequentie te bepalen van grote delen van een bepaald gen, zoals het MPN141 gen. In plaats daarvan kan men volstaan met het bepalen van een enkel 'single-nucleotide polymorfisme' (SNP) in bijvoorbeeld het MPN528a gen of het MPN141 gen. Een techniek die hiervoor gebruikt kan worden is pyrosequencing. Pyroseqeuncing is een zogenaamde 'real-time sequencing by synthesis' techniek, die geschikt is om kleine delen van een genoom te bepalen met een maximum van ongeveer 50 bp. Recent zijn twee pyrosequencing assays beschreven voor het bepalen van het subtype van *M. pneumoniae*.[13] Voordelen van deze techniek is de relatief hoge snelheid en de directe toepasbaarheid op patiëntenmateriaal.

MLVA- typering

Er is recent ook een studie gepubliceerd waarin Multilocus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) is toegepast om *M. pneumoniae* te typeren.[22] Deze MLVA assay is ontworpen op basis van de beschikbare genoom sequentie van de M129 stam (zie 'genetische variatie in *M. pneumoniae*'), waarin een aantal tandem repeats worden beschreven. In totaal worden voor deze MLVA assay vijf verschillende tandem repeats gebruikt om *M. pneumoniae* te kunnen typeren. In totaal kunnen hiermee 26 MLVA types (A-Z) van *M. pneumoniae* worden onderscheiden. Deze types kunnen in één van twee groepen worden onderscheiden zijn die overeen komen met het subtype waartoe een stam behoort.[22] Deze techniek kan dus ook gebruikt worden voor het bepalen van het subtype. Inmiddels is deze techniek gemodificeerd zodat deze direct toegepast kan worden op patiëntenmateriaal.[20]

MPN141 typering

Omdat subtypering overal in het genoom mogelijk is, is het niet nodig een bepaald gen te typeren. Desondanks zijn de meeste typeringstechnieken gericht op het MPN141 gen. Daarnaast kan het bepalen van het MPN141 type belangrijk zijn omdat het een hoger discriminerend vermogen heeft en een hogere resolutie kan hebben dan de subtype bepaling. Omdat het aantal varianten van P1 dat theoretisch gevormd kan worden zeer groot is (zoals hierboven beschreven is) zal de sequentie bepaald moeten worden van het gehele variabele deel van het P1 gen, dus zowel het RepMP2/3 als RepMP4 element zal gesequenced moeten worden. Dit kan eenvoudig bewerkstelligd worden door PCR en sequencing.Een aantal technieken hebben zich echter gericht op de detectie van een beperkt aantal sequentie varianten van het P1. Deze technieken zijn gebaseerd op real-time PCR in combinatie met High Resolution Melt analysis en op PCR en sequencing van slechts een beperkt deel van het gen.

Tabel. Verschillende technieken en targets van de afgelopen 10 jaar.

Target	Moleculaire techniek	Soort typering	Literatuur	Jaar
MPN141/MPN528a	Pyrosequencing	Subtypering	Spuesens et al.	2010
MPN142/RepMP5	PCR, sequencing	Subtypering MPN142	Spuesens et al.	2010
MPN141	Real-time PCR, HRM	MPN141	Schwartz et al.	2009
MPN141	Real-time PCR, HRM	Subtypering	Schwartz et al.	2009
MPN141/RepMP4/ RepMP2/3	PCR, sequencing	Subtypering MPN141	Spuesens et al.	2009
Tandem Repeats	MLVA	Subtypering	Dégrange et al.	2009
MPN141	PCR-RFLP, sequencing	MPN141	Kenri et al	2008
MPN141	PCR-RFLP, sequencing	MPN141	Pereyre et al.	2007
MPN141/ Householdgenes	MLST, RFLP	Subtypering	Dumke et al.	2003
MPN141, genoom	RFLP, PFGE	Subtypering	Cousin-Allery et al.	2000
MPN141	PCR-RFLP	MPN141	Dorigo-Zetsma et al.	2000

PCR Poymerase Chain Reaction, HRM High Resolution Melt Analysis, MLVA Multi Locus Variable Number Tandem Repeat Analysis, RFLP Restriction Fragment Length Polymorphism, MLST MultiLocus Sequence Typing, PFGE Pulsed Field Gel Electrophoresis.

Ten slotte kan de vraag gesteld worden wat de epidemiologische en evolutionaire waarde is van het bepalen van het MPN141 type. Immers, in elke bacterie generatie kan de sequentie van het P1 gen via homologe DNA recombinatie veranderen. Een dergelijke verandering kan weliswaar grote gevolgen hebben voor het P1 gen, maar deze verandering heeft geen invloed op de rest van het bacteriële genoom. Hiermee kunnen bacteriën uit dezelfde klonale bacterielijn dus sterk verschillende P1 sequenties hebben.

Classificatie van *M. pneumoniae* stammen op basis van MPN141 typering

Om de variatie in het MPN141 gen eenduidig te kunnen beschrijven en resultaten van MPN141 typeringsstudies beter te kunnen vergelijken hebben wij een classificatie systeem opgezet, waarmee zowel bekende variatie in het MPN141 kan worden aangeduid, maar ook nieuwe variatie op een structurele manier kan worden beschreven.[3, 4, 11, 12] Voordat dit systeem bestond werden nieuwe MPN141 varianten (*M. pneumoniae* stammen met variatie in het MPN141 gen) vaak aangeduid met cijfers en letters (zoals 2a, 2b, 2c etcetera). Met deze aanduiding kunnen echter onmogelijk alle mogelijke varianten worden beschreven en bovendien geeft de aanduiding geen enkele informatie over de sequentie van het gen. In het nieuwe classificatie systeem wordt van elke stam niet alleen aangegeven tot welk subtype deze behoort, maar ook welke RepMP4,

RepMP2/3 elementen in het MPN141 voorkomen en welke RepMP5 elementen in het MPN142 gen voorkomen. Elk RepMP4, RepMP2/3 en RepMP5 element heeft daarvoor een extra lettercodering gekregen op volgorde van het voorkomen in het genoom (a tot en met h voor RepMP4, a-j voor RepMP2/3 en a-h voor RepMP5). Een subtype 1 stam met een onveranderd MPN141 gen en een onveranderd MPN142 gen krijgt in deze classificatie de codering: 1-P1(4-c, 2/3-d, 5-c). De eerste '1' geeft het subtype aan. Dit wordt gevolgd door 'P1', hetgeen betrekking heeft op het P1 operon waarin zowel MPN141 als MPN142 is gelegen. Vervolgens kan tussen haakjes de volgorde van de verschillende RepMP elementen in de twee genen worden aangegeven. Een subtype 1 stam met een recombinatie tussen het RepMP4-c en het RepMP4-h element kan als volgt worden aangeduid: 1-P1(4-c[h]c, 2/3-d, 5-c). De term '4-c[h]c' geeft aan dat het originele 4-c element ten dele is vervangen door het RepMP4-h element. Deze term kan worden aangevuld met de genoom posities van de verschillende elementen, zodat ook duidelijk wordt hoe groot de sequentie is, die is overgedragen naar het RepMP4-c element in het MPN141 gen.

MOLECULAIRE TYPERING VAN *M. PNEUMONIAE* IN DE TOEKOMST

Om een onderscheid te maken tussen verschillende *M. pneumoniae* stammen wordt op dit moment zowel de standaard subtypering, MPN141 typering als wel de recent beschreven MLVA-typering gebruikt. Afgezien van de vraag welke techniek het beste onderscheid maakt tussen verschillende stammen, zijn in de toekomst een aantal aspecten van typering van *M. pneumoniae* van belang. Allereerst dienen de gevonden verschillen tussen stammen nauwkeurig beschreven te worden. Dit is met name van belang bij het gebruik van de MPN141 typering. Het classificatiesysteem zoals hier nogmaals kort werd beschreven kan hierin een belangrijke rol spelen. Op de tweede plaats wordt het in de toekomst belangrijk om het moleculaire type (subtype, MPN141-type of MLVA-type) van *M. pneumoniae* te relateren aan kliniek. Tot op heden is nog weinig bekend over de relatie tussen moleculair (sub) type van *M. pneumoniae* en (de ernst van) klinische verschijnselen.

CONCLUSIE

In dit artikel hebben we aan de hand van de kennis die er bestaat over de genetische variatie van *M. pneumoniae*, de actuele moleculaire typeringstechnieken voor *M. pneumoniae* beschreven. De belangrijkste conclusies die hierbij getrokken kunnen worden zijn de volgende: 1. de genetische variatie van *M. pneumoniae* is zeer uitgebreid

(ondanks dat deze bacterie een 'minimaal' genoom heeft); 2. *M. pneumoniae* stammen kunnen worden onderverdeeld in twee genetische subtypes (subtype 1 en subtype 2); 3. de *M. pneumoniae* subtypes kunnen worden onderscheiden met behulp van een grote verscheidenheid aan typeringsmethodes; 4. het bepalen van de variatie in het MPN141 gen is van beperkte betekenis voor het bepalen van de epidemiologische of evolutionaire verwantschap tussen *M. pneumoniae* stammen. Ten slotte is het noodzakelijk dat toekomstig onderzoek aan *M. pneumoniae* zich onder meer richt op de relatie tussen bacterieel genotype en zowel de verspreiding als de virulentie van deze bacterie.

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Chapter 3

Sequence variations in RepMP elements of 23 *Mycoplasma pneumoniae* strains

Emiel Spuesens

This chapter is largely a modified version of the manuscript entitled:
Sequence variations in RepMP2/3 and RepMP4 elements reveal intragenomic homologous DNA recombination events in *Mycoplasma pneumoniae*. Spuesens EB, Oduber M, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C. *Microbiology*. 2009 Jul; 155(Pt 7): 2182-96

ABSTRACT

The gene encoding major adhesin protein P1 of *Mycoplasma pneumoniae*, MPN141, contains two DNA sequence stretches, designated RepMP2/3 and RepMP4, which display variation among strains. This variation allows strains to be differentiated in two major P1 genotypes (1 and 2) and several variants. Interestingly, multiple variants of the RepMP2/3 and RepMP4 elements exist at other sites within the bacterial genome. Because these variants are closely related in sequence, but not identical, it has been hypothesized that they have the capacity to recombine with their counterparts within MPN141, and thereby serve as a source of sequence variation of the P1 protein. In order to determine the variation within the RepMP2/3 and RepMP4 elements, both within the bacterial genome and among strains, we analyzed the DNA sequences of all RepMP2/3 and RepMP4 elements within the genomes of 23 *M. pneumoniae* strains. Our data demonstrate that: recombination is likely to have occurred between two RepMP2/3 elements in four of the strains, and all previously described P1 genotypes can be explained by inter-RepMP recombination events. Moreover, the difference between the two major P1 genotypes is present in all RepMP elements, such that subtype 1 and 2 strains can be differentiated on the basis of sequence variation in each RepMP element. This suggests that subtype 1 and subtype 2 strains represent evolutionary diverged strain lineages.

INTRODUCTION

Mycoplasma pneumoniae is a human pathogen from the bacterial class of *Mollicutes* that represents one of the smallest self-replicating species with respect to both genome size and cellular dimensions [1]. The limited size of the genome of this species is generally considered to be the result of a gradual loss of genome information from a common gram-positive ancestor [2]. Hitherto, the complete genomic sequence of a single *M. pneumoniae* strain (M129) has been determined. This sequence was reported to have a length of 816,394 base pairs (bp), containing 689 open reading frames (ORFs) [3, 4]. In general, *M. pneumoniae* is regarded as a genetically highly stable microorganism. This view is based on the finding of limited sequence diversity between strains as determined by various molecular typing techniques, which allowed strains to be divided in two major groups (subtype 1 and 2). The molecular discrimination of *M. pneumoniae* isolates in these two subtypes was first described by Dallo *et al.* [5] and Su *et al.* [6], who showed the

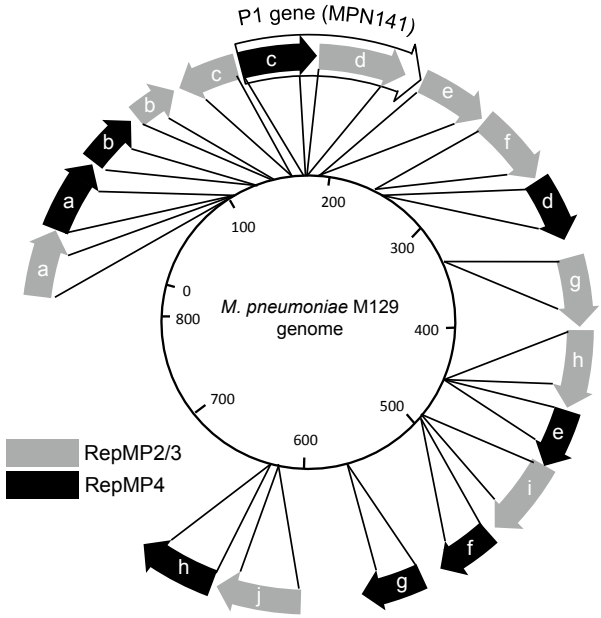


Fig. 1. Distribution of RepMP2/3 and RepMP4 elements.
Structure of the *M. pneumoniae* MPN141 ORF and distribution of RepMP2/3 and RepMP4 elements within the *M. pneumoniae* genome. The location and orientation of all RepMP2/3 and RepMP4 elements within the *M. pneumoniae* M129 genome is indicated by gray and black boxes, respectively 3. The elements are labeled 'a' to 'j' for RepMP2/3 and 'a' to 'h' for RepMP4. The location of the P1 gene is indicated by the arrow at the top. The nucleotide positions of the elements within the genome are listed in Table S1 (supplementary tables).

existence of two different sequence variants of the gene encoding the major adhesin of *M. pneumoniae*, i.e. the P1 protein. The sequence differences between these variants were found to reside in two regions within the P1 (or MPN141) gene [7]. Interestingly, multiple versions of these regions, which were termed RepMP4 and RepMP2/3, respectively, were found at genome sites outside of the P1 gene [3, 8, 9]. In total, 8 variants of the RepMP4 element and 10 variants of the RepMP2/3 element were found throughout the genome of strain M129 (Fig. 1) [3, 9]. Because the different variants of a given RepMP element are similar in sequence, but not identical, it was hypothesized that homologous recombination between the RepMP elements within the P1 gene and those elsewhere in the genome could generate alternative sequence variants of the P1 gene [9, 10]. Obviously, variations within the P1 gene can result in altered P1 proteins at the surface of the bacterium. As the P1 protein is the most important immunological protein[11-14], alteration of the P1 protein could have a major impact on the virulence and/or spread of *M. pneumoniae*.

Despite the relatively high intrinsic potential for variation of the P1 gene by means of homologous recombination between the RepMP elements, only a limited number of sequence variants of the P1 gene have currently been identified among isolates of *M. pneumoniae*. Apart from the two major subtypes (1 and 2) described above, one variant of subtype 1 (variant 1, strain Mp4817) [15] and two variants of subtype 2 (2a and 2b) [15-18] have been reported, in addition to a single aberrant isolate (Mp3896) that could not be classified as a typical subtype 1 or 2 strain [18]. Many questions remain as to the origin of the variation within the P1 gene of *M. pneumoniae* strains, including the strains mentioned above. There are two main reasons for this: the RepMP sequences both within and outside of the P1 gene have not yet been studied systematically, and only a single *M. pneumoniae* genome sequence is currently available. Nevertheless, a study on a variant 2a strain (strain 309) by Kenri and coworkers [16] suggested that inter-RepMP2/3 element recombination had occurred in this strain. This was based on the similarity between the variant 2a-specific sequence within the P1 gene and a RepMP2/3 element elsewhere in the genome [16]. Homologous recombination events have also been suggested to underlie the atypical sequences of the P1 gene of strains Mp3896 and Mp4817, although the occurrence as well as sequences of RepMP2/3 and RepMP4 elements outside of the P1 gene were not reported for these strains [15, 18].

In order to increase our understanding of the occurrence as well as dynamics of intragenomic recombination of RepMP2/3 and RepMP4 elements in *M. pneumoniae*, and to shed light on the origins of the subtype 1 and 2 differences among strains, we have determined the sequences of all 10 RepMP2/3 and all 8 RepMP4 elements in a set of 23 different *M. pneumoniae* isolates. Our data show that the occurrence of intragenomic recombination of RepMP2/3 elements is a relatively common phenomenon in *M.*

pneumoniae and that all RepMP2/3 and RepMP4 elements carry either a subtype 1- or subtype 2-specific signature sequence. As a consequence, the *M. pneumoniae* genome possesses either subtype 1- or subtype 2-specific sequences, but not both, indicating that these subtypes represent evolutionary separated strain lineages.

METHODS

Strains

We used 23 different *M. pneumoniae* strains as listed in Table 1. These strains were described previously by Dorigo-Zetsma and coworkers [15, 19], and were kindly provided by Dr. S.A. Zaat (Academic Medical Center, Amsterdam). Since the complete genome

Table 1. Names and P1 genotypes of the *M. pneumoniae* strains used in this study.

No.	Strain	P1 genotype
1	M129 (ATCC 29342)	1
2	MAC (ATCC 15492)	2
3	P1 1428 (ATCC 29085)	1
4	Mp5	1
5	Mp22	1
6	Mp72	1[a]
7	Mp1042	1[a]
8	Mp1116	1
9	Mp1286	1[a]
10	Mp1397	1[a]
11	Mp1842	2
12	Mp2004	2[a]
13	Mp2018	1[a]
14	Mp2157	1[a]
15	Mp4599	2[a]
16	Mp4817	'Variant 1'
17	Mp5181	2[a]
18	Mp5191	1[a]
19	Mp5192	1[a]
20	Mp5194	2
21	Mp5196	2[a]
22	Mp5245	1[a]
23	Ofo	2[a]

[a] Determined in this study. The genotype of the other strains was described previously [19].

sequence is currently only available for strain M129 (ATCC 29342; a subtype 1 strain), we used this strain as a reference for the sequences of the RepMP2/3 and RepMP4 elements [3, 4]. The P1 gene sequence, however, is available for a few other strains. Of these, we used PI 1428 (ATCC 29085) and Mp22 as representatives of subtype 1 strains. Strain Mp4817 was employed as the prototype 'variant 1' strain, whereas MAC (ATCC 15492) and Mp1842 were regarded as subtype 2 strains.

M. pneumoniae cultures

All strains were cultured in Mycoplasma medium containing 1.4% Difco™ PPLO broth (Becton Dickinson), 0.15% Difco™ TC Yeastolate, UF (Becton Dickinson), 1.4% glucose, 20% horse serum, 1,000 U/ml Penicillin G, 500 U/ml Polymyxine B, and 0.02 mg/ml phenol red. The pH of the medium was adjusted to 7.8-8.0 using a solution of 2 N NaOH, followed by filter-sterilization. Strains were grown in 3 ml of medium at 37°C/5% CO_2 in 25 cm^2 tissue culture flasks (Greiner). Cells were harvested upon color change of the medium (from red/orange to yellow).

Genomic DNA purification

Genomic DNA was isolated from *M. pneumoniae* cells as follows. Cultures (3 ml) were harvested by centrifugation for 10 min in a microcentrifuge at full speed. The cell pellet was resuspended and washed in 1 ml of 0.9% NaCl, followed by centrifugation. The resulting pellet was resuspended in 400 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). Then, 70 µl of 10% sodium dodecyl sulfate (SDS) and 5 µl of Proteinase K (10 mg/ml) was added. After incubation for 10 min at 65°C, 100 µl of 5 M NaCl was added, followed by the addition of 100 µl of a solution of 10% N-cetyl-N,N,N-trimethylammonium bromide (CTAB) in 0.7 M NaCl. The solution was incubated for 10 min at 65°C, after which the DNA was extracted from the solution using 500 µl of chloroform/isoamyl alcohol (24:1; vol:vol). The DNA-containing aqueous phase was transferred to a fresh tube and the DNA was precipitated by the addition of 360 µl of isopropanol. Following incubation at -20°C for 30 min, the DNA was precipitated by centrifugation for 10 min at full speed in a microcentrifuge. After a wash step with 70% ethanol, the DNA was dried, and resuspended in 10 µl of H_2O.

Amplification and cloning of RepMP2/3 and RepMP4 elements. In order to sequence all 10 RepMP2/3 and all 8 RepMP4 elements from each strain, 18 specific PCR primer sets were designed based on the genome sequence of *M. pneumoniae* strain M129 (GenBank accession no. U00089). The sequences of the primers, which were synthesized by and purchased from Eurogentec, are listed in Table S1. Of all strains, purified chromosomal DNA was used to amplify each RepMP2/3 and RepMP4 element by high-fidelity PCR. The PCR mixture (25 µl) contained either 0.5 µM or 1.0 µM primer 1, either 0.5 µM or 1.0 µM primer 2, 0.2 mM of each dNTP (Fermentas), 0.02 U/µl *Pfu*

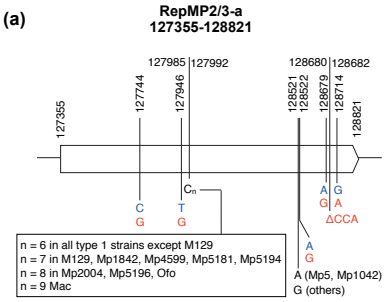
DNA polymerase (Fermentas), 1´*Pfu* buffer containing $MgSO_4$ (Fermentas) and 10 ng of purified *M. pneumoniae* DNA. For the majority of the PCR reactions, the following conditions were used: 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 52°C, and 4 min at 72°C. For amplification of the RepMP2/3-h element, the annealing temperature was lowered to 45°C. The resulting PCR fragments, of which the lengths are depicted in Table S1, were purified by ethanol precipitation and cloned into *Hin*cII-digested vector pBluescript SK$^-$ (Stratagene). In some cases, purified PCR products were used directly in DNA sequencing reactions (below).

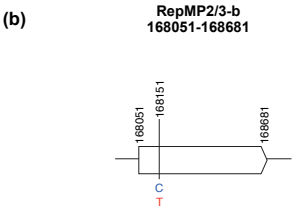
Sequencing of RepMP2/3 and RepMP4 elements. Automated DNA sequencing was performed using ABI PRISM® BigDye™ Terminator (BDT) Cycle Sequencing Ready Reaction Kits and a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing reactions (10 µl), contained 1µl BDT Ready Reaction premix (Applied Biosystems), 5´ sequencing buffer (Applied Biosystems), 3.2 µM of either the M13 reverse or forward primer (Eurogentec), and 1 µl of plasmid DNA or PCR product (150-300 ng). Since most of the RepMP elements are larger than 1 kb, new primers were designed in order to close all sequences double-stranded using a 'primer-walking' strategy. Raw DNA sequence data was processed using Chromas version 2.33 software (Technelysium Pty Ltd) and assembled using the application SeqMan™ II (DNASTAR).

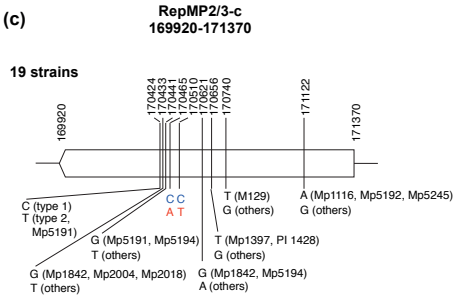
Nucleotide sequence accession numbers. The GenBank accession numbers are depicted in Table S2.

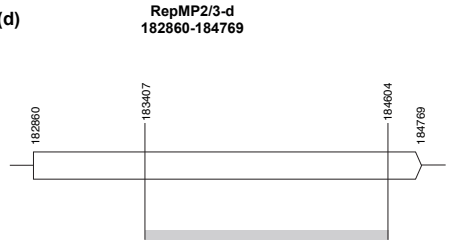
RESULTS

Amplification and sequencing of RepMP2/3 and RepMP4 elements. In order to determine the nucleotide sequences of all RepMP2/3 and RepMP4 elements of the 23 *M. pneumoniae* strains, we designed 18 element-specific PCR primer sets (Table S1). The sequences of the primers were derived from the genome sequence of strain M129, and were chosen such as to include the complete RepMP2/3 and RepMP4 elements as defined by Himmelreich and coworkers [3]. Each primer set gave rise to specific PCR products for each of the 23 strains. Thus, a total of 414 PCR products were generated that were each cloned into *E. coli* plasmid pBluescript SK$^-$ and sequenced. If unique sequence differences were found between the congruent elements from different strains, these differences were confirmed by re-sequencing (using newly amplified DNA fragments). In each of these cases, we could confirm the previously observed differences. As a consequence, we did not detect any mutations induced by PCR amplification. We did find several examples of putative sequence errors, however, in previously deposited GenBank files; these errors are listed in the legends to Fig. 2-5.

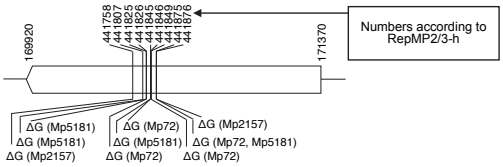


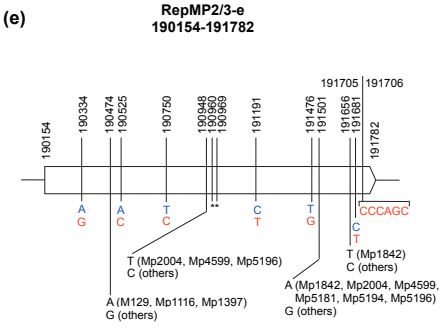


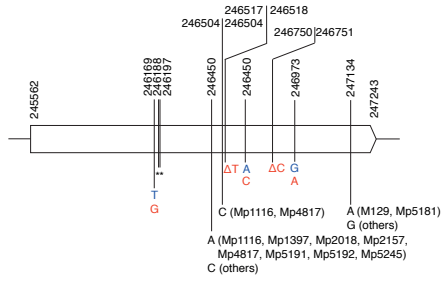




4 strains: Mp5, Mp72, Mp2157, Mp5181:
Sequence identical to RepMP2/3-h (441237 - 442786)







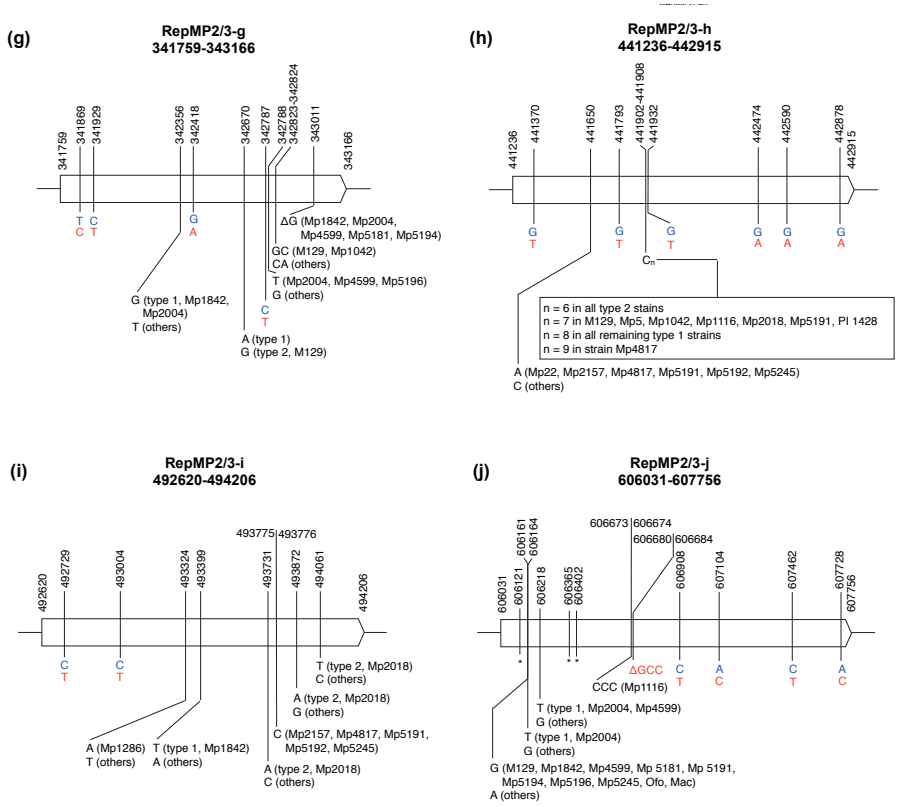


Fig. 2. Sequence variation among the 10 RepMP2/3 elements within the 23 *M. pneumoniae* strains. The sequences of the elements RepMP2/3-a to RepMP2/3-j are schematically represented in (a) to (j), respectively. The boxes indicate the length (drawn to scale) and orientation of each element and only list those nucleotide sequences within a given element that differ among the strains or between any strain and sequenced strain M129 [3]. Below the name of each element, its position within the genome of M129 is listed, as derived from GenBank accession no. U00089. The other positions in the figure also refer to this sequence. Nucleotides, insertions and deletions (Δ) that are found in all subtype 1 and all subtype 2 strains are indicated in blue and red, respectively. Sequence variations that are not subtype-specific are indicated in black. At some positions, indicated with an asterisk (*), the M129 sequence from GenBank differed from the sequences of M129 and all other strains as determined in this study. We therefore assume that the GenBank file is not correct at these positions. The incorrect nucleotides are: in RepMP2/3-b, a T at position 166239 (should be a C) and an A at position 166249 (should be a C); in RepMP2/3-e, a T at position 190960 (should be a C); in RepMP2/3-e, a G at position 190969 (should be an A); in RepMP2/3-f, a T at position 246188 (should be a C) and a T at position 246197 (should be a C); in RepMP2/3-j, a T at position 606121 (should be a C), an A at position 606365 (should be a T), and a C at position 606402 (should be a G). Since element RepMP2/3-d, which is localized within the P1 gene, is highly variable, the sequence variations within this element are depicted in a separate figure (Fig. 3).

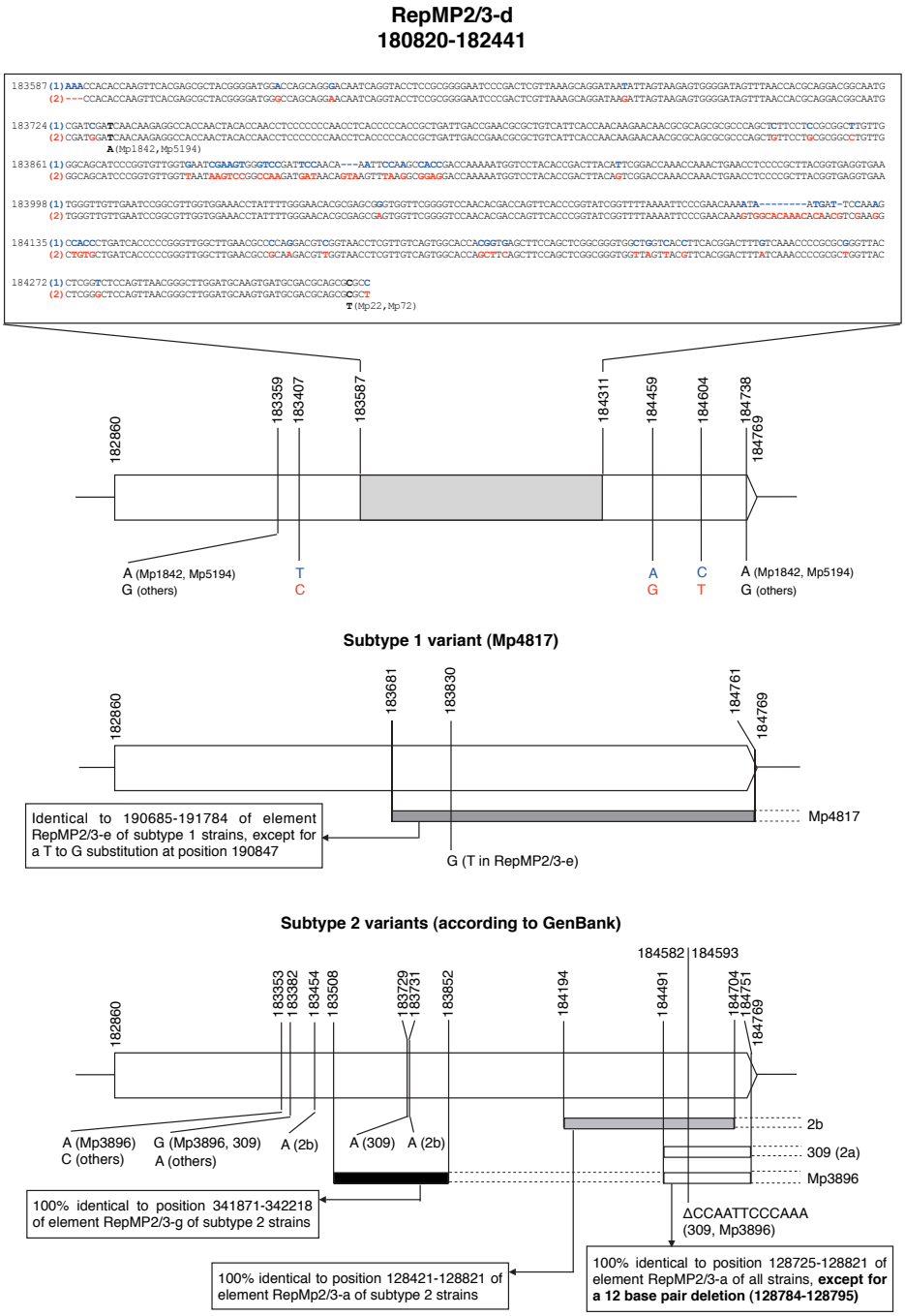


Fig. 3. Sequence variation of element RepMP2/3-d within *M. pneumoniae* strains. All nucleotide positions are taken from GenBank file U00089. Nucleotides, insertions and deletions (Δ) that are found in all subtype 1 and all subtype 2 strains are indicated in blue and red, respectively. The box at the top represents the

sequence as found in most strains; the most variable part of this sequence is detailed in the inset at the top, in which the upper line shows the sequence of subtype 1 strains (1) and the lower line represents sequences of subtype 2 strains (2). The lower two boxes show the sequence organization as found in subtype 1 variant strain Mp4817 and in the subtype 2 variants (309, 2b and Mp3896). The insets describe the putative origin of sequences that are not found in the RepMP2/3-d elements of most *M. pneumoniae* strains. The published sequence of the RepMP2/3-d element from strain MAC (GenBank accession no. AF290001) was found to contain several errors (as was inferred from the sequences that we generated for MAC as well as all other subtype 2 strains); these errors were found at position 182872 (G should be A), 183622 (A should be G), 183631 (G should be A), 183727 (C should be T), 183728 (C should be G), 183730 (G should be A), 183746 (T should be C), 183842 (C should be G), 183848 (T should be G), 183855 (T should be C), and 183882 (G should be T). A single-nucleotide error was also found in the published sequence of element RepMP2/3-d of strain Mp1842 (GenBank accession no. AF290002); at position 182872, a G should be an A.

In order to distinguish between the 10 RepMP2/3 and 8 RepMP4 elements, we employed a novel systematic nomenclature for the elements (Fig. 1 and Table S1) that is based on their appearance within the genome and the current numbering of the *M. pneumoniae* M129 genome in the GenBank database. This novel nomenclature was necessary because the current genome numbering differs from that originally reported by Himmelreich et al. [3]. Previously, the elements were not named systematically in the literature [16]. In order to avoid confusion with this previous labeling, we have proposed a nomenclature that includes the suffixes –a to -j for the 10 RepMP2/3 elements and -a to -h for the 8 RepMP4 elements.

The sequence variation within RepMP2/3 elements among *M. pneumoniae* strains. The sequences of the RepMP2/3 elements are shown in Fig. 2. One of the most significant findings from the analysis of these sequences is that each element possesses either a subtype 1 signature (the nucleotides indicated in blue in Fig. 2) or a subtype 2 signature (the nucleotides indicated in red). Thus, a subtype 1 strain exclusively contains subtype 1-specific RepMP2/3 elements and a subtype 2 strain only contains subtype 2-specific RepMP2/3 elements. The subtype-specificity is defined by single-nucleotide polymorphisms (SNPs) (in each element in Fig. 2), by deletions of one nucleotide (twice in RepMP2/3-f) or three nucleotides (in RepMP2/3-a and RepMP2/3-j), as well as by an insertion of six nucleotides (in RepMP2/3-e). Besides these subtype-specific differences, almost all elements also differ among strains in a subtype-independent fashion. In most cases, these differences are SNPs. The highest level of variation among strains was observed in two of the RepMP2/3 elements, *i.e.* in RepMP2/3-d, which is located within the MPN141 gene (Fig. 3), and in RepMP2/3-c (Fig. 2(c)). Most of the nucleotide sequence differences within element RepMP2/3-d are subtype-specific. As described above, these differences have previously been employed for the classification of strains as subtype 1, 2, 2a and 2b. It has been suggested that the RepMP2/3-d element from subtype 2a strain 309 contains sequences that are derived from another RepMP2/3

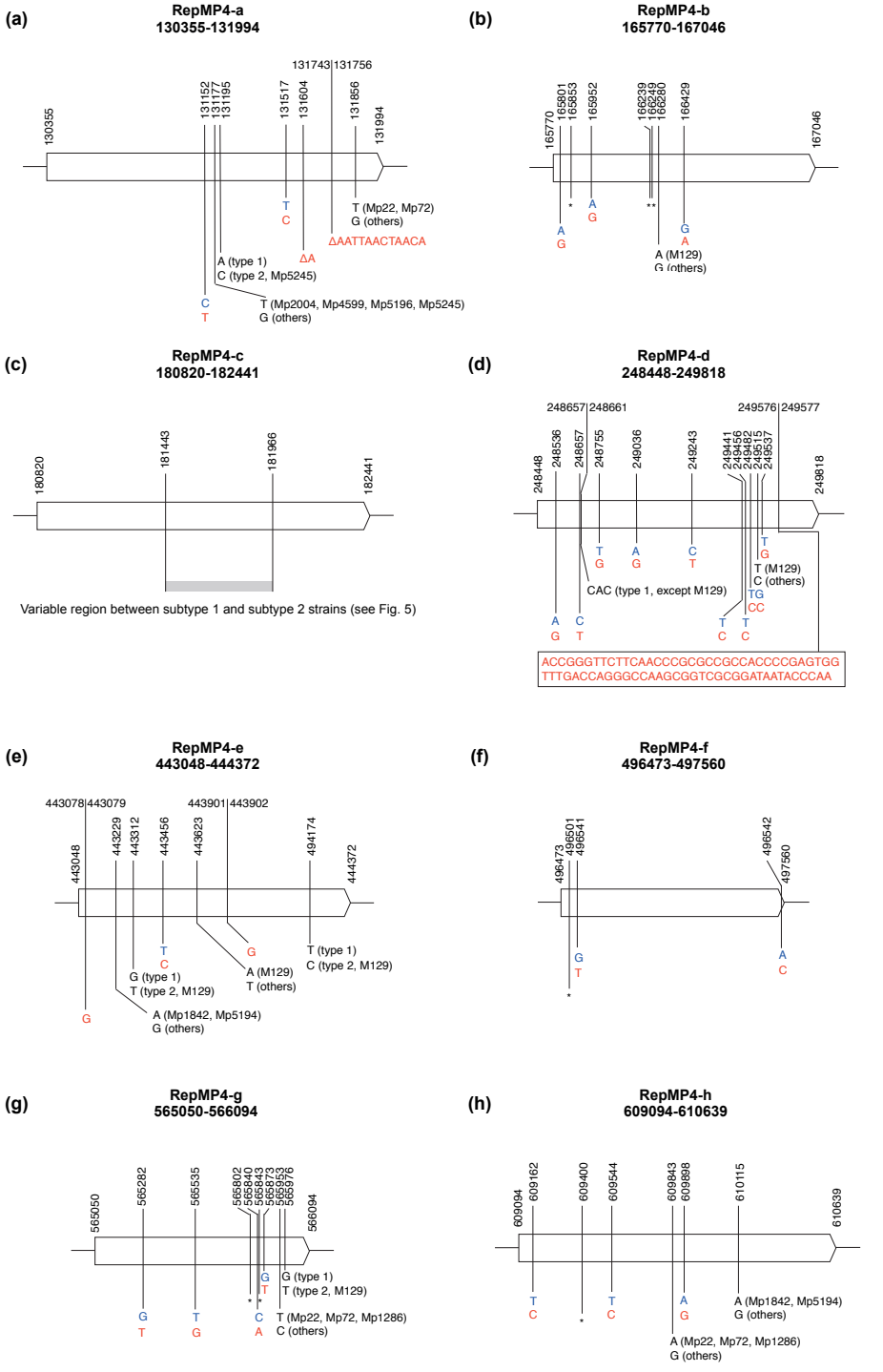

(a)
RepMP4-a
130355-131994
130355
131152
131177
131195
131517
131604
131743
131756
131856
131994
T
C
T (Mp22, Mp72)
G (others)
ΔAATTAACTAACA
ΔA
A (type 1)
C (type 2, Mp5245)
C
T
T (Mp2004, Mp4599, Mp5196, Mp5245)
G (others)
(b)
RepMP4-b
165770-167046
165770
165801
165853
165952
166239
166249
166280
166429
167046
*
A
G
**
A
G
G
A
A (M129)
G (others)
(c)
RepMP4-c
180820-182441
180820
181443
181966
182441
Variable region between subtype 1 and subtype 2 strains (see Fig. 5)
(d)
RepMP4-d
248448-249818
248657
248661
249576
249577
248448
248536
248657
248755
249036
249243
249441
249456
249486
249515
249537
249818
T
G
A
G
C
T
T (M129)
C (others)
CAC (type 1, except M129)
TG
CC
A
G
C
T
T
C
T
C
ACCGGGTTCTTCAACCCGCGCCGCCACCCCGAGTGG
TTTGACCAGGGCCAAGCGGTCGCGGATAATACCCAA
(e)
RepMP4-e
443048-444372
443078
443079
443901
443902
443048
443229
443312
443456
443623
494174
444372
T
C
G
T (type 1)
C (type 2, M129)
G (type 1)
T (type 2, M129)
A (M129)
T (others)
G
A (Mp1842, Mp5194)
G (others)
(f)
RepMP4-f
496473-497560
496473
496501
496541
496542
497560
G
T
*
A
C
(g)
RepMP4-g
565050-566094
565050
565282
565535
565802
565840
565843
565873
565953
565976
566094
G
T
G
T
*
*
G (type 1)
T (type 2, M129)
G
T
T
G
C
A
T (Mp22, Mp72, Mp1286)
C (others)
(h)
RepMP4-h
609094-610639
609094
609162
609400
609544
609843
609888
610115
610639
T
C
*
T
C
A
G
A (Mp1842, Mp5194)
G (others)
A (Mp22, Mp72, Mp1286)
G (others)

Fig. 4. Sequence variation among the 8 RepMP4 elements within the 23 *M. pneumoniae* strains. The sequences of the elements RepMP4-a to RepMP2/3-h are schematically represented in (a) to (h), respectively. The boxes indicate the length (drawn to scale) of each element and only list those nucleotide sequences within a given element that differ among the strains or between any strain and sequenced strain M129 [3]. Below the name of each element, its position within the genome of M129 is listed. The other positions in the figure also refer to this sequence. Nucleotides, insertions and deletions (Δ) that are found in all subtype 1 and all subtype 2 strains are indicated in blue and red, respectively. Sequence variations that are not subtype-specific, are indicated in black. At some positions, indicated with an asterisk (*), the M129 sequence from GenBank differed from the sequences of M129 and all other strains as determined in this study. We therefore assume that the GenBank file is not correct at these positions. The incorrect nucleotides are: in RepMP4-b, a G at position 165853 (should be a C); in RepMP4-f, a T at position 496501 (should be an A); in RepMP4-g, a G at position 565802 (should be a C) and a T at position 565843 (should be a G); in RepMP4-h, a T at position 609400 (should be a G). Because element RepMP4-c, which is localized within the P1 gene, is highly variable, the sequence variations within this element are depicted in a separate figure (Fig. 5).

element, *i.e.* RepMP2/3-a [16]. Indeed, we also found the sequence between position 184491 and 184751 within RepMP2/3-d from subtype 2a strains to be identical to a sequence within RepMP2/3-a; this sequence is identical in subtype 1 and 2 strains (Fig. 3).

Interestingly, the availability of the sequences of all RepMP2/3 elements from both subtype 1 and 2 strains enabled the tracing of the putative origin of 'aberrant' sequences within the MPN141 gene of the other variant strains, *i.e.* strains Mp4817, Mp3896 and 2b strains. As shown in Fig. 3, the variable part of the RepMP2/3-d element in strain Mp4817 is identical, except for a single nucleotide substitution, to sequences from element RepMP2/3-e as found in all subtype 1 strains, including Mp4817. Consequently, in contrast to what has previously been suggested [15], the RepMP2/3-d element from Mp4817 exclusively contains subtype 1-specific sequences and is not a hybrid of subtype 1- and subtype 2-specific sequences. Within strain Mp3896, the RepMP2/3-d element contains sequences that are identical to those of two other RepMP2/3 elements, *i.e.* RepMP2/3-g from subtype 2 strains (between position 183508 and 183852) and RepMP2/3-a (between position 184491 and 184751, similarly as found in subtype 2a strains) (Fig. 3). Finally, subtype 2b strains contain a relatively large sequence within their RepMP2/3-d elements (from position 184194 to 184704) that is identical to a sequence from element RepMP2/3-a as found in all investigated subtype 2 strains (Fig. 2(a)).

The second highly variable RepMP2/3 element within our strain collection is RepMP2/3-c. In four of the strains, *i.e.* Mp5, Mp72, Mp2157 and Mp5181, this element contains a 1.5-kb sequence that is identical to the sequence of RepMP2/3-h from the same strain, except for four single-nucleotide deletions in both Mp72 and Mp5181, and 2 single-nucleotide deletions in Mp2157. As a consequence, these strains carry two RepMP2/3-h sequences, one of which replaces the RepMP2/3-c

sequence typically found in other strains. These data strongly support the hypothesis that RepMP2/3 elements can recombine in a nonreciprocal, unidirectional fashion. Furthermore, these results show that recombination between these elements can occur at genome sites other than MPN141. It is noteworthy that the putative recombination event involving RepMP2/3-c and RepMP2/3-h has taken place independently in four different strains. This is inferred from the presence of differences between these strains in their RepMP2/3-h sequences, which are also copied to their putative second location at the RepMP2/3-c locus. Moreover, these four strains include three subtype 1 strains (Mp5, Mp72 and Mp2157) and one subtype 2 strain (Mp5181), indicating that this DNA rearrangement is not a subtype-specific event.

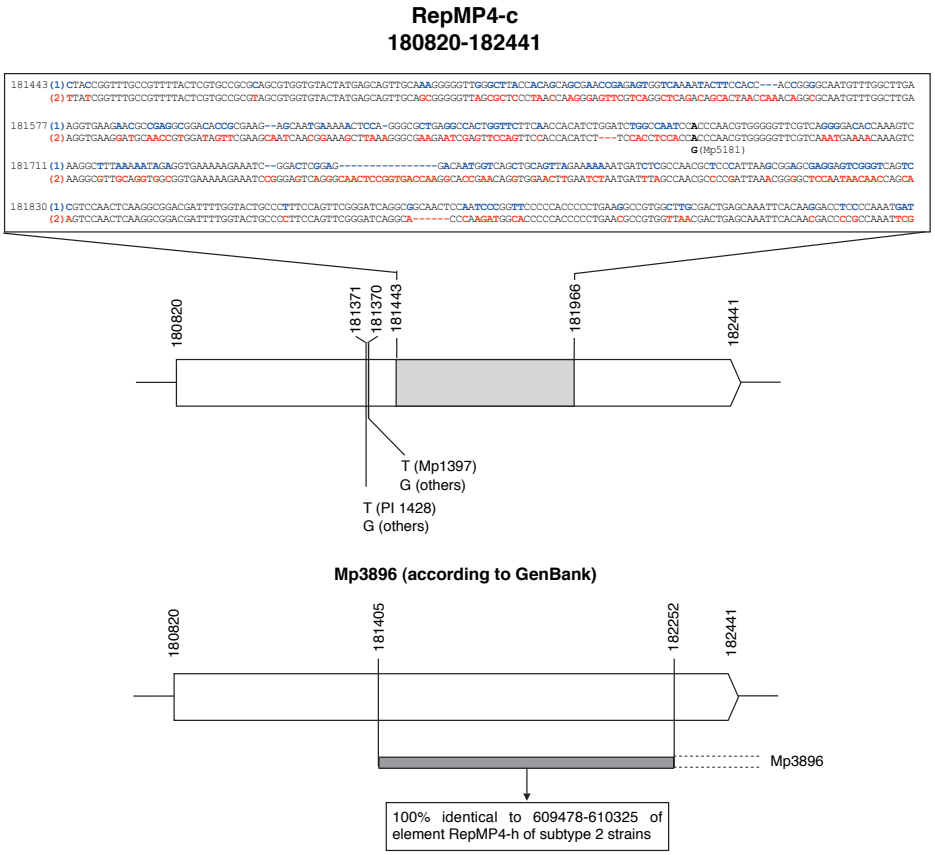


Fig. 5. Sequence variation of element RepMP4-c within *M. pneumoniae* strains. All nucleotide positions are taken from GenBank file U00089. Nucleotides, insertions and deletions (Δ) that are found in all subtype 1 and all subtype 2 strains are indicated in blue and red, respectively. The box at the top illustrates the sequence of most strains, whereas the box at the bottom describes the sequence of strain Mp3896. The inset at the bottom describes the putative origin of the sequence between position 181405 and 182252 of the RepMP4-c element of this strain.

The sequence variation within RepMP4 elements among *M. pneumoniae* strains. The sequences of the eight RepMP4 elements from the 23 strains are summarized in Fig. 4. Similarly as was found for the RepMP2/3 elements, each RepMP4 element possesses either a subtype 1 signature (the nucleotides indicated in blue in Fig. 4) or a subtype 2 signature (the nucleotides indicated in red). The subtype-specificity is principally defined by SNPs (in each RepMP4 element), but also by deletions of one and 12 nucleotides (both in RepMP4-a) and by insertions of three and 72 nucleotides (both in RepMP4-d). We found these 72 nucleotides to also be present in two other elements of subtype 2 strains, *i.e.*, in RepMP4-a (between positions 131502 and 131573, with a single mismatch at position 131522 (C>G)) and in RepMP4-g (between positions 565811 and 565852, with a mismatch at position 565840 (C>A)).

In addition to these subtype-specific differences, most of the elements also display subtype-independent SNPs, albeit to a far lesser extent than do the RepMP2/3 elements. The element that shows the highest level of sequence variability is the one located within the MPN141 ORF, *i.e.* RepMP4-c (Fig. 5). The variations within this element are numerous and are centered on the middle part of the element; these variations have previously been used to distinguish between subtype 1 and subtype 2 strains. Indeed, the vast majority of the variations found in this element are subtype-specific. Unlike the RepMP2/3 elements, the RepMP4 elements within the 23 strains do not show any indication of a previous inter-element recombination event. Nevertheless, the obtained sequences of the RepMP4 elements did allow us to explain the structure of the aberrant MPN141 gene within strain Mp3896, which could previously not be classified as either a subtype 1 or subtype 2 strain [18]. A region of 848 bp within the RepMP4-c element of Mp3896 was found to be identical to the sequence between position 609478 and 610325 of the RepMP4-h element as found in all subtype 2 strains (Fig. 4(h) and Fig. 5). Thus, it is likely that strain Mp3896 has acquired RepMP4-h sequences within its MPN141 gene by a recombination event in which the RepMP4-h element was used as donor sequence. Taken together with the sequence data on the RepMP2/3 elements described above, the 'aberrant' strain Mp3896 can be described as a subtype 2 strain in which the MPN141 gene has undergone three different homologous recombination events, one involving its RepMP4 sequence and two involving its RepMP2/3 sequence.

DISCUSSION

The analysis of the DNA sequences of the RepMP2/3 and RepMP4 elements of various *M. pneumoniae* strains has revealed the following three major findings: (i) the genetic differences between subtype 1 and subtype 2 strains are reflected in each of the RepMP2/3 and RepMP4 elements within the *M. pneumoniae* genome, (ii) a similar

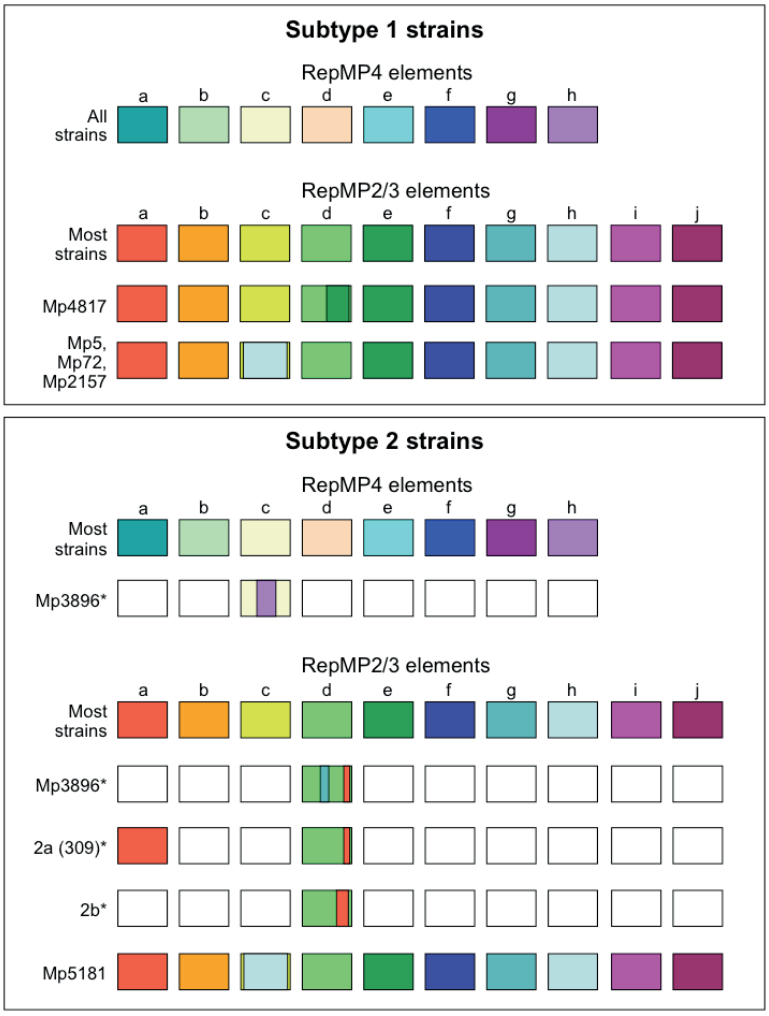


Fig. 6. Schematic representation of the configuration and structure of RepMP2/3 and RepMP4 elements in all *M. pneumoniae* strains published to date. The top panel describes subtype 1 strains, whereas the lower panel describes subtype 2 strains. Each colored block represents the sequence of a particular RepMP element, either RepMP4-a to -h (at the top within each panel) or RepMP2/3-a to -j (at the bottom). The blocks are not drawn to scale. A color change within a specific block indicates a putative DNA rearrangement event in which sequences from a specific element have been transferred to another element. For instance, within strain Mp4817, RepMP2/3-e sequences (in dark green) have been transferred to RepMP2/3-d (in light green). The colors of the elements labeled as 'All strains' or 'Most strains' were used as reference. In the 'Subtype 1' panel, 'All strains' refers to all subtype 1 strains that have been examined in this study. 'Most strains' refers to all subtype 1 strains (top panel) or subtype 2 strains (bottom panel) that were studied, with the exception of the strains named separately in each panel. For the strains marked with an asterisk (*), *i.e.* strain 309 (2a (309)) [16], the 2b strains [17] and Mp3896 [18], the sequences of only a limited number of RepMP elements have been determined. These elements include those located within the P1 gene and, in the case of strain 309, also the RepMP2/3-a element. The elements that have not been sequenced have been left blank.

homologous recombination event has occurred between two RepMP2/3 elements in four of the examined strains, and (iii) all previously described P1 genotypes can be explained by inter-RepMP recombination events.

M. pneumoniae subtype 1 and 2 strains represent separate genetic lineages.

With regard to the first finding listed above, it is important to discriminate between the 'P1 genotype' and the 'subtype' of a strain. This is crucial because two *M. pneumoniae* isolates with a virtually identical genetic composition can have a significantly different P1 genotype as a consequence of a single recombination event between a RepMP element within the P1 gene and an element elsewhere in the genome. For instance, strain Mp4817 has previously been classified as an aberrant 'variant 1a' strain, because the DNA sequence of the RepMP2/3 element within its P1 gene ('P1 genotype') was considerably different from that of other strains [15]. Due to the unavailability of the DNA sequences of other RepMP elements within this strain, the unusual structure of this RepMP element was even considered to be the result of a combination of subtype 1 and subtype 2 sequences [15]. By contrast, our data indicate that strain Mp4817 is a typical subtype 1 strain with respect to the sequences of all of its RepMP2/3 and RepMP4 elements. This strain does, however, differ from other subtype 1 strains because its RepMP2/3-e element has recombined with, and donated sequences to, the RepMP2/3-d element within the P1 gene. Similar considerations can be made for the other variant strains that have been isolated thus far, as summarized in Fig. 6.

Thus, while strain Mp4817 should be classified as a subtype 1 strain, the variant 2a and 2b strains, as well as strain Mp3896, should be classified as subtype 2 strains. Nevertheless, because these variant strains carry a P1 gene with a structure different from that of other subtype 2 strains, we have proposed a *M. pneumoniae* strain classification method in which subtype annotation is separated from the annotation of the P1 gene structure, as outlined in Table 2. Because the sequence data strongly indicate that subtype 1 and subtype 2 strains of *M. pneumoniae* represent separate and relatively conserved genetic entities, it is highly unlikely that isolates of a mixed subtype will be found in the near future.

In order to correctly type *M. pneumoniae* isolates, it seems sufficient to determine the sequences of the variable (RepMP) regions within the P1 gene and compare those to the sequences of the RepMP elements described in this study. Based upon the high level of sequence conservation that was found among the different RepMP elements within both subtype 1 and subtype 2 strains, we anticipate that the origins of 'novel' sequences within the P1 gene of new isolates can be traced back using the sequence library of RepMP elements that we have generated.

Table 2. Classification of *M. pneumoniae* strains on the basis of the origins of the RepMP sequences within the MPN141 or P1 gene.

Strain/ isolate	Subtype[b]	Classification	
		Sequence type[c]	Short type[d]
M129	1	1-P1(4-c; 2/3-d)	1-P1(4-c; 2/3-d)
PI 1429	1	1-P1(4-c; 2/3-d)	1-P1(4-c; 2/3-d)
Mp4817	1	1-P1(4-c; 2/3-d[183681]e[190685-191784;G190847] d[184761])	1-P1(4-c; 2/3-d[e]d)
Mp5[a]	1	1-P1(4-c; 2/3-d)	1-P1(4-c; 2/3-d)
Mp72[a]	1	1-P1(4-c; 2/3-d)	1-P1(4-c; 2/3-d)
Mp2157[a]	1	1-P1(4-c; 2/3-d)	1-P1(4-c; 2/3-d)
MAC	2	2-P1(4-c; 2/3-d)	2-P1(4-c; 2/3-d)
Mp3896	2	2-P1(4-c[181405]h[609478-610325]c[182252]; 2/3-d[183508]g[341871-342218]d[183852-184491]a[128725-128821;Δ128784-128795]d[184751])	2-P1(4-c[h]c; 2/3-d[g] d[a]d)
309 (2a)	2	2-P1(4-c; 2/3-d[184491]a[128725-128821;Δ128784-128795] d[184751])	2-P1(4-c; 2/3-d[a]d)
2b	2	2-P1(4-c; 2/3-d[184194]a[128421-128821]d[184704])	2-P1(4-c; 2/3-d[a]d)
Mp5181[a]	2	2-P1(4-c; 2/3-d)	2-P1(4-c; 2/3-d)

[a] Although these strains carry a recombined RepMP2/3 element (as summarized in Fig. 6), this element is not located within the P1 gene. Hence, these recombination events are not reflected in their 'sequence type'.
[b] The subtype 1 and 2 annotation listed here is based on sequence differences found between subtype 1 and 2 strains in each RepMP2/3 and RepMP4 element.
[c] A sequence type is composed of a subtype number (either 1 or 2), followed by the arrangement of RepMP sequences (between brackets) within the P1 gene; '4-c' refers to the RepMP4-c element and '2/3-d' refers to the RepMP2/3-d element. If these elements contain sequences derived from other RepMP elements, the nucleotide positions of the insertion sites are indicated between brackets; the inserted sequences are indicated by their positions within the RepMP element of origin (between brackets). In the sequence type of strain Mp4817, 'G_{190847}' indicates that the nucleotide at position 190847 is replaced by a G nucleotide. The identity of the RepMP elements of origin are indicated by the letters 'a' to 'j' for RepMP2/3 and by 'a' to 'h' for RepMP4, as introduced in Table S1.
[d] The short sequence types only list the origins of the RepMP sequences and not their full sequence details. Thus, elements with inserted sequences of different lengths, such as 309 and the 2b strains, have the same name. The letters in brackets refer to the origin of the inserted sequences; *e.g.* for strain Mp4817, '2/3-d[e] d' indicates that sequences from element RepMP2/3-e are inserted into RepMP2/3-d sequences.

Nevertheless, we cannot exclude the possibility that novel, deviant RepMP2/3- and RepMP4-like elements will be found in the future. It is also important to note that we did not thoroughly investigate the putative presence of RepMP elements at genome positions other than those known for reference strain M129.

Homologous recombination between RepMP elements.

In four of the investigated strains, including three subtype 1 strains (Mp5, Mp72, Mp2157) and one subtype 2 strain (Mp5181), sequences from element RepMP2/3-h appeared to be transferred to the RepMP2/3-c element. As the original sequence of element RepMP2/3-c seems to be lacking in these strains and the information from the RepMP2/3-h element is copied to the 'RepMP2/3-c location', we infer that these elements may have recombined by gene conversion. To our knowledge, it is the first time that putative homologous recombination events between RepMP2/3 elements outside of the P1 gene have been detected. As these DNA rearrangements do not include known operons or expressed genes, it is tempting to generalize that recombination among RepMP elements within the *M. pneumoniae* genome is governed solely by similarity in DNA sequence and is not executed in a directed fashion. Since a similar recombination event has occurred independently in four different strains, which represented 17% of all strains in this study, it is possible that this event is highly favorable, either because the local genomic structure surrounding elements RepMP2/3-c and RepMP2/3-h is easily accessible for the enzymatic recombination machinery or because these elements share a higher level of sequence similarity with each other than with any other RepMP2/3 element within the genome. However, there is no support for this latter notion, as all elements share significant similarity in at least part of their sequences.

Gene conversion events were also hypothesized to have occurred in strain Mp4817 (described above) and strain 309, in which element RepMP2/3-d was found to carry sequences derived from element RepMP2/3-a [16]. Although the sequences of the RepMP elements outside of the P1 gene have not yet been determined for '2b' strains and strain Mp3896, it is likely that gene conversion-like events have also taken place in these strains. In more general terms, the current data on the dynamics of the exchange of sequence information among RepMP2/3 and RepMP4 elements suggests that these DNA rearrangements occur by (unidirectional) gene conversion and not by reciprocal exchange of homologous DNA sequences.

In conclusion, our data strongly support the hypothesis that the recombination of RepMP2/3 and RepMP4 elements forms the basis of antigenic variation of the P1 protein of *M. pneumoniae* [15, 16]. The RepMP elements that are scattered around the bacterial genome appear to constitute a wealthy source for P1 sequence variability.

It is therefore highly likely that novel variants of the P1 gene will continue to appear. This view stresses the importance of the availability of an adequate, universal typing method for *M. pneumoniae* strains as described in this study. It is clear, however, that the biological and evolutionary constraints on a recombination event within the P1 ORF are stringent, as the encoded P1 protein plays an essential role in the life cycle of *M. pneumoniae*. Each recombination event within the P1 ORF must therefore eventually result in the expression of a functional P1 protein. Nevertheless, we assume that recombination

among RepMP elements within the P1 gene may occur far more frequently than would be deduced from the relatively infrequent detection of P1 sequence variation among either subtype 1 or subtype 2 strains. Clearly, recombination events leading to non-functional P1 proteins will have a biological disadvantage and not be selected for. The extent to which RepMP recombination occurs within the *M. pneumoniae* genome will therefore have to be studied in a context in which biological selection against 'unfavorable' DNA rearrangements is reduced to a minimum. We are currently exploring such contexts.

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Chapter 4

Variation in a surface-exposed region of the *Mycoplasma pneumoniae* P40 protein as a consequence of homologous dna recombination between RepMP5 elements

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SUMMARY

Mycoplasma pneumoniae is a human pathogen that causes a range of respiratory tract infections. The first step in infection is adherence of the bacteria to the respiratory epithelium. This step is mediated by a specialized organelle, which contains several proteins (cytadhesins) that have an important function in adherence. Two of these cytadhesins, P40 and P90, represent the proteolytic products from a single 130-kDa protein precursor, which is encoded by the MPN142 gene. Interestingly, MPN142 contains a repetitive DNA element, termed RepMP5, of which homologs are found at seven other loci within the *M. pneumoniae* genome. It has been hypothesized that these RepMP5 elements, which are similar but not identical in sequence, may recombine with their counterpart within MPN142 and thereby provide a source of sequence variation for this gene. As this variation may give rise to amino acids changes within P40 and P90, the recombination between RepMP5 elements may constitute the basis of antigenic variation and, possibly, immune evasion by *M. pneumoniae*. To investigate the sequence variation of MPN142 in relation to inter-RepMP5 recombination, we determined the sequences of all RepMP5 elements in a collection of 25 strains. The results indicate that: (i) inter-RepMP5 recombination events have occurred in seven of the strains, and (ii) putative RepMP5 recombination events involving MPN142 have induced amino acid changes in a surface-exposed part of the P40 protein in two of the strains. We conclude that recombination between RepMP5 elements is a common phenomenon that may lead to sequence variation of MPN142-encoded proteins.

INTRODUCTION

Mycoplasma pneumoniae is a common pathogen of the human respiratory tract that can cause both upper and lower respiratory tract infections, in particular in childhood and adolescence [1]. The first and crucial step in colonization and infection with *M. pneumoniae* is attachment of the bacterium to the epithelium of the respiratory tract. An essential role in this process is played by a specialized attachment organelle at the tip of *M. pneumoniae* cells. This attachment organelle (also referred to as 'terminal tip') is composed of a complex of adherence and accessory proteins. The loss of function of one of the proteins from this complex almost invariably results in a non-functional attachment organelle and, hence, a non-virulent phenotype of the bacteria [2]. One of the most prominent adherence proteins in the attachment organelle is the ~170-kDa P1 protein. This protein forms a complex with two accessory proteins, called P40 and P90 [3], which function at the distal tip of the attachment organelle and are required for the correct localization of P1 [4, 5]. In line with this close functional relationship, it was shown that the genes encoding the P1, P40 and P90 proteins are linked at both the transcriptional and translational level and are located in a single operon [6]. In this operon, the MPN141 gene encodes the P1 protein, whereas the MPN142 gene encodes a 130-kDa precursor protein which is cleaved into an 'N-terminal' protein of ~40 kDa (P40) and a 'C-terminal' protein of ~90 kDa (P90) [5, 7, 8].

Apart from the functional linkage of MPN141 and MPN142, these genes also share an important structural feature. Both genes contain stretches of DNA of which homologous sequences exist at other loci within the *M. pneumoniae* genome. These stretches of DNA and their homologous counterparts are known as RepMP elements (or repetitive DNA elements). The MPN141 gene contains a RepMP4 element and a RepMP2/3 element, of which respectively seven and nine versions are present at other loci in the genome. The MPN142 contains a RepMP5 element of which seven other versions are present throughout the genome of *M. pneumoniae* strain M129 [9, 10] (Fig. 1). These elements range in size from 1825 to 3360 bp (supplementary Table S1) and share 48% to 91% identity among each other (supplementary Table S2). It has been hypothesized that these elements could rearrange with their homologous counterparts by means of homologous DNA recombination. Obviously, such rearrangements could generate sequence variation within the MPN141 and MPN142 genes and potentially give rise to amino acid changes in the proteins they encode [11, 12]. As the surface-exposed P1, P40 and P90 proteins are immunogenic [5, 8, 13-17], it is tempting to speculate that amino acid changes within these proteins could play a role in immune evasive strategies of *M. pneumoniae*.

Currently, there is strong evidence that recombination among RepMP2/3 elements and RepMP4 elements indeed occurs in *M. pneumoniae* strains [18, 19] and that these recombination events are characterized by a unidirectional DNA sequence transfer

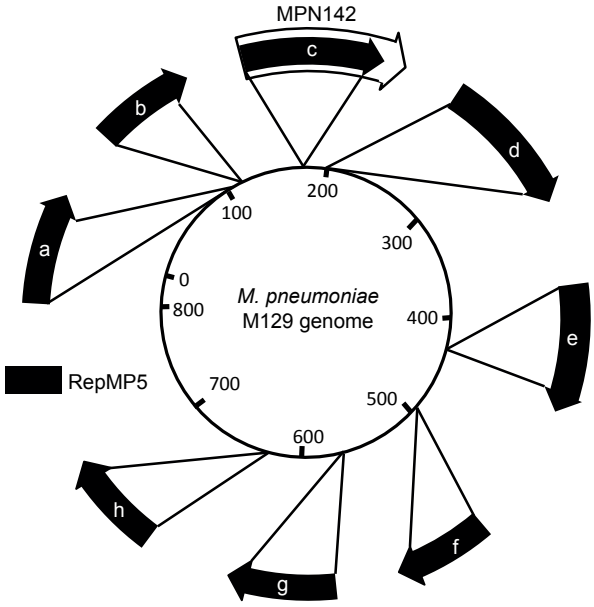


Fig. 1. The localization and orientation of all RepMP5 elements from *M. pneumoniae* strain M129 (GenBank file U00089.2) [9]. The RepMP5 elements are drawn as black boxes and labeled 'a' to 'h'. Specific nucleotide positions of the RepMP5 elements are shown in Table S1 and Fig. 2.

mechanism reminiscent of gene conversion [18-21]. Moreover, in several clinical strains such recombination events have likely led to sequence changes within the MPN141 gene, resulting in amino acid sequence alterations within the P1 protein [18-22]. While there is considerable knowledge on the mechanism underlying sequence variation of the MPN141 gene, not much is known about the dynamics of variation of the MPN142 gene. The potential role of the MPN142-localized RepMP5 element in this process is yet also unclear. This is remarkable, because this element, which has a length of ~2.7 kb, spans a significant part of the 5' end of the MPN142 open reading frame (ORF), and includes the sequence that encodes the proposed cleavage site that separates the P40 and P90 proteins (Fig. 3) [5, 7, 8, 23]. Nevertheless, homologous recombination between RepMP5 elements has previously been hypothesized to occur on the basis of two observations: (i) the finding that the sequence of the MPN142-borne RepMP5 element from strain FH differs significantly from that of strain M129, and (ii) the finding that the variant sequence of this element from strain FH was also found at a site distant from MPN142 within the bacterial genome, in both strain FH and M129 [23]. In this regard, it is important to note that strain FH is a so-called subtype 2 strain, whereas strain M129 is a subtype 1 strain. Subtype 1 and subtype 2 strains represent distinct evolutionary lineages of *M. pneumoniae* that can be distinguished on the basis of DNA sequence differences (mostly single-nucleotide polymorphisms, small deletions and insertions) throughout the bacterial

genome; these differences between subtype 1 and 2 strains were also found in all variants of RepMP2/3 and RepMP4 [18].

To investigate the sequence variation among RepMP5 elements in *M. pneumoniae,* as well as the putative role of these elements in antigenic variation of the surface-exposed P40 and P90 proteins, we have determined the sequences of all eight RepMP5 elements in a collection of 25 *M. pneumoniae* strains. Our data give novel insights into the antigenic variation potential of two surface exposed proteins and the actual protein sequence variation of one surface exposed protein in *M. pneumoniae*.

METHODS

Strains. A total of 25 different *M. pneumoniae* strains, including 15 subtype 1 and 10 subtype 2 strains, were used in this study. The names and characteristics of these strains are listed in Table 1. All strains, except strain R003, were described previously by Dorigo-Zetsma and coworkers [22] and were isolated in Denmark and the Netherlands between 1962 and 1995. Strain R003 was isolated in Germany in 1991 and was described previously by Dumke *et al.* and Maquelin *et al.* [16, 24]. The complete genome sequence of *M. pneumoniae* strain M129 (ATCC 29342; a subtype 1 strain) was used as a reference [9, 10]. In addition, we used strain PI 1428 (ATCC 29085) as a representative of subtype 1 and Mac (ATCC 15492) and FH (ATCC 15531) as representatives of subtype 2 strains.

M. pneumoniae cultures and genomic DNA purification. Strains were grown in 3 ml Mycoplasma medium containing 1.4% DifcoTM PPLO broth (Becton Dickinson), 0.15% DifcoTM TC Yeastolate, UF (Becton Dickinson), 1.4% glucose, 20% horse serum, 1,000 U/ml Penicillin G, 500 U/ml Polymyxine B, and 0.02 mg/ml phenol red, at 37°C/5% CO_2 in 25 cm^2 tissue culture flasks (Greiner). Cells were harvested upon color change of the medium (from red/orange to yellow). Genomic DNA was isolated from *M. pneumoniae* cells as described by Spuesens *et al.* [18].

Amplification and cloning of RepMP5 elements. Eight specific PCR primer sets were designed on the basis of the genome sequence of *M. pneumoniae* strain M129 (GenBank file U00089.2). The primers, which were synthesized by and purchased from Eurogentec, are listed in Table S1. Of all strains, purified chromosomal DNA was used to amplify each RepMP5 element by high-fidelity PCR. For most elements, the PCR mixture (25 µl) contained 0.4 µM primer 1, 0.4 µM primer 2, 0.2 mM of each dNTP (Fermentas), 0.02 U/µl *Pfu* DNA polymerase (Fermentas), 1×*Pfu* buffer containing $MgSO_4$ (Fermentas) and 10 ng of purified *M. pneumoniae* DNA. For the majority of the PCR reactions, the following conditions were used: 5 min at 94°C, followed by 35 cycles

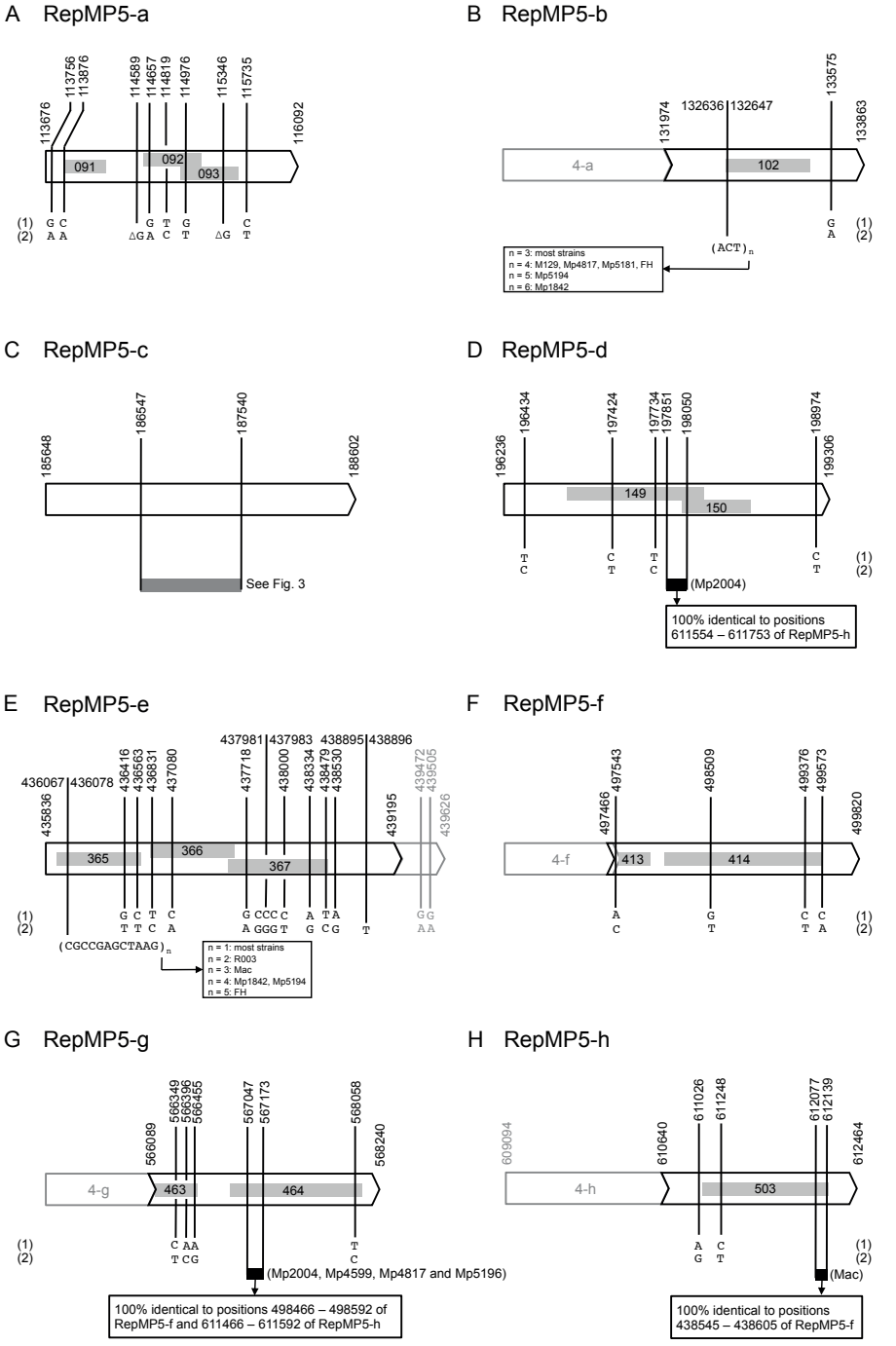


Fig. 2. Sequence variation among the eight RepMP5 elements in 25 *M. pneumoniae* strains. Nucleotide positions are derived from GenBank file U00089.2. The sequences of RepMP5-a to -h are schematically

represented in figures (a) to (h), respectively. Only subtype-specific polymorphisms, tandem repeats and putative inter-RepMP5 recombination events are shown. Subtype-independent sequence polymorphisms are available online (supplementary table). The arrowed boxes indicate the length (to scale) and orientation of each element. Elements RepMP5-b, -f, -g and -h are directly flanked by a RepMP4 element, whereas RepMP5-e is flanked by a RepMP1 element; these flanking elements are indicated in grey. A detailed description of RepMP5-c is shown in Fig. 3. Open reading frames (ORFs) that were identified within the elements are shown as grey bars (including their ORF numbers). Subtype-specific polymorphisms are indicated below the arrowed boxes in two rows. The top row represents the subtype 1 signature sequence (1) and the bottom row the subtype 2 sequence (2). A 'Δ' indicates a deletion. Putative recombination events were found in four elements (RepMP5-c, -d, -g and -h) in seven strains and are represented by black bars accompanied by insets stating the putative donor sequence; details on RepMP5-c are listed in Fig. 3.

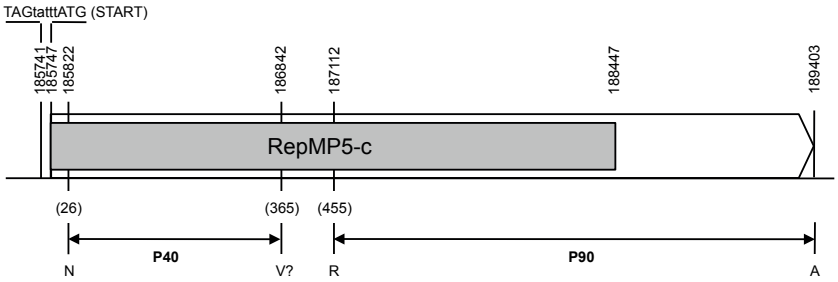
Table 1. Names, genotype and P1 subtype of *M. pneumoniae* strains used in this study

No.	Strain	Genotype or subtype[a]	P1 operon subtype[b]
1	M129 (ATCC 29342)	1	1-(4-c; 2/3-d; 5-c)
2	Mac (ATCC 15492)	2	2-(4-c; 2/3-d; 5-c)
3	PI1428 (ATCC 29085)	1	1-(4-c; 2/3-d; 5-c)
4	Mp5	1	1-(4-c; 2/3-d; 5-c)
5	Mp22	1	1-(4-c; 2/3-d; 5-c[a/f]c)
6	Mp72	1	1-(4-c; 2/3-d; 5-c[a/f]c)
7	Mp1042	1	1-(4-c; 2/3-d; 5-c)
8	Mp1116	1	1-(4-c; 2/3-d; 5-c)
9	Mp1286	1	1-(4-c; 2/3-d; 5-c)
10	Mp1397	1	1-(4-c; 2/3-d; 5-c)
11	Mp1842	2	2-(4-c; 2/3-d; 5-c)
12	Mp2004	2	2-(4-c; 2/3-d; 5-c)
13	Mp2018	1	1-(4-c; 2/3-d; 5-c)
14	Mp2157	1	1-(4-c; 2/3-d; 5-c)
15	Mp4599	2	2-(4-c; 2/3-d; 5-c)
16	Mp4817	1	1-(4-c; 2/3-d[e]d; 5-c)
17	Mp5181	2	2-(4-c; 2/3-d; 5-c)
18	Mp5191	1	1-(4-c; 2/3-d; 5-c)
19	Mp5192	1	1-(4-c; 2/3-d; 5-c)
20	Mp5194	2	2-(4-c; 2/3-d; 5-c)
21	Mp5196	2	2-(4-c; 2/3-d; 5-c)
22	Mp5245	1	1-(4-c; 2/3-d; 5-c)
23	Ofo	2	2-(4-c; 2/3-d; 5-c)
24	FH (ATCC 15531)	2	2-(4-c; 2/3-d; 5-c)
25	R003	2	2-(4-c; 2/3-d[a]d; 5-c)

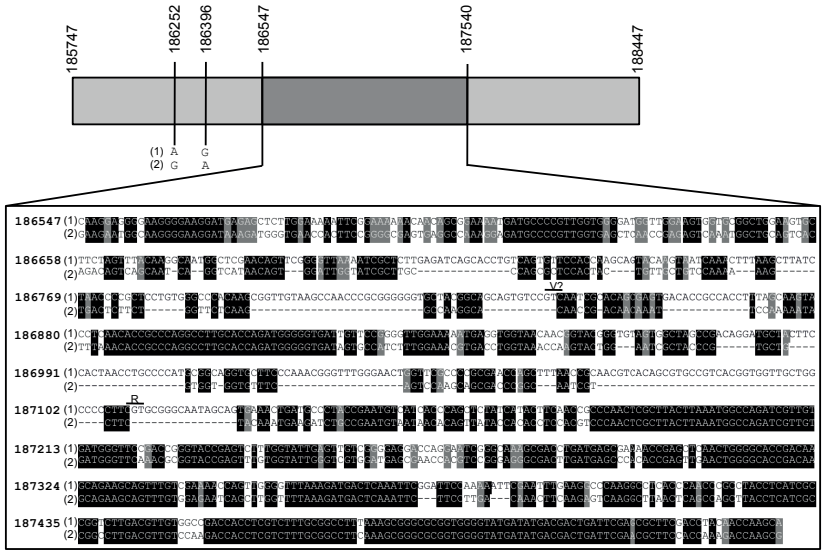
[a]The genotype or subtype of the strains was previously determined by Dorigo Zetsma *et al.* [22], Dumke *et al.* [16], and Spuesens *et al.* [18].

[b]The 'P1 operon subtype' was defined according to the classification scheme of Spuesens *et al.*. [18].

A MPN142 (185747 – 189403)



B RepMP5-c (185747 – 188447) subtype-dependent DNA sequence differences



C RepMP5-c subtype-dependent amino acid sequence differences

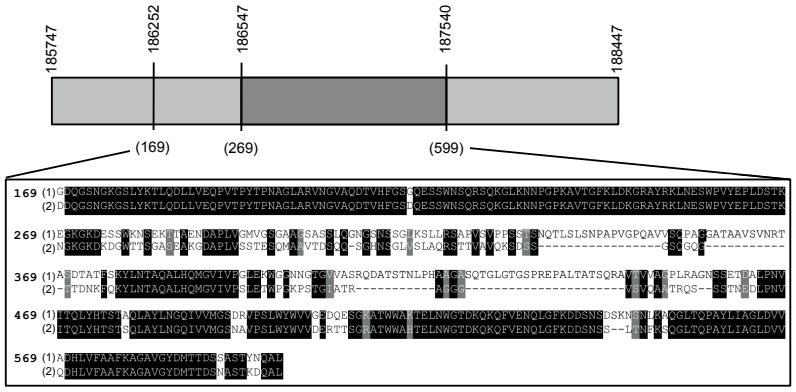


Fig. 3. Sequence variation in the RepMP5-c element of *M. pneumoniae* strains. (a) Schematic representation of the MPN142 gene (the arrowed box). The nucleotide positions are derived from GenBank file U00089.2. The (positions of the) N- and C-terminal amino acids of the P40 and P90 proteins, which are encoded by MPN142, are shown below the arrowed box. The C-terminal amino acid of the P40 protein (V?) was inferred from the determined molecular mass of the P40 protein [7]. (b) Sequence variation within the RepMP5-c element of *M. pneumoniae* strains. Only subtype-specific polymorphisms are shown. Strain-specific sequence polymorphisms are listed online (supplementary table). The inset shows the sequence between position 186547 and 187540, which displays the highest variability between subtype 1 strains (the top line) and subtype 2 strains (the bottom line). The putative C-terminal amino acid of the P40 protein (V?) and the N-terminal amino acid of the P90 protein (R) are indicated above the corresponding DNA sequences, i.e. the codons GTC and CGT, respectively. Identical nucleotides are boxed. (c) The amino acid sequences encoded by the RepMP5-c sequence of subtype 1 (top line) and subtype 2 strains (bottom line) between position 169 and 599. Identical amino acids are boxed.

of 30 sec at 94°C, 30 sec at 55°C, and 5 min at 72°C. For amplification of the three elements with an expected length of >3 kb (i.e. RepMP5-d, RepMP5-e and RepMP5-h), a long template PCR protocol was used, in which the PCR mixture (25 µl) contained both Thermostable DNA polymerase and *Pfu* DNA polymerase in a ratio of 10:1. Specifically, this mixture contained 0.4 µM of primer 1, 0.4 µM of primer 2, 0.2 mM of each dNTP (Fermentas), 0.04 U/µl of Thermostable DNA polymerase (Integro), 0.004 U/µl of *Pfu* DNA polymerase (Fermentas), 1× reaction buffer (Integro), 2 mM $MgCl_2$ and 10 ng of purified *M. pneumoniae* DNA. The conditions for the long template protocol were as follows: 5 min at 94°C, followed by 25 cycles of 10 sec at 94 °C, 30 sec at 55 °C, and 4 min at 68 °C, with an increment of 10 sec per cycle for the last 15 cycles. All resulting PCR fragments, of which the lengths are depicted in Table S1, were purified by ethanol precipitation and cloned into *Hinc*II-digested vector pBluescript SK$^-$ (Stratagene). In some cases, purified PCR products were used directly in DNA sequencing reactions (as described below).

Sequencing of RepMP5 elements. Automated DNA sequencing was performed using ABI PRISM® BigDye™ Terminator (BDT) Cycle Sequencing Ready Reaction Kits and a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing reactions (10 µl), contained 1µl BDT Ready Reaction premix (Applied Biosystems), 5× sequencing buffer (Applied Biosystems), 3.2 pmol of either the M13 reverse or forward primer (Eurogentec), and 1 µl of plasmid DNA or PCR product (150-300 ng). Since most of the RepMP5 elements are larger than 2 kb, new primers were designed in order to close all sequences double-stranded using a 'primer-walking' strategy. Raw DNA sequence data was processed and assembled using the application SeqManTM II (DNASTAR). Alignments were made with the online multiple sequence alignment program ClustalW, available at http://www.ebi.ac.uk/clustalw/ClustalW/.

RESULTS

Amplification and sequencing of RepMP5 elements. To determine the nucleotide sequences of the RepMP5 elements within the genomes of the 25 *M. pneumoniae* strains, we designed eight element-specific PCR primer sets (Table S1). The sequences of the primers were derived from the complete genome sequence of strain M129 [9], and were selected such as to allow specific amplification of all known RepMP5 elements. Since no systematic nomenclature for the RepMP5 elements was available, the elements were provided with a suffix, i.e. -a to -h, respectively, similarly as described previously for the RepMP2/3 and RepMP4 elements [18] (Table S1). This nomenclature is based on the consecutive appearance of each repetitive element within the genome of *M. pneumoniae* strain M129 (GenBank file U00089.2) (Himmelreich *et al.*, 1996).

During the selection of appropriate PCR primer sequences, we found the DNA sequence homology between some of the RepMP5 elements to extend beyond the boundaries that were initially determined for these elements (Himmelreich *et al.*, 1996). Consequently, the borders of some of the elements (RepMP5-d and -e) had to be redefined such that these elements turned out to be considerably larger than reported previously (Table S1). In addition, for two of the RepMP5 elements (RepMP5-e and -h) it was not possible to design unique PCR primers targeting the exact borders of the elements, as these elements were joined at one side to another repetitive element (a RepMP1 and a RepMP4 element, respectively). The PCR primers targeting elements RepMP5-e and -h were therefore designed in such a way that RepMP5-e would be amplified jointly with the neighboring RepMP1 element, and RepMP5-h would be amplified together with the adjacent RepMP4 element.

All eight RepMP5 element-specific primer sets gave rise to PCR products of expected lengths for all 25 investigated strains. Thus, a total of 200 PCR products were produced. Subsequently, the PCR products were cloned into *E. coli* plasmid pBluescript SK$^-$ and sequenced using a 'primer walking' strategy. To minimize the occurrence of sequencing errors due to infidelity of the (proofreading) *Pfu* DNA polymerase, duplicate sequencing reactions were performed on independently produced PCR fragments in case a unique polymorphism was encountered in a single strain.

Strain subtype-dependent sequence variation among RepMP5 elements. The sequences of the RepMP5 elements from all 25 strains are represented schematically in Fig. 2. Similarly as described previously for the RepMP2/3 and RepMP4 elements within the *M. pneumoniae* genome [18], each RepMP5 element was found to have either a subtype 1-specific or a subtype 2-specific sequence. This subtype differentiation corresponded completely with that derived previously from the P1 gene sequences of these strains (Table 1). This signifies that subtype 1 and subtype 2 strains can be distinguished on the basis of single-nucleotide polymorphisms (SNP) in all RepMP5 elements as well as by

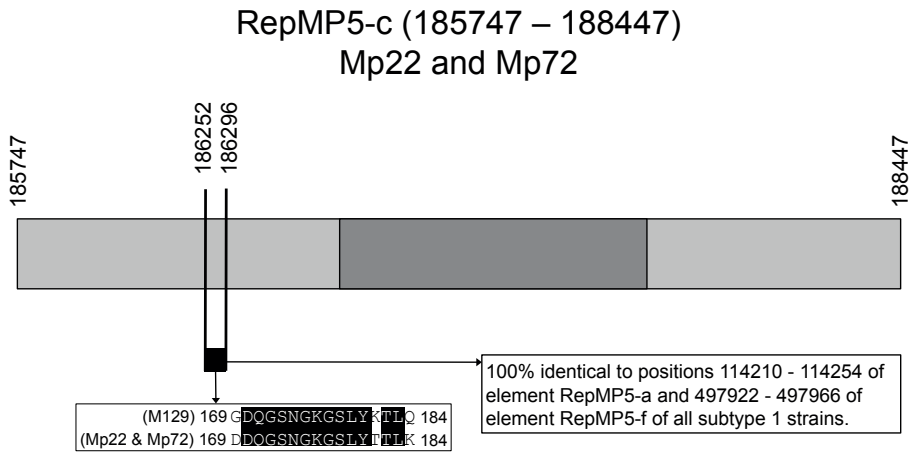


Fig. 4. Structure of the RepMP5-c element from strains Mp22 and Mp72. A putative recombination event, which introduced sequences from either RepMP5-a or RepMP5-f into the RepMP5-c element, is indicated by the black box. The amino acid sequence changes that are predicted to occur in the P40 protein due to this recombination event are depicted at the bottom of the figure, in which the amino acid sequences from strains Mp22 and Mp72 are aligned to those of strain M129. Identical amino acids are boxed.

single-nucleotide deletions (in RepMP5-a; Fig. 2a) and a single insertion (in RepMP5-e; Fig. 2e). The most abundant subtype-specific variation was observed in the RepMP5-c element, which is located in the MPN142 gene (Fig. 3). This variation was previously also observed in a study in which the RepMP5-c sequences from strains M129 and FH were compared [23]. The sequence differences between subtype 1 and subtype 2 strains give rise to significant differences in the predicted amino acid sequences of the MPN142-encoded P40 and P90 proteins (Fig. 3c).

Subtype-independent sequence differences in RepMP5 elements. The sequences of the RepMP5 elements also differ among strains in a subtype-independent fashion. The observed differences (or mutations) include nucleotide transitions and transversions, as well as insertions and deletions (Table S2). Taken together, 83.4% of the mutations was unique to a given RepMP5 element within a strain, whereas 16.6% of the mutations was shared with the corresponding element from at least one other strain. It is interesting to note that differences were also found between strain M129 sequences deposited in GenBank and those determined in this study in four of the eight RepMP5 elements (RepMP5-e to -h). These differences may originate either from sequencing errors or from the accumulation of mutations within the genome of laboratory strain M129 during prolonged culturing. Nevertheless, repeated amplification and sequencing of elements RepMP5-e to -h from strain M129 did not indicate the occurrence of amplification and/or sequencing errors in this study.

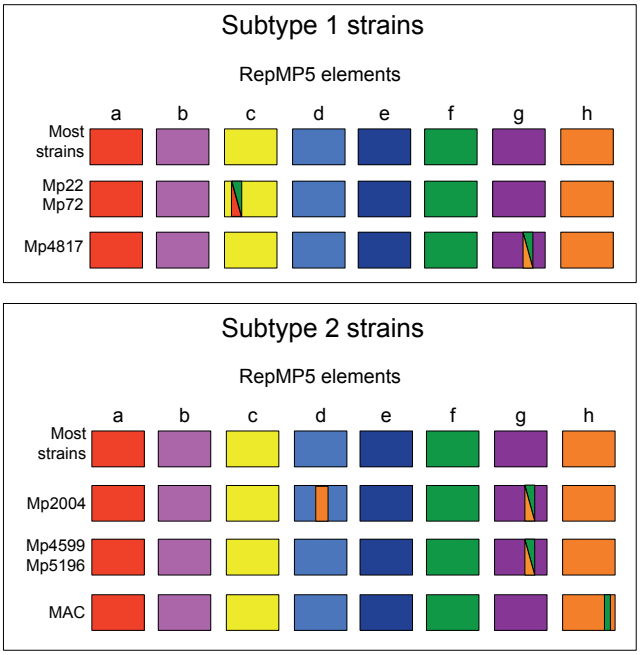


Fig. 5. Schematic representation of the structure of the RepMP5 elements in 25 *M. pneumoniae* strains. This representation is based on the scheme published previously for the RepMP2/3 and RepMP4 elements within the *M. pneumoniae* genome [18]. Each box represents the sequence of a single RepMP5 element. Each row of boxes represents all RepMP5 elements as found in one or more strains (subtype 1 strains in the top panel and subtype 2 strains in the bottom panel). The different patterns within the boxes indicate putative recombination events; each pattern refers to the donor element from which a particular sequence may have been derived. The presence of a box with two different patterns indicates that the sequences may have originated from either of two different donor

Another interesting observation that was made in two of the eight RepMP5 elements was the presence of tandem repeats of varying lengths. Within element RepMP5-b, the sequence 5′-ATC-3′ was found to be present in tandems of either three (in 19 strains), four (in four strains), five (in one strain) or six (in one strain) (Fig. 2b). Another tandem repeat was identified in element RepMP5-e. While a single copy of this 12-bp repeat (5′-CGCCGAGCTAAG-3′) was found in 20 of the strains, two to five tandem copies of this repeat were detected in the remaining five strains (Fig. 2e).

Recombination between RepMP5 elements. Within four of the eight RepMP5 elements (RepMP5-c, -d, -g, and -h) from seven of the 25 strains, sequence stretches were identified that differed significantly from those of their corresponding elements in other strains. Conversely, these sequence stretches were found to be identical to sequences found in other RepMP5 elements from the same strain. Specifically, the RepMP5-d element from subtype 2 strain Mp2004 contains a 199-bp fragment that is identical to a sequence found in element RepMP5-h (Fig. 2d). In this strain, as well as in three other strains (the

subtype 1 strain Mp4817 and subtype 2 strains Mp4599 and Mp5196), the RepMP5-g element was found to contain a 127-bp sequence that is identical to a sequence from either RepMP5-f or RepMP5-h (these two elements are identical in this sequence; Fig. 2g). Furthermore, in subtype 2 strain Mac, a 61-bp sequence was identified in RepMP5-h that is identical to a sequence from the RepMP5-e element (Fig. 2h). The most significant finding, however, was the identification of a 45-bp aberrant sequence within the MPN142-localized RepMP5-c element of two subtype 1 strains (Mp22 and Mp72); this sequence was found to be identical to a sequence from either RepMP5-a or RepMP5-f (these two elements are identical in this sequence; Fig. 4). Together, these data strongly support the hypothesis that RepMP5 elements may rearrange within the *M. pneumoniae* genome by virtue of homologous DNA recombination. In addition, as the 'donor' DNA elements appeared to have remained unaltered by the presumed recombination events, the results also suggest that the rearrangement of RepMP5 sequences may have occurred in a nonreciprocal, unidirectional fashion, similarly as proposed previously for the RepMP2/3 and RepMP4 elements [18, 19]. The putative homologous recombination events that have occurred in the investigated *M. pneumoniae* strains are summarized schematically in Fig. 5.

P40 amino acid sequence variation induced by RepMP5 rearrangements. As the putative recombination events that involved the RepMP5-c elements of strains Mp22 and Mp72 could influence the protein coding capacity of the MPN142 genes of these strains, we compared the predicted amino acid sequences derived from these genes with those derived from the MPN142 gene of reference strain M129. As shown in Fig. 4, the MPN142-encoded amino acid sequence from strains Mp22 and Mp72 differs from that of strain M129 at three positions within a 16-amino acid stretch, between amino acid positions 169 and 184 of the P40 protein. The three mutations include a Gly to Ala substitution at position 169, a Lys to Thr substitution at position 181, and a Gln to Lys substitution at position 184. Importantly, these amino acid residues are located in a surface-exposed region of the P40 protein [5, 8]. These findings therefore indicate that the rearrangement of RepMP5 elements can result in modification of surface-exposed parts of the *M. pneumoniae* P40 protein, which may influence the recognition of P40 by human antibodies.

DISCUSSION

The characterization of the RepMP5 elements from a set of 25 *M. pneumoniae* strains has led to the following main conclusions: (i) the genetic difference between subtype 1 and subtype 2 strains of *M. pneumoniae* can be observed in each RepMP5 element within the bacterial genome, (ii) DNA sequences can be transferred from one RepMP5 element to

another, most likely by unidirectional (non-reciprocal) homologous DNA recombination, and (iii) RepMP5 rearrangements can lead to amino acid sequence changes in surface-exposed regions of the P40 protein, which may contribute to antigenic variation of *M. pneumoniae*. Although the actual occurrence of recombination events between RepMP5 elements was not monitored in 'real time' in this study, the aforementioned conclusions are justified because the sequences of the RepMP5 sequences among subtype 1 strains and among subtype 2 strains are relatively stable.

Strain subtype-specific sequence differences among repetitive DNA elements were previously also reported for each copy of the RepMP2/3 and RepMP4 elements from *M. pneumoniae* [18]. In most RepMP elements, the sequence differences between subtype 1 and subtype 2 strains are represented by SNPs. However, within the RepMP elements located within the P1 operon (i.e., RepMP4-c and RepMP2/3-d within ORF MPN141 and RepMP5-c within MPN142), the sequence differences between the two subtypes are much more pronounced. Nevertheless, it is clear that the difference between these subtypes can already be observed at the single-nucleotide level, a notion that is useful in the design of molecular typing assays [25]. The genetic differences between the subtypes are, however, not solely restricted to RepMP elements, as differences are also found in 'regular' ORFs, such as MPN528a [26, 27].

Apart from the subtype-specific differences between strains in the sequences of their RepMP5 elements, several subtype-independent as well as strain-specific differences were found among these elements. The most prominent subtype-independent difference that was observed was the occurrence of variable numbers of tandem repeats in two of the elements. The significance of (the variable number of) these repeats is yet unknown, in particular because the elements in which they reside have not been shown to be translated into protein. However, it is interesting to note that the part of RepMP5-b that contains the trinucleotide tandem repeat from M129 has previously been cloned within the RepMP5-c element such as to mimic a putative recombination event within the MPN142 gene [28]. The recombinant *M. pneumoniae* strain carrying this modified MPN142 gene was found to be virulent in an animal model and to express both the P40 and P90 protein [28]. Regardless of the putative function of the tandem repeats in the pathogenesis of *M. pneumoniae* infection, trinucleotide repeats have previously also been described within genes of other microorganisms, such as the TAC repeats within the *iaaL* gene from *Pseudomonas savastanoi* pv. savastanoi [29].

Although the RepMP5 elements that are located outside the P1 operon all harbor one or more ORFs (Fig. 2) it is currently unknown whether some or all of these ORFs are translated into protein. The RepMP5-c element appears to be the only RepMP5 sequence that is actually protein-coding. In line with current hypotheses, the other seven RepMP5 elements could serve as a source of variant sequences, which may be transferred to

other RepMP5 elements (including RepMP5-c) by homologous recombination. Indeed, we were able to identify a total of four different putative recombination events that had occurred in seven of the 25 investigated strains. It was previously shown that the region of the P40 protein containing such a recombination event, is extracellularly expressed and carries antigenic determinants [5]. Whether or not the sequence may actually be targeted by the humoral immune response is not known because putative epitopes within this region have not yet been identified. Regardless, this finding is the first to demonstrate a modification of the MPN142 sequence, as well as the MPN142-encoded amino acid sequence, by virtue of the exchange of RepMP5 sequences.

Previously, a recombination event was hypothesized to have occurred in the MPN142 gene of *M. pneumoniae* strain FH [23]. It was suggested that the difference between strain M129 (a subtype 1 strain) and strain FH (a subtype 2 strain) in their RepMP5-c sequences was caused by the introduction of sequences from a distal RepMP5 element (the donor element) into the RepMP5-c element of strain FH. The donor element was found to be present within the genomes of both strain FH and strain M129 [23]. Indeed, we found this putative donor element, which is RepMP5-h, to be present in all investigated *M. pneumoniae* strains. However, because all subtype 2 strains analyzed to date have a similar RepMP5-c sequence (Fig. 3), this putative recombination event is likely to have taken place early in the evolution of *M. pneumoniae*, concurrently with, or immediately following, the divergence of the two main strain lineages of *M. pneumoniae* (subtype 1 and subtype 2). In contrast, the putative recombination events of RepMP5 elements that were identified in this study are likely to have occurred more recently.

It is interesting to note that two of the investigated strains that harbor putative RepMP5 rearrangements (Mp4817 and Mp72) were previously also found to contain rearrangements in other repetitive elements [18]. While strain Mp4817 was found to have sequences from RepMP2/3-e inserted into RepMP2/3-d (which is localized within MPN141), strain Mp72 was reported to contain RepMP2/3-h sequences within RepMP2/3-c [18]. The putative recombination events that occurred in these strains were probably not linked because the elements that were involved in these events are located at distant sites within the *M. pneumoniae* genome.

As the primary structure of the MPN142 ORF of a given *M. pneumoniae* isolate may have clinical and/or epidemiological consequences, it may be useful to include information on this structure in the classification system that was previously proposed for *M. pneumoniae* strains [18]. This system contains data on both the subtype of a strain as well as the structure of the MPN141 gene [18, 25, 26]. On the basis of the current knowledge on the organization of the MPN142 gene, we expanded the *M. pneumoniae* classification system to address the overall structure of all three RepMP elements within the P1 operon. For instance, strains Mp22 and Mp72 can be classified as 1-P1(4-c; 2/3-d; 5-c[a/f]c). In this classification, '1' refers to the general subtype of the strains (i.e., subtype 1) and

'P1(4-c; 2/3-d; 5-c[a/f]c)' refers to the structure of the P1 operon, in which RepMP4-c ('4-c') and RepMP2/3-d ('2/3-d') do not contain significant sequence rearrangements as opposed to reference strain M129, whereas element RepMP5-c contains an insertion of a sequence identical to that of either element RepMP5-a or RepMP5-f ('5-c[a/f]c'). In Table 1 this classification system is applied to all strains from this study.

In conclusion, we have found that putative recombination events between RepMP5 elements can result in amino acid sequence changes in surface-exposed parts of the *M. pneumoniae* P40 protein. Because the RepMP5-c element spans ~74% of the MPN142 ORF and overlaps with sequences encoding both P40 and P90, it is likely that recombination events involving RepMP5-c can also lead to amino acids changes in the P90 protein. We hypothesize that such events, as well as other, novel inter-RepMP recombination events, will continue to emerge among clinical isolates of *M. pneumoniae*.

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Chapter 5

A classification system for genetic variants of *Mycoplasma pneumoniae*

Emiel Spuesens

This chapter is adapted from:
Identification and classification of P1 variants of *Mycoplasma pneumoniae*. Spuesens EB, Hartwig NG, van Rossum AM, Vink C. *J Clin Microbiol.* 2010 Feb;48(2):680; author reply 680.

Sequence variation within the P1 gene of *Mycoplasma pneumoniae*. Spuesens EB, Hartwig NG, van Rossum AM, Vink C. *J Clin Microbiol.* 2011 Oct;49(10):3723; author reply 3723-4.

CLASSIFICATION OF *M. PNEUMONIAE* STRAINS

Our data in chapter 2 and 3 have indicated that *M. pneumoniae* strains can genetically and evolutionary be divided into two lineages, i.e. a subtype 1 and a subtype 2 lineage[1, 2]. The major variations in sequences within the MPN141 gene that have previously been detected appear to be due solely to intra-genomic, inter-RepMP element recombination events. The variation in sequences of the RepMP elements that we have described allows for strains to be classified, analyzed and named in a more comprehensive and systematic fashion than was previously possible. We designed a new classification system in which we described the subtype and RepMP organization. For example, strain M129 may be classified as a strain of 'sequence type' 1-P1(4-c; 2/3-d), in which '1' indicates the subtype of the strain (in this case subtype 1) and P1(4-c; 2/3-d) denotes the RepMP4 (4-c) and RepMP2/3 (2/3-d) organization within the P1 (MPN141) gene. Strain Mp4817 may be described as 1-P1(4-c; 2/3-d[$_{183681}$] e[$_{190685\text{-}191784}$;G$_{190847}$]d[$_{184761}$]), in which 'd[$_{183681}$]' and 'd[$_{184761}$]' indicate the nucleotide positions of the RepMP2/3-d element between which sequences from an RepMP2/3-e element (e[$_{190685\text{-}191784}$;G$_{190847}$]) have been inserted; 'G$_{190847}$' indicates the presence of a G nucleotide (instead of a T nucleotide) at position 190847 of the inserted sequence. In short, strain Mp4817 may be referred to as a 1-P1(4-c; 2/3-d[e]d) strain. A short name may be particularly useful when different elements have donated sequences to a single RepMP element within the MPN141 gene. For example, strain Mp3896 may be classified in short as a 2-P1(4-c[h]c; 2/3-d[g]d[a]d) strain. We applied this classification system to the strains studied in the previous chapters. The classification is depicted in table 2 of chapter 3 and table 1 of chapter 4.

Clear advantages of this method of classification is that: it relies directly on DNA sequence information, and it is also applicable to newly identified strains possessing a yet unknown arrangement of RepMP sequences within their P1 gene. At least two publications by other authors underline the value of this classification system. First, Schwartz et al.[3] reported on a new real time PCR high resolution melting (HRM) technique that allows the identification of P1 variants of *M. pneumoniae*. With this technique they identified a new genetic P1 variant, which was then sequenced. On the basis of the sequence data, the isolate was suggested to exhibit an "intermediate" genotype between subtypes 1 and 2. It was subsequently referred to as 'isolate 3'. Subsequently, using our proposed classification system and available RepMP sequence data, we determined that the new P1 gene found by Schwartz et al. contained a recombination event identical to a variant which was already described by Dumke et al., and referred to as strain 2b. In this strain, a putative homologous DNA recombination event has transferred sequences from element RepMP2/3-a to the RepMP2/3 element within the P1 gene, i.e., RepMP2/3-d.

The strain can be classified as 2-P1(2/3-d[a]d). The RepMP4 element sequence was not available.

Second, Zhao et al.[4] described the sequence of the P1 gene from 60 *Mycoplasma pneumoniae* strains. Within these strains they identified novel P1 gene sequences. When further characterized it was determined that a rearrangement of RepMP4 sequences had taken place, which resulted in the novel P1 gene sequence. Moreover, this study underlines the importance to clearly distinguish the "subtype" to which an *M. pneumoniae* strain belongs from the "P1 sequence type" of this strain. *M. pneumoniae* strains can be classified as subtype 1 or subtype 2 strains; these subtypes can be discriminated on the basis of relatively small sequence differences that are found throughout the bacterial genome. However, the variation of the P1 sequence type is likely determined by homologous DNA recombination events among RepMP4 elements and RepMP2/3 elements, which are subtype-independent processes (and can occur in both subtype 1 and subtype 2 strains). This clear distinction leaves no room for ambiguity and confusion in the molecular typing of *M. pneumoniae*. More importantly, it leaves no room for the suggestion by Zhao et al. that a subtype 1 variant strain would harbor subtype 2-specific sequences. As described previously, the P1 gene sequence variation of this subtype 1 strain is merely the result of a recombination event between RepMP2/3 sequences and is not the result of horizontal DNA transfer between a subtype 1 strain and a subtype 2 strain.

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Chapter 6

Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* by pyrosequencing

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ABSTRACT

The first choice antibiotics for treatment of *Mycoplasma pneumoniae* infections are macrolides. Several recent studies, however, have indicated that the prevalence of macrolide (ML)-resistance, which is determined by mutations in the bacterial 23S rRNA, is increasing among *M. pneumoniae* isolates. Consequently, it is imperative that ML-resistance in *M. pneumoniae* is rapidly detected to allow appropriate and timely treatment of patients. We therefore set out to determine the utility of pyrosequencing as a convenient technique to assess ML-resistance. In addition, we studied whether pyrosequencing could be useful for molecular typing of *M. pneumoniae* isolates. To this end, a total of four separate pyrosequencing assays were developed. These assays were designed such as to determine a short genomic sequence from four different sites, i.e. two locations within the 23S rRNA gene, one within the MPN141 (or P1) gene and one within the MPN528a gene. While the 23S rRNA regions were employed to determine ML-resistance, the latter two were used for molecular typing. The pyrosequencing assays were performed on a collection of 108 *M. pneumoniae* isolates. The ML-resistant isolates within the collection (n=4) were readily identified by pyrosequencing. Moreover, each strain was correctly typed as either a subtype 1 or subtype 2 strain by both the MPN141 and MPN528a pyrosequencing test. Interestingly, two recent isolates from our collection, which were identified as subtype 2 strains by the pyrosequencing assays, were found to carry novel variants of the MPN141 gene, having rearrangements in each of the two repetitive elements (RepMP4 and RepMP2/3) within the gene. In conclusion, pyrosequencing is a convenient technique for ML-resistance determination as well as molecular typing of *M. pneumoniae* isolates.

INTRODUCTION

Mycoplasma pneumoniae is a common bacterial agent of upper and lower respiratory tract infections (RTIs), especially in children. About 20-40% of all community-acquired pneumonias is caused by this small, cell wall-less bacterium [1]. Effective treatment of *M. pneumoniae* infections is usually achieved by the use of macrolides (MLs), which are generally considered as the first choice antibiotics in both children and adults for two reasons: (1) *M. pneumoniae* lacks a cell wall and is therefore intrinsically resistant to cell wall targeting drugs such as penicillin, and (2) MLs have little side effects and are easy to administer, which is of particular importance in children. However, the widespread empirical use of these antibiotics in treatment of RTIs may eventually lead to an increase in the prevalence of ML-resistance of *M. pneumoniae*. Indeed, a recent study [2] reported a dramatic increase of ML-resistance among *M. pneumoniae* isolates, from 0% in 2002 to over 40% in 2008, following the increased use of ML in Japan. In concurrence with this finding, ML-resistant *M. pneumoniae* isolates have also been reported to emerge in other countries [3-5]. Given the apparent increase in the prevalence of ML-resistance, it is crucial that ML-resistant isolates are detected in a timely fashion to allow targeted treatment adjustments for *M. pneumoniae*-induced RTIs and to create more awareness of this upcoming problem in the general clinical community.

The ML-resistant phenotypes are defined by specific point mutations in the V domain of the single-copy 23S rRNA gene of *M. pneumoniae*. The mutations in this gene that induce a high-level of ML-resistance include an A to G transition at position 2063 and an A to C transversion at position 2064 [6]. Low-level ML-resistance is induced by an A to G transition at position 2067 and a C to G or A transversion at position 2617 of the 23S rRNA gene [7, 8]. The presence of these mutations can be detected by PCR of the 23S rRNA gene followed by dideoxy chain termination sequencing. This procedure, however, can be laborious and time-consuming. Other methods, such as those based on real-time PCR followed by (high resolution) melt analysis, have clear advantages over previous assays, in particular regarding the speed at which data can be obtained [3, 9]. Nevertheless, these methods often rely on the execution of multiple rounds of PCR and require ample experience in interpretation of the results. It is therefore important to seek novel, convenient molecular tests for the detection of ML-resistance.

Because molecular diagnostic tests have already proven their value in both detection and genotyping of *M. pneumoniae*, it would be useful to design assay platforms in which these tests can be combined with those aimed at the determination of ML-resistance. Currently, real-time PCR is the predominant technique used for the detection of *M. pneumoniae* in clinical microbiological laboratories. The methods and approaches used for genotyping of *M. pneumoniae*, however, are more diverse. The majority of these methods are aimed at the detection of variation within the MPN141 gene. This gene, which

encodes the immunodominant and essential cytadhesin P1, contains two DNA elements, termed RepMP4 and RepMP2/3, which are variable among *M. pneumoniae* isolates [10-14]. It is hypothesized that recombination between these elements and their homologs located at other positions in the bacterial genome is the cause of this variation. In total, the genome of *M. pneumoniae* strain M129 was found to include 8 variants of RepMP4 and 10 variants of RepMP2/3 [15]. In principle, the determination of the structure of the variable regions (RepMP4 and RepMP2/3) within the MPN141 gene can address two separate questions. First, it addresses whether sequences from another RepMP element within the genome have been introduced into the MPN141 gene [14]. This information may be clinically and epidemiologically useful if the changes on the DNA level lead to an altered P1 amino acid sequence, which could have an influence on the virulence of *M. pneumoniae* isolates. Second, the sequences of the RepMP elements within the MPN141 gene indicate to which of the two major *M. pneumoniae* subtypes (subtype 1 and 2) an isolate can be classified. However, the sequence differences between subtype 1 and 2 strains can be observed throughout the genome of *M. pneumoniae*, including all RepMP elements, and are not restricted to the MPN141 gene [14, 16, 17]. The two subtypes of *M. pneumoniae* therefore appear to represent two separate evolutionary lineages [14]. In this respect, it is important to discriminate between determination of the subtype of isolates on the one hand, and determination of the MPN141 genotype on the other. In principle, a 'genotype switch' of MPN141 can occur at any given moment in a single bacterium within a population due to a RepMP recombination event. This switch can occur irrespective of the subtype of the bacterium. However, a bacterium cannot switch from subtype 1 to subtype 2 (or the reverse). From an evolutionary point of view it is therefore primarily important to classify isolates on the basis of their subtype. With this in mind, we set out to evaluate whether the application of the pyrosequencing technique would be useful in the design of a simple and reliable molecular procedure aimed at: (1) the determination of the subtype of *M. pneumoniae* isolates, and (2) the detection of 23S rRNA mutations responsible for ML-resistance. The pyrosequencing technique, which is a real-time sequencing-by-synthesis method, is of particular interest because it is highly suitable for the generation of relatively short DNA sequences.

In this study, we describe the design and evaluation of a set of four different pyrosequencing assays. Two of these were aimed at the determination of the subtype of *M. pneumoniae* strains by exploiting single-nucleotide polymorphisms (SNPs) between subtype 1 and subtype 2 strains. The other two assays were used for the detection of the aforementioned ML-resistant genotypes. We report that these assays are convenient and reliable, providing a 100% correlation with conventional molecular methods for typing and ML-resistance determination. In addition, we describe the identification of two *M. pneumoniae* isolates carrying novel MPN141 variants, having rearrangements in both the RepMP2/3 and RepMP4 element of the MPN141 gene.

MATERIALS AND METHODS

M. pneumoniae strains, culturing and DNA isolation. A total of 108 *M. pneumoniae* isolates were used in this study. These isolates included the three reference strains M129 (ATCC 29342), MAC (ATCC 15492) and FH (ATCC 15531) and 100 clinical isolates, obtained between 1973 and 2005, (kindly provided by R. Dumke) [17, 18]. Briefly, these included 69 subtype 1 strains (one of which carrying an MPN141 rearrangement) and 31 subtype 2 strains (four of which with an MPN141 rearrangement). Three of the isolates were found to have a ML resistant phenotype (MIC > 100 µg/µl). In addition, five *M. pneumoniae*-positive clinical specimens (throat swabs and nasopharyngeal washings) were used that were recently collected in the Netherlands. The names of all isolates are shown in Table 1. The culturing of, and DNA extraction from *M. pneumoniae* strains was performed as previously described [14]. DNA isolation from clinical specimens was performed using the QIAamp DNA mini kit (QIAGEN, the Netherlands) according to the manufacturer's recommendations, except that phocine herpesvirus was added before isolation as an internal control for real-time PCR [19]. The DNA was eluted with elution buffer AE (10 mM Tris-HCl pH 9.0, 0.5 mM EDTA) in a volume of 50 µl.

M. pneumoniae detection and quantification of bacterial load. The clinical specimens used in this study were first investigated for the presence of *M. pneumoniae* DNA by an in-house developed, quantitative real-time (TaqMan®) PCR assay. This assay targets a conserved region of the MPN141 gene and produces an amplicon of 73 bp. Primer and probe sequences are listed in Table 2. Reactions were performed in a final volume of 25 µl, containing 0.4 µM forward primer, 0.6 µM reverse primer, 0.2 µM probe, 1´Taqman® Universal Master Mix containing UNG (Applied Biosystems) and 5 µl of template DNA. The cycling conditions for each PCR were as follows: 2 min at 50°C, 10 min at 90°C, followed by 40 cycles of 5 sec at 90°C and 10 sec at 60°C.

For quantification of the load of *M. pneumoniae* in clinical specimens, the real-time PCR amplicon obtained from strain M129 was cloned into *Hin*cII-digested vector pBluescript SK$^-$ (Stratagene). The resulting construct, pBSK$^-$P1-73, was used in serial dilutions in order to quantify purified genomic DNA of strain M129 by real-time PCR. Ten-fold serial dilutions of the quantified genomic M129 DNA were subsequently used in every PCR run in order to determine the bacterial DNA load in clinical specimens [19]. These bacterial loads are expressed as *M. pneumoniae* genome copies per ml original sample. The real-time PCR assay was used to determine the limits of detection of the pyrosequencing assays described below.

M. pneumoniae genotyping by RFLP and sequencing. The genotype of most clinical isolates was previously determined by PCR-restriction fragment-length polymorphism (PCR-RFLP) analysis and sequencing of the MPN141 gene [17]. A similar PCR-RFLP method was performed on *M. pneumoniae* isolates cultured from five novel clinical specimens.

Table 1. Subtyping and pyrosequencing results of all *M. pneumoniae* isolates used in this study.

Strain	Subtype[a]	ML-susc.[b]	Pyrosequencing[c]					
			MPN141	MPN528a	23S rRNA 1			23S rRNA 2
			184,991	650,584	2,063	2,064	2,067	2,617
M129	1	S	T	A	A	A	A	C
MAC	2	S	C	C	A	A	A	C
FH	2	S	C	C	A	A	A	C
M155/98	2	S	C	C	A	A	A	C
M192/98	2	S	C	C	A	A	A	C
M885/97	2	S	C	C	A	A	A	C
R035[g]	1	ND	T	A	A	C	A	C
M384/98	2	S	C	C	A	A	A	C
M234/98	1	S	T	A	A	A	A	C
M13/98	2	S	C	C	A	A	A	C
D3142	1	S	T	A	A	A	A	C
M88460/99	1	S	T	A	A	A	A	C
M88408/99	1	S	T	A	A	A	A	C
R003	2[d]	S	C	C	A	A	A	C
R022	1	S	T	A	A	A	A	C
R023	1	S	T	A	A	A	A	C
R024	1	S	T	A	A	A	A	C
R013	1	S	T	A	A	A	A	C
R027	1	S	T	A	A	A	A	C
R008	1	S	T	A	A	A	A	C
R014	1	S	T	A	A	A	A	C
R002	1	S	T	A	A	A	A	C
R007	1	S	T	A	A	A	A	C
R010	1	S	T	A	A	A	A	C
R004	1	S	T	A	A	A	A	C
R011	1	S	T	A	A	A	A	C
R033	1	S	T	A	A	A	A	C
R028	1	S	T	A	A	A	A	C
R018	1	S	T	A	A	A	A	C
R005	1	S	T	A	A	A	A	C
R026	1	S	T	A	A	A	A	C
R034	2	S	C	C	A	A	A	C
R006	2	S	C	C	A	A	A	C
R025	1	S	T	A	A	A	A	C
R030	1	S	T	A	A	A	A	C

Table 1. Subtyping and pyrosequencing results of all *M. pneumoniae* isolates used in this study. (continued)

Strain	Subtype[a]	ML-susc.[b]	Pyrosequencing[c]					
			MPN141	MPN528a	23S rRNA 1			23S rRNA 2
			184,991	650,584	2,063	2,064	2,067	2,617
R012	1	S	T	A	A	A	A	C
R015	1	S	T	A	A	A	A	C
R009	1	S	T	A	A	A	A	C
R020	1	S	T	A	A	A	A	C
R031	1	S	T	A	A	A	A	C
R019	1	S	T	A	A	A	A	C
R021	1	S	T	A	A	A	A	C
M88521/99	1	S	T	A	A	A	A	C
M79060/00	1	S	T	A	A	A	A	C
M79692/00	2[d]	S	C	C	A	A	A	C
M88558/99	1	S	T	A	A	A	A	C
M88566/99	1	S	T	A	A	A	A	C
M88579/99	1	S	T	A	A	A	A	C
M88587/99	1	S	T	A	A	A	A	C
M88708/99	2	S	C	C	A	A	A	C
M79378/00	1	S	T	A	A	A	A	C
D 91/00	2	S	C	C	A	A	A	C
P 2530	2	S	C	C	A	A	A	C
P 535	2	S	C	C	A	A	A	C
R016	1	S	T	A	A	A	A	C
M79461/00	2	S	C	C	A	A	A	C
M814/97	2	S	C	C	A	A	A	C
Mp4817	1[e]	S	T	A	A	A	A	C
R017	1	S	T	A	A	A	A	C
M759/97	1	S	T	A	A	A	A	C
PIF59	2[d]	S	C	C	A	A	A	C
M667/97	1	S	T	A	A	A	A	C
M602/97	1	S	T	A	A	A	A	C
M762/97	1	S	T	A	A	A	A	C
M726/97	1	S	T	A	A	A	A	C
P05/132[b]	1	R	T	A	A	G	A	C
D479	2	S	C	C	A	A	A	C
M727/97	1	S	T	A	A	A	A	C
M826/98	1	S	T	A	A	A	A	C
M765/98	2	S	C	C	A	A	A	C

Table 1. Subtyping and pyrosequencing results of all *M. pneumoniae* isolates used in this study. (continued)

Strain	Subtype[a]	ML-susc.[b]	Pyrosequencing[c]					
			MPN141	MPN528a	23S rRNA 1			23S rRNA 2
			184,991	650,584	2,063	2,064	2,067	2,617
M729/97	1	S	T	A	A	A	A	C
M586/97	1	S	T	A	A	A	A	C
D22	1	S	T	A	A	A	A	C
R032	1	S	T	A	A	A	A	C
M529/97	1	S	T	A	A	A	A	C
M713/96	1	S	T	A	A	A	A	C
M509/97	1	S	T	A	A	A	A	C
R001	2[d]	S	C	C	A	A	A	C
T71	1	S	T	A	A	A	A	C
T72	1	S	T	A	A	A	A	C
T73	1	S	T	A	A	A	A	C
T74	1	S	T	A	A	A	A	C
PIA75	1	S	T	A	A	A	A	C
PIA76	1	S	T	A	A	A	A	C
T77	1	S	T	A	A	A	A	C
T78	2	S	C	C	A	A	A	C
T79[b]	2	R	C	C	G	A	A	C
T80	2	S	C	C	A	A	A	C
T81	2	S	C	C	A	A	A	C
T82	1	S	T	A	A	A	A	C
T83	2	S	C	C	A	A	A	C
T84	2	S	C	C	A	A	A	C
F51	2	S	C	C	A	A	A	C
F52	1	S	T	A	A	A	A	C
F54	2	S	C	C	A	A	A	C
F55	1	S	T	A	A	A	A	C
F56	1	S	T	A	A	A	A	C
F57	2	S	C	C	A	A	A	C
F58	2	S	C	C	A	A	A	C
F60	2	S	C	C	A	A	A	C
M468/97	1	S	T	A	A	A	A	C
M688/98[b]	2	R	C	C	G	A	A	C
R029	1	S	T	A	A	A	A	C
R1108/01	2[f,g]	ND	C	C	A	A	A	C
R0109/01	2[f,g]	ND	C	C	A	A	A	C

Table 1. Subtyping and pyrosequencing results of all *M. pneumoniae* isolates used in this study. (continued)

Strain	Subtype[a]	ML-susc.[b]	Pyrosequencing[c]					
			MPN141	MPN528a	23S rRNA 1			23S rRNA 2
			184,991	650,584	2,063	2,064	2,067	2,617
R0609/02	1[f]	ND	T	A	A	A	A	C
H002	1[f]	ND	T	A	A	A	A	C
H003	1[f]	ND	T	A	A	A	A	C

[a] The subtyping of strains was performed previously by PCR-RFLP and sequencing. Subtype 2 strains are highlighted in grey.
[b] ML susceptibility (S = sensitive, R = resistant, ND = not determined) of the strains (Dumke et al. 2010). Strains R035, M688/98, P05/132 and T79 contain point mutations associated with ML-resistance.
[c] The positions of the indicated nucleotides are derived from either the complete genome sequence of *M. pneumoniae* M129 (for the P1 and MPN528a assays) (GenBank file U00089.2) or the 23S rRNA gene sequence of M129 (for the 23S rRNA 1 and 2 assays) (GenBank file X68422.1). Nucleotide positions 2,063, 2,064, 2,067 and 2,617 correspond to nucleotide positions 122,119, 122,120, 122,123 and 122,673, respectively, of GenBank file U00089.2.
[d] Strains R003, M79692/00, PIF59 and R001 contain a RepMP2/3-d[a]d rearrangement as described previously in detail (Kenri, et al, 1999, Spuesens, et al, 2009).
[e] Strain 4817 contains a RepMP2/3-d[e]d rearrangement as described previously in detail (Spuesens, et al, 2009).
[f] Strains R1108/01, R0109/01, R0609/01, H002 and H003 are isolated in this study.
[g] Strains R1108/01 and R0109/01 contain rearrangements in both RepMP4-c and RepMP2/3-d and are described in this study in detail.

Briefly, the PCR-RFLP was carried out as follows. The RepMP2/3 and RepMP4 elements from the MPN141 gene were first amplified as described previously [14] (the primers used are listed in Table 2). The resulting PCR fragments were then digested with *Hae*III and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide-staining. Isolates R1108/01 and R0109/01 could not be correctly classified as either subtype 1 or 2 based on this RFLP method (data not shown). The PCR products that were obtained from these strains were therefore sequenced by the dideoxy chain termination method. The GenBank accession numbers of these new sequences are: HM447036 (R1108/01, RepMP4-c), HM447037 (R1108/01, RepMP2/3-d), HM447038 (R0109/01, RepMP4-c) and HM447039 (R0109/01, RepMP2/3-d).

Determination of ML-resistant genotypes and subtype of *M. pneumoniae* isolates by pyrosequencing. To identify ML-resistant genotypes, two pyrosequencing assays were developed that target domain V of the 23S rRNA gene. The first assay (23S rRNA 1) was developed to detect five point mutations at three positions within the gene, i.e. A2063G (in which the 'normal' A nucleotide at position 2063 is changed into a G), A2063C, A2064G, A2064C and A2067G. The second assay (23S rRNA 2) was developed to detect two mutations at a single position, i.e. C2617A and C2617G.

Table 2. Oligonucleotides used in this study and parameters of amplification and sequencing reactions.

Assay	Primer/probe	Sequence (5'>3')	5' label	3' label	[primer/probe] (μM)	[Mg] (mM)	Nucleotide positions[a]	Amplicon size (bp)
Real-time PCR								
MPN141	Forward primer	AAG CAC GAG TGA CGG AAA CAC			0.4	NA	184,877 – 184,949	73
	Reverse Primer	CTA ATA CGG GGA ATG ATG TGG TG			0.6			
	Probe	CTC CAC CAA CAA CCT CGC GCC TA	FAM	TAMRA	0.2			
PCR and pyrosequencing								
MPN141	Forward primer	GAA TGA TGT GGT GGG GGT TG			0.6	1	184,937 – 185,020	84
	Reverse primer	GGG GTG CGT ACA ATA CCA TCA A	Biotin		0.6			
	Sequence primer	CAA CGC CGC AAA GAT			0.3			
MPN528a	Forward primer	ATC TAC CGA TTC AAC CAA CTG CT			0.4	3.5	650,499 – 650,664	166
	Reverse primer	GCT AAC TGC GCT AGA GCA AAA T	Biotin		0.8			
	Sequence primer	AGA AAT CGA AAA CTG ACT AT			0.3			
23S rRNA 1	Forward primer	TCG GTG AAA TCC AGG TAC G			0.6	2	122,068 – 122,197 (2,012 – 2,141)	130
	Reverse primer	CAT CGA TTG CTC CTA CCT ATT CTC	Biotin		0.8			
	Sequence primer	AGG CGC AAC GGG ACG			0.3			
23S rRNA 2	Forward primer	TTC AAA CCG TCG TGA GAC AG	Biotin		0.8	1.5	122,646 – 122,758 (2,590 – 2,702)	113
	Reverse primer	AAC TGG AGC ATA AGA GGT GTC CT			0.8			
	Sequence primer	CTA CGG GCA CAA TAG AT			0.3			
Conventional PCR								
RepMP4-c	Forward primer	att gcc aaa agc aaa ttg ctg			0.4	2	180,820 – 182,441	1,622
	Reverse primer	ata gcg cac cct aaa gac ac			0.4			
RepMP2/3-d	Forward primer	gtc caa tcc aaa atg tgg ttg			0.4	2	182,860 – 184,769	1,910
	Reverse primer	act act gag gtt acc act act cg			0.4			

[a] The genome positions and sequences were derived from the complete genome sequence of *M. pneumoniae* M129 (GenBank file U00089.2) and from the 23S rRNA gene sequence of this strain (GenBank file X68422.1) (between brackets). NA, not applicable.

To discriminate between *M. pneumoniae* isolates on the basis of their subtype (i.e. subtype 1 and 2), two different pyrosequencing assays were developed. The first one targets a subtype-specific nucleotide at position 184,991 in the genomic sequence of *M. pneumoniae* M129; at this position, which is located in a conserved region near the 3' end of the MPN141 gene, a T is found in subtype 1 strains and a C in subtype 2 strains. The second pyrosequencing assay is targeted at a subtype-specific nucleotide in the MPN528a gene, at position 650,584 of the M129 genome, which is an A in subtype 1 strains and a C in subtype 2 strains. PCR and sequence primers for all pyrosequencing assays were designed using PyroMark™ Assay Design Software 2.0 and are listed in Table 2.

The pyrosequencing procedure. In short, the pyrosequencing procedure consists of four general steps: (1) generating 5'-biotinylated PCR products, (2) processing the PCR products to obtain single-stranded (ss)DNA, (3) annealing of the sequence primer to the ssDNA, and (4) sequencing, followed by analysis of the results. The PCR reactions from step 1 are performed as follows. PCR mixtures (25 µl) contained 0.4-0.8 µM of each primer (one of which is biotinylated; see Table 2 for the detailed conditions of each of the four PCR reactions), 0.2 mM of each dNTP, 1-3.5 mM $MgSO_4$ (Table 2), 0.02 U/µl *Pfu* DNA polymerase (Fermentas), 1´*Pfu* buffer (Fermentas) and either 5 µl of purified *M. pneumoniae* DNA or 5 µl of a diluted suspension of an *M. pneumoniae* culture in H_2O. The following cycling conditions were used for all assays: 5 min at 94°C, followed by 30 cycles of 10 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C.

The resulting, biotinylated PCR products were immobilized to Streptavidin Sepharose™ High Performance beads (GE healthcare) and processed to yield high quality ssDNA using the PyroMark™ Vacuum Prep Workstation, according to the manufacturer's instructions. Briefly, 10 µl of PCR product was added to a mixture of 2 µl streptavidin beads, 40 µl of binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6) and 28 µl H_2O and mixed for 15 minutes by shaking. After immobilization to the beads, PCR products were washed successively with 70% ethanol (for 10 sec), 0.2 M NaOH (10 sec), and 10 mM Tris-acetate (pH 7.6) (30 sec). Single-stranded DNA was then released into a PSQ™ HS 96 Plate containing 0.3 µM sequence primer in 12 µl annealing buffer (20 mM Tris-acetate, 2 mM Mg-Acetate, pH 7.6) per well. The plate was heated for 2 min at 80°C and slowly cooled down to room temperature to allow sequence primer annealing. The pyrosequencing reactions and sequence analysis were performed using the PyroMark™Q96MD sequencer (Biotage) and accompanying software.

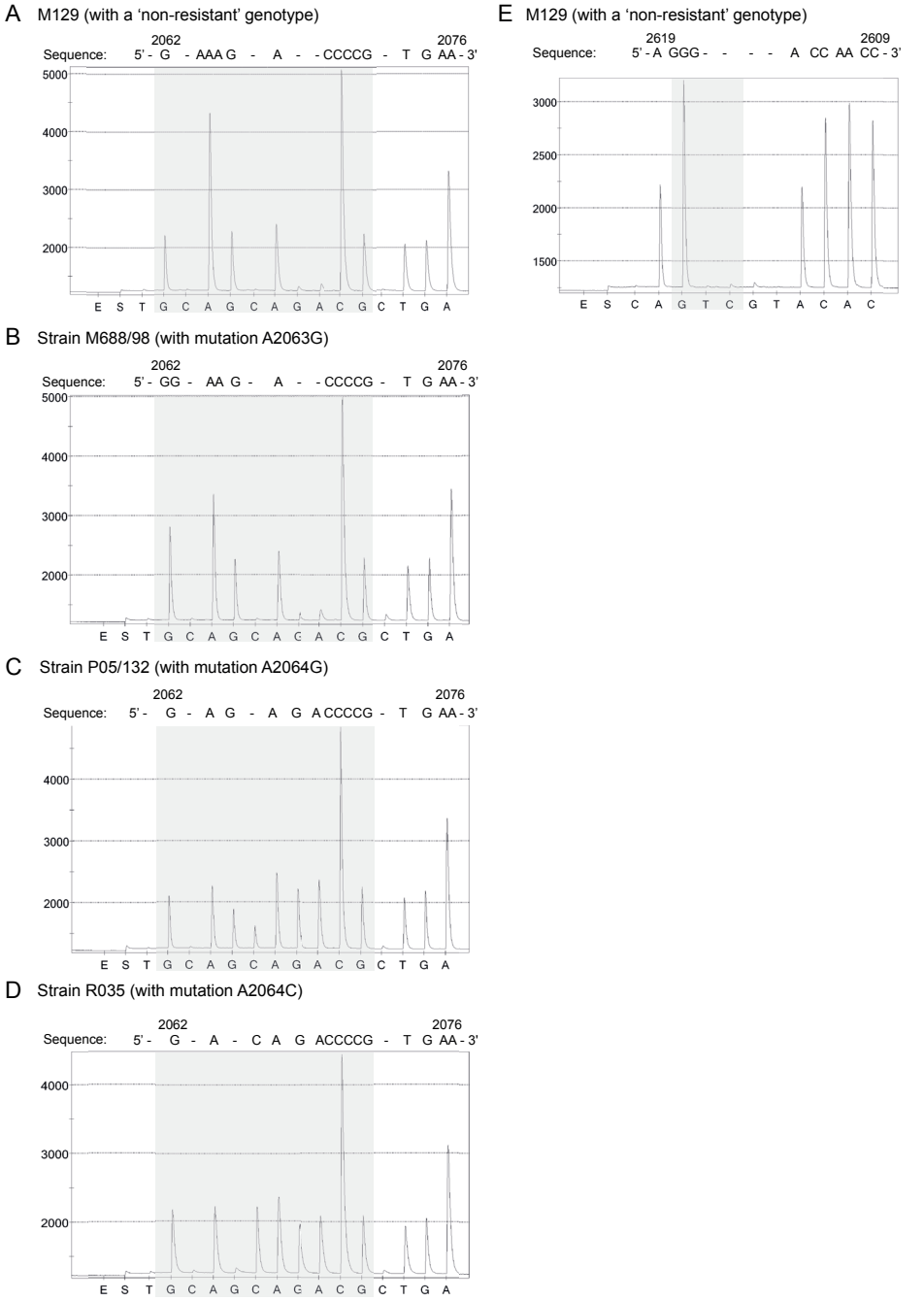

Fig. 1. Pyrograms obtained with the 23S rRNA 1 (A-D) and 23S rRNA 2 (E) pyrosequencing reactions. Pyrograms from the 23S rRNA 1 assay are executed on isolates M129, M688/98, P05/132 and R035. The pyrogram in (A) shows the 23S rRNA gene sequence that is typical of 'normal', ML-sensitive *M. pneumoniae* isolates at positions 2061-2075. The pyrograms in (B-D) show the corresponding sequences

obtained with isolates M688/98, P05/132 and R035, displaying the ML-resistance mutations A2063G, A2064G and A2064C, respectively. On the X-axis (below the figures) the nucleotide (A, C, G and T) dispensing order is depicted. E and S show the dispensing of the enzyme solution and substrate solution, respectively. The values on the Y-axis represent relative light units, as detected by the PyroMark™Q96MD sequencer. Above the figures, the actual sequence that was derived from the pyrograms by the PyroMark™ software is shown. The numbers above the first and last nucleotide are corresponding positions in the 23S rRNA gene. The regions within the pyrograms containing possible SNP-related peaks are highlighted in grey. The pyrogram in (E) shows the 23S rRNA gene sequence that is typical of 'normal' ML-sensitive *M. pneumoniae* isolates at position 2617. Here the pyrogram from the 23S rRNA 2 assay performed on isolate M129 is shown. The 23S rRNA 2 assay targets the region surrounding the nucleotide at position 2617 of the *M. pneumoniae* 23S rRNA gene. The labeling of the figure is similar as in (A-D). The results that were obtained with the 23S rRNA 1 assay and 23S rRNA 2 assay for all *M. pneumoniae* isolates used in this study are listed in Table 1.

RESULTS

Identification of ML-resistant genotypes in *M. pneumoniae* by pyrosequencing. In the design of a pyrosequencing procedure that is suitable for the detection of all 23S rRNA mutations known to be involved in ML-resistance, two different pyrosequencing reactions were developed. This was required because pyrosequencing produces DNA sequences with lengths up approximately 60 nucleotides, whereas the mutations involved in ML-resistance are situated approximately 550 bp apart, around positions 2065 and 2617 of the 23S rRNA. The first pyrosequencing reaction (termed 23S rRNA 1; Table 2) was therefore targeted at the region around position 2065 (to detect putative point mutations at positions 2063, 2064 and 2067), while the second reaction (23S rRNA 2) was targeted at position 2617.

The two assays were performed on a total of 108 *M. pneumoniae* isolates. Three of the isolates (T79, M688/98 and P05/132) were previously reported to have an ML-resistant genotype as well as phenotype, as defined by their resistance to erythromycin at a minimum inhibitory concentration (MIC) value of >100 µg/ml [3]. As shown in Fig. 1 and Table 1, the 23S rRNA 1 pyrosequencing reaction could readily identify these ML-resistant isolates in the collection. The pyrograms showed an A2063G transition in strains T79 and M688/98 (Fig. 1B) and an A2064G transition in strain P05/132 (Fig. 1C). The sequences that were obtained for these isolates corresponded completely with those that were determined previously by conventional methods [3, 7]. Another isolate, R035, was also diagnosed as an ML-resistant isolate by the 23S rRNA 1 test. This strain was found to harbor an A2064C mutation in the 23S rRNA (Fig. 1D). Although the actual antibiotic resistance phenotype of this isolate was not tested, the A2064C mutation was previously reported to induce a MIC value of 100 µg/ml erythromycin in other strains [7]. The other strains from the collection were not found to carry any of the ML-resistance mutations around position 2065 of the 23S rRNA gene (Table 1).

The 23S rRNA 2 reaction produced similar results for each of the 108 isolates in the collection, demonstrating that none of the isolates possessed a mutation at position 2617 in the 23S rRNA (Table 1). A typical pyrogram of the 'normal' 23S rRNA gene sequence around this position is shown in Fig. 1E. Because of the unambiguous nature of the results obtained for each of the strains using the 23S rRNA 2 assay, it should readily allow the identification of isolates carrying mutations at position 2617.

Both the 23S RNA 1 and 23S RNA 2 assay were performed on DNA isolated from bacterial cultures and required a minimal bacterial DNA load of 5×10^2 *M. pneumoniae* genome copies/ml. This DNA load corresponds to a cycle threshold (C_t) value of 35 in our real-time PCR assay.

Subtype determination of *M. pneumoniae* by pyrosequencing. To evaluate the usefulness of pyrosequencing in determination of the subtype of *M. pneumoniae* isolates, we designed two separate pyrosequencing reactions. Both reactions were devised such as to identify SNPs between subtype 1 and subtype 2 strains. The first of these assays, the MPN141 assay, was targeted at the identification of the nucleotide at position 184,991 (within the MPN141 gene). At this position, a T is present in subtype 1 strains and a C in subtype 2 strains. The second assay, the MPN528a assay, was targeted at the nucleotide at position 650,584 of the *M. pneumoniae* genome, which is located within the MPN528a gene. We sequenced this gene, which encodes a homologue of RecU Holliday junction resolving enzymes (data not shown), from three subtype 1 and three subtype 2 strains and found the subtype 1 strains to have an A at position 650,584 and the subtype 2 strains to have a C at this position. The evaluation of the MPN528a pyrosequencing assay was therefore primarily aimed at addressing whether or not this putative SNP in the MPN528a gene would be characteristic for all isolates in our collection.

As shown in Fig. 2A and Table 1, the MPN141 pyrosequencing assay readily divided the isolates into two groups, i.e. those with a T nucleotide and those with a C nucleotide at position 184,991 within the MPN141 gene. These results corresponded with the sequence information that was previously obtained for these strains by conventional molecular methods [14, 17]. Also, the five novel clinical isolates were correctly typed as either subtype 1 isolates (isolates R0609/01, H002 and H003; Table 1) or subtype 2 isolates (R1108/01 and R0109/01). Thus, the MPN141 pyrosequencing reaction was able to differentiate unambiguously between subtype 1 and subtype 2 *M. pneumoniae* isolates.

The subtyping results obtained with the MPN141 pyrosequencing assay corresponded completely with those obtained with the MPN528a assay (Fig. 2B and Table 1). All isolates classified as subtype 1 strains by the MPN141 pyrosequencing assay were found to carry an A at position 650,584 within the MPN528a gene, whereas all isolates defined as subtype 2 strains were found to have a C at this position. The implications

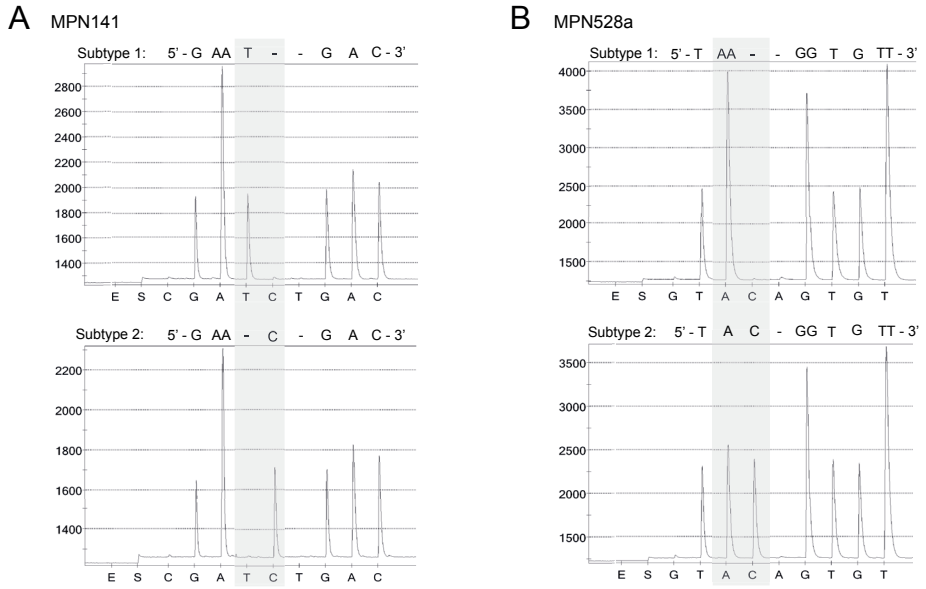

Fig. 2. Pyrograms obtained with the MPN141 and MPN528a pyrosequencing assays. (A) Pyrograms from the MPN141 assay executed on isolates M129 (top; a subtype 1 strain) and MAC (bottom; a subtype 2 strain). (B) Pyrograms from the MPN528a assay executed on isolates M129 (top; a subtype 1 strain) and MAC (bottom; a subtype 2 strain). The labeling of the figures is similar as in Fig. 1. The results obtained with the MPN141 assay and MPN528a assay for all *M. pneumoniae* isolates used in this study are listed in Table 1.

of these results are twofold. First, the SNP at position 650,584 of the MPN528a gene can be used to discriminate between subtype 1 and subtype 2 *M. pneumoniae* strains. Second, subtype 1 strains are unable to express a full-length protein product from the MPN528a gene, because the nucleotide at position 650,584 in this gene forms part of a TAA translation termination codon. Subtype 2 strains, however, are able to express a full-length protein from the MPN528a open reading frame, as they contain a TAC codon (encoding tyrosine) at the position of the TAA codon in subtype 1 strains. The consequences of this crucial difference between subtype 1 and 2 strains is described elsewhere[20].

In conclusion, both the MPN141 assay and MPN528a assay are convenient assays that are suitable for the classification of *M. pneumoniae* isolates as either subtype 1 or subtype 2 strains. Because these assays target highly conserved sequences within either subtype 1 or subtype 2 strains, they are not influenced by putative recombination events that may have occurred within the RepMP4 and/or RepMP2/3 elements of the MPN141 gene. Thus, every *M. pneumoniae* isolate, including isolates carrying rearrangements of the RepMP2/3 and/or RepMP4 element within the MPN141 gene, such as strains R001, R003, M79692/00, PIF59, an Mp4817 (Table 1), can be classified

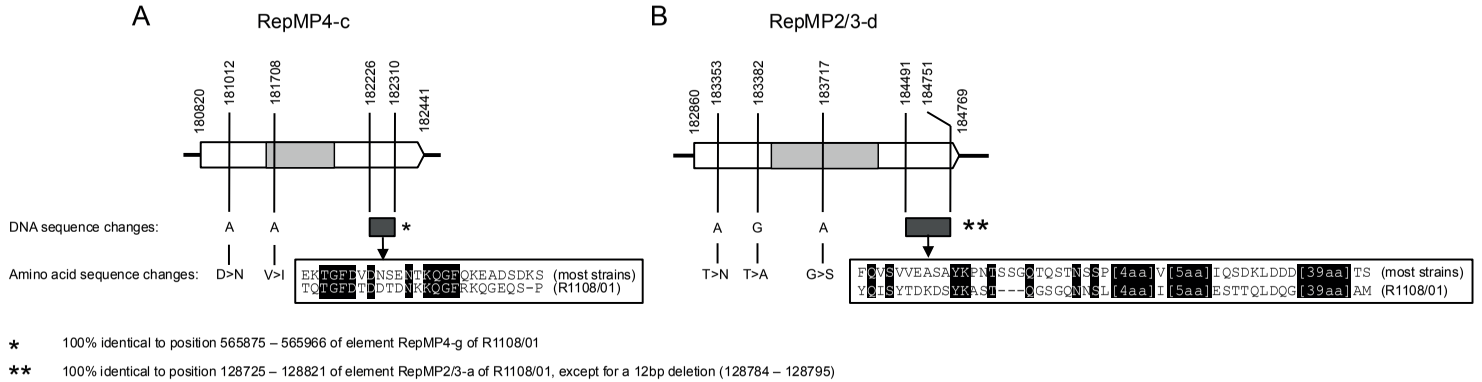

Fig. 3. Schematic representation of the structure of the RepMP4-c element (A) and RepMP2/3-d element (B) wihin the MPN141 gene of strains R1108/01 and R0109/01. The arrow bars represent the RepMP4-c and RepMP2/3-d elements as sequenced in this study. Since a complete genome sequence of a subtype 2 strain is not yet available, all nucleotide positions, indicated by vertical lines, are derived from the full genome sequence of *M. pneumoniae* subtype 1-strain M129 (GenBank file U00089.2). The grey areas in the arrow bars indicate the regions within the RepMP4-c and RepMP2/3-d elements that are rich in subtype-specific sequences; these regions have been analyzed before in detail (Spuesens, et al, 2009). Strains R1108/01 and R0109/01 have a subtype-2 signature and are identical to other subtype 2 strains except for the strain-specific SNPs indicated by the vertical lines. In addition, as illustrated by the black boxes, strains R1108/01 and R0109/01 contain rearrangements as compared to most other subtype 2 strains. A part of RepMP4-c is replaced by sequences from RepMP4-g (A) and a part of RepMP2/3-d is replaced by sequences from RepMP2/3-a (B). The latter DNA rearrangement is identical to that reported previously for *M. pneumoniae* strain 309 (Kenri, et al, 1999). The amino acid sequences of the putative P1 proteins from strains R1108/01 and R0109/01 that differ from the P1 sequences of other subtype 2 strains are detailed at the bottom of the figure. The insets show the amino acid (aa) sequence changes that result from the described DNA rearrangements. In both insets, the upper sequence is the one found in most subtype 2 strains, whereas the lower sequence is the one found in both R1108/01 and R0109/01. Black boxes in the alignments indicate identical amino acids.

unambiguously by these assays. Both the MPN141 assay and MPN528a assay were found to have a similar lower detection limit as the ML-resistance assays described above (5×10^2 *M. pneumoniae* genome copies/ml sample).

DNA template requirements for pyrosequencing. The results described above were obtained after amplification and pyrosequencing of DNA purified form *M. pneumoniae* cultures. To investigate whether the assays could also be performed directly on colonies or liquid cultures of *M. pneumoniae* without prior DNA purification, the following experiments were performed. Colonies of *M. pneumoniae* grown on PPLO agar were picked and resuspended in 100 µl of phosphate-buffered saline (PBS). In parallel, grown liquid cultures (4 ml) of bacteria were centrifuged and the bacterial pellets were resuspended in 100 µl PBS. Then, five µl of a $1/10^4$ dilution of these suspensions was included directly in the four different pyrosequencing PCR reactions. An amplicon was obtained in each PCR assay for each bacterial suspension. The pyrosequencing results obtained with these amplicons were highly reliable, and were of similar quality as the results obtained after PCR and sequencing of purified *M. pneumoniae* DNA (data not shown). The pyrosequencing assays can therefore be performed directly on diluted colonies or diluted broth of *M. pneumoniae* without the need for DNA purification.

The identification of isolates carrying novel MPN141 rearrangements. As described above, the five novel clinical isolates that were investigated in this study were each readily subtyped by the pyrosequencing assays. For three of these strains (the subtype 1 strains), the typing data corresponded with those obtained by PCR-RFLP (Table 1). However, for the other two strains (R1108/01 and R0109/01), which were classified as subtype 2 strains by pyrosequencing, an aberrant PCR-RFLP banding pattern was found. These strains could therefore not be classified correctly by PCR-RFLP. Because the aberrant PCR-RFLP results indicated that these strains harbored variations in their RepMP4-c and RepMP2/3-d elements (the RepMP elements located in the MPN141 gene), these elements were completely sequenced and compared to all known RepMP elements by BLAST analysis. While the RepMP4-c elements and RepMP2/3-d elements of both strains clearly contained *M. pneumoniae* subtype 2-specific sequences, novel MPN141 sequences were found between position 182,226 and 182,310 within RepMP4-c and between position 184,491 and 184,751 within RepMP2/3-d. BLAST analysis of the 'novel' sequence within RepMP4-c revealed this sequence to be 100% identical to part of the RepMP4-g element of subtype 2 strains. The 'novel' sequence within RepMP2/3-d was found to be 100% identical, apart from a 12-bp deletion, to sequences from the RepMP2/3-a element of subtype 2 strains [14]. These results indicated that the RepMP2/3-d element and RepMP4-c element of strains R1108/01 and R0109/01 were both rearranged as a consequence of homologous DNA recombination. Interestingly, the rearrangement within the RepMP2/3-d element of these strains was found to be identical to that reported for strain 309, which was previously described as a '2a' strain [11].

The structures of the RepMP4-c and RepMP2/3-d elements of strains R1108/01 and R0109/01 are shown schematically in Fig. 3. This figure also illustrates the consequences of the rearrangements within these elements for the P1 amino acid sequences that are encoded by the MPN141 genes of strains R1108/01 and R0109/01. The introduction of RepMP4-g sequences within RepMP4-c resulted in 15 amino acid changes within a stretch of 25 amino acids of the P1 protein. As described before for strain 309, the changes in RepMP2/3-d resulted in modification of 29 out of 87 amino acids. Thus, the rearrangements within RepMP4-c and RepMP2/3-d have a major influence on the amino acid sequence of the P1 protein of strains R1108/01 and R0109/01.

To further investigate the nature of the RepMP rearrangements that occurred in strains R1108/01 and R0109/01, we determined the sequences of the donor elements that were hypothesized to be involved in these DNA rearrangements, i.e. the RepMP4-g and RepMP2/3-a elements. The sequences of these elements were found to be identical to those of the RepMP4-g and RepMP2/3-a elements from most other subtype 2 strains, as determined previously [14]. Thus, the novel, aberrant sequences within the MPN141 genes of both isolate R1108/01 and R0109/01 are hypothesized to be the result of unidirectional homologous DNA recombination events. Based on the classification scheme of Spuesens et al. [14], the two strains with the novel MPN141 structure, which were both isolated in Rotterdam, The Netherlands, can be classified as 2-P1(4-c[g]c; 2/3-d[a]d) strains.

DISCUSSION

In this study, we have demonstrated that pyrosequencing is highly useful for the genetic analysis of *M. pneumoniae*. Specifically, we showed that pyrosequencing can have two important diagnostic applications, i.e. (i) the detection of mutations within the 23S rRNA gene that are known to be involved in ML resistance of *M. pneumoniae*, and (ii) the molecular typing (or subtyping) of *M. pneumoniae* isolates. The main advantages of pyrosequencing as opposed to other sequencing protocols are that: (i) the technique is rapid; a regular pyrosequencing run will produce results within less than 15 min (irrespective of the number of samples, with a maximum of 96 samples per run), and (ii) only a very short DNA sequence is produced, which can easily be processed, in particular when handling a large number of samples. It is also important to note that up to 96 samples can be sequenced simultaneously on the PyroMark™Q96MD pyrosequencing platform and that different sequencing reactions can be analyzed in parallel using a microtiter plate format. In the current workflow of the microbiological diagnostic laboratory, the pyrosequencing assays will generally only be performed after a clinical specimen has been tested as *M. pneumoniae*-positive by both (real-time) PCR and culture. Clearly,

the pyrosequencing tests could also be applied directly on DNA purified form clinical samples. This, however, will require an increased sensitivity of the PCR reactions that precede the actual sequencing steps. Attempts to improve this sensitivity are currently underway.

Because ML-resistance is governed by specific mutations in the V domain of the 23S rRNA and is not influenced by, for instance, the inactivation and/or efflux of antibiotics, it is appropriate to apply molecular methods in the detection of these mutations. Previously, several of these methods have been reported to be suitable for the detection of these mutations within the *M. pneumoniae* genome. These techniques rely either on amplification and dideoxy chain termination sequencing of the entire 23S rRNA gene [2] or on PCR of relatively small parts of the 23S rRNA gene, followed by (high-resolution) melt curve analysis [3, 5, 9]. Although these techniques are useful, they have significant disadvantages. The major disadvantage of the dideoxy sequencing technique is that it is rather time-consuming and generates considerably more information than required. The drawback of melt curve analysis is that the data can be difficult to interpret, in particular in cases where novel mutations have emerged in the 23S rRNA gene. In contrast to these techniques, pyrosequencing is a convenient technique that generates unambiguous results, as the output is a short DNA sequence. Moreover, the evaluation and interpretation of the sequence data is performed directly by the PyroMark™ software, and does not require any interpretation by the operator of the pyrosequencing machine.

The 23S rRNA 1 and 2 pyrosequencing assays were designed such as to detect all mutations known to be involved in ML-resistance. These mutations are localized around positions 2065 and 2617 of the 23S rRNA gene. Although two separate pyrosequencing reactions are required to target these two regions, this is not a problem, because the PCR and pyrosequencing reactions were devised such as to employ a universal cycling and pyrosequencing protocol. In effect, these two assays only differ in some reaction parameters (Table 2), but can be executed in the same PCR and pyrosequencing runs. Another advantage of pyrosequencing is that it has the capacity to detect mutations at the target sites that differ from all known mutations. Thus, novel point mutations that may arise in the future will also be detected by the 23S rRNA 1 and 2 assays.

The recent characterization of all RepMP4 and all Rep2/3 elements in 23 different *M. pneumoniae* isolates has demonstrated that the differences between subtype 1 and 2 strains are reflected in the sequences of each of these elements [14]. Moreover, these differences are also found outside of the RepMP elements in the *M. pneumoniae* genome, as inferred from the results of bacterial typing studies and the sequences of individual genes, such as the MPN528a gene described here. We found this gene to have a subtype-specific SNP that allowed us to differentiate between subtype 1 and subtype 2 isolates. This SNP was therefore found to be an ideal target for a pyrosequencing assay aimed at discrimination between the two *M. pneumoniae* subtypes. Another pyrosequencing

assay that we designed, the MPN141 assay, was similarly able to make the distinction between subtype 1 and 2 isolates; this assay was directed at a subtype-specific SNP within a conserved part of the MPN141 gene. The results obtained with both assays correlated with subtyping data obtained using other, conventional molecular methods. Both the MPN528a assay and MPN141 assay can therefore be used individually in order to conveniently determine the subtype of *M. pneumoniae* isolates.

It is obvious that the MPN528a and MPN141 subtyping assays are not aimed at the identification of rearrangements of RepMP elements, which may give rise to sequence variations in the RepMP elements of the MPN141 gene. However, as any of the 8 RepMP4 elements and any of the 10 RepMP2/3 elements have the capacity to recombine with their counterparts within the MPN141 gene, it is practically unfeasible to design a simple assay aimed at the identification of all potential variations within the MPN141-localized RepMP elements. These variations can only be identified unambiguously by sequencing the complete RepMP4 and RepMP2/3 elements within the MPN141 gene (RepMP4-c and RepMP2/3-d, respectively), as discussed previously [14]. Again, it is important to emphasize that the differentiation between *M. pneumoniae* isolates on the basis of their subtype should be distinguished from the discrimination between isolates on the basis of differences within their MPN141-localized RepMP elements [13]. This notion was stressed again by the finding of two isolates with a novel MPN141 structure. While these isolates were readily identified as subtype 2 strains by the MPN528a and MPN141 pyrosequencing assays, they were found to have an aberrant structure of the MPN141 gene as determined by a PCR-RFLP technique targeted at the MPN141 gene. Both strains (R1108/01 and R0109/01) were found to have a novel organization within the RepMP4-c element and a known variation ('2a') of the RepMP2/3-d element of the MPN141 gene. While the RepMP4-c element had acquired sequences derived from element RepMP4-g, the RepMP2/3-d element contained sequences derived from element RepMP2/3-a [14]. This is the first time that RepMP4-g sequences are reported to be involved in DNA rearrangement events in *M. pneumoniae*. This rearrangement further represents the second homologous recombination event ever observed in any RepMP4 element. The first isolate that was identified carrying an 'aberrant' sequence within element RepMP4-c was strain Mp3896 [12], in which sequences from the RepMP4-h element were likely transferred to RepMP4-c [12, 14].

The discovery of two strains with yet another, unique configuration of the MPN141 gene once again demonstrates that the exchange of homologous DNA can occur between any two RepMP elements within the *M. pneumoniae* genome. It is therefore likely that novel genotypes will continue to emerge due to inter-RepMP recombination events. Consequently, it is not sufficient that assays directed at the determination of the 'P1 genotype' are only capable of detecting known genotypes. Regarding the novel recombination events in isolates R1108/01 and R0109/01, it is important to mention

that these events appear to be the result of a unidirectional DNA recombination event, such as gene conversion. This observation is in line with all other RepMP recombination events that have previously been analyzed, which were found to be the result of a unidirectional transfer of genetic information [11, 14].

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Part 2

Clinical aspects of *Mycoplasma pneumoniae*

Chapter 7

Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: a cross-sectional study

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ABSTRACT

Background

Mycoplasma pneumoniae is thought to be a common cause of respiratory tract infections (RTIs) in children. The diagnosis of *M. pneumoniae* respiratory tract infections currently relies on serological methods and/or the detection of bacterial DNA in the upper respiratory tract (URT). It is conceivable, however, that these diagnostic methods also yield positive results if *M. pneumoniae* is carried asymptomatically in the URT. Positive results from these tests may therefore not always be indicative of a symptomatic infection. The existence of asymptomatic carriage with *M. pneumoniae* has not been established. We hypothesized that asymptomatic carriage in children exists and investigated whether colonization and symptomatic infection could be differentiated by current diagnostic methods.

Methods and Findings

This study was conducted at the Erasmus MC-Sophia Children's Hospital and the out-of-hours General Practitioners Cooperative (GPC) in Rotterdam, The Netherlands. Asymptomatic children (n = 405) and 321 children with RTI symptoms aged 3 months-16 years were enrolled in a cross sectional study from July 2008 – November 2011. Clinical data, pharyngeal and nasopharyngeal specimens, and serum samples were collected. The primary objective was to differentiate between colonisation and symptomatic infection with *M. pneumoniae* by current diagnostic methods, especially real-time PCR. *M. pneumoniae* DNA was detected in 21.2% (95% CI 17.2% - 25.2%) of the asymptomatic children and in 16.2% (95% CI 12.2% - 20.2%) of the symptomatic children (p = 0.11). Neither serology nor quantitative PCR or culture differentiated asymptomatic carriage from infection. A total of 202 children were tested for the presence of other bacterial and viral pathogens. Two or more pathogens were found in 56% (63/112) of the asymptomatic children and in 55.5% (50/90) of the symptomatic children. Finally, longitudinal sampling showed persistence of *M. pneumoniae* in the URT for up to four months. Fifteen of the 21 asymptomatic children and 19 of the 22 symptomatic children in this longitudinal follow-up tested negative after one month.

Conclusions

Although our study has limitations such as a single study site and limited numbers, our data clearly indicate that the presence of *M. pneumoniae* in the URT is common in asymptomatic children. The current diagnostic tests for *M. pneumoniae* are unable to differentiate between asymptomatic carriage and symptomatic infection.

INTRODUCTION

Mycoplasma pneumoniae is considered a major cause of upper and lower respiratory tract infections (RTIs) and disease (RTD) in humans, and particularly in children. Over one third of the childhood cases of community-acquired pneumonia that require hospitalisation are thought to be caused by *M. pneumoniae* [1, 2]. The current diagnosis of *M. pneumoniae* infections relies on the detection of either serum antibodies against *M. pneumoniae* or bacterial DNA in samples of the upper respiratory tract (URT) as recommended in the guidelines published by the British Thoracic Society and the Infectious Disease Society of America [1, 2].

PCR based methods are increasingly used in daily clinical practice, as well as in clinical studies for the detection of *M. pneumoniae*, because they provide fast and sensitive results in the acute phase of an infection [3-5]. However, RTD caused by other common bacterial pathogens (such as *Streptococcus pneumoniae*) cannot yet be diagnosed by PCR, because these pathogens are asymptomatically carried in the human population at high rates. Likewise, if *M. pneumoniae* would be commonly carried asymptomatically in the URT of children, the detection of this bacterial species may not indicate a symptomatic infection. This would have major implications for the interpretation of the results of current diagnostic methods for *M. pneumoniae* RTIs and their use in clinical management. Clinical management of *M. pneumoniae* RTI in children mainly consists of administration of macrolides, because *M. pneumoniae* is not susceptible to penicillins. In an increasing number of countries resistant strains of *M. pneumoniae* are rapidly emerging, and these are associated with prolonged disease. In Asia, up to 90% of *M. pneumoniae* derived from clinical samples is currently macrolide-resistant [6]. The frequent use of macrolides in children probably contributes significantly to the selection of macrolide-resistant strains [7]. Decreasing the use of macrolides by better (interpretation of) diagnostic methods might therefore help to prevent macrolide resistance.

In contrast to numerous published studies on carriage of *S. pneumoniae* in children, studies that specifically address asymptomatic carriage with *M. pneumoniae* have hitherto not been performed. To our knowledge, this is the first study in which the current state of the art diagnostic method for *M. pneumoniae*, i.e. PCR, is evaluated using a symptomatic and an asymptomatic group of children during a three years period of sampling. To our knowledge, this is also the first study to investigate the presence of *M. pneumoniae* in both symptomatic and asymptomatic children in a longitudinal fashion, including data on the occurrence of symptomatic infection during carriage. Although previous studies have reported the presence of *M. pneumoniae* in seemingly healthy individuals, these studies all suffered from drawbacks related to the study design (such as the lack of an appropriate control group and/or the lack of a follow-up study), or to limitations of the diagnostic assays that were employed [8-15]. As a consequence, clear

conclusions concerning the existence and dynamics of carriage with *M. pneumoniae* could thus far not be drawn. We hypothesized that asymptomatic carriage in children exists and investigated whether colonisation and symptomatic infection could be differentiated by current diagnostic methods. Secondary objectives of the present study were to assess the possible association between acute symptomatic *M. pneumoniae* infection and children's age, and to determine the influence of *M. pneumoniae* genotype as well as viral and bacterial co-infections on the severity of *M. pneumoniae* RTIs.

METHODS

Ethics statement

This cross-sectional study was approved by the Medical Ethics Review Board of the Erasmus MC (NL20418.078.08) and conducted at the Erasmus MC-Sophia Children's Hospital and the out-of-hours General Practitioners Cooperative (GPC) in Rotterdam, The Netherlands. Written informed consent was obtained from all parents and from children above the age of 12.

Study design and population

Study participants, aged 3 months to 16 years were enrolled between July 2008 and November 2011 in two groups. The first group, which will be referred to as 'the asymptomatic group', was enrolled during admission for a planned elective surgical procedure, unrelated to RTD at the short-stay department of the hospital. Exclusion criteria were a current respiratory tract infection (based on questionnaires and physical examination by the attending anaesthesiologist), the use of antibiotics in the past two days (seven days for azithromycin), and severe concomitant disease (e.g. chronic lung disease, cardiovascular disease, neoplasia, liver disease, kidney disease, metabolic disease or psychomotor impairment). Baseline characteristics (Table 1) and information about RTIs in the previous two months were recorded using a standardized questionnaire. Two study-team members collected respiratory and blood samples just prior to the start of the surgical procedure while the child was under general anaesthesia. Three to four weeks later, a questionnaire was completed by phone on development of respiratory tract infections in the weeks after enrolment.

In the second group, i.e. 'the symptomatic group', children diagnosed with RTI were enrolled at either the Emergency Department (ED) of the hospital or the out-of-hours GPC. Exclusion criteria were the same as for the asymptomatic group, except for having a current RTI. A standardized questionnaire was used to record baseline characteristics, clinical symptoms and diagnosis at discretion of the attending physician. Respiratory specimens and a capillary blood sample were collected. Three to four weeks later, a

Table 1. Baseline characteristics of 726 study participants

	Asymptomatic group	Symptomatic group		
	Total N = 405	Total N = 321	ED n = 131	GPC n = 190
Age	5.17 (4.76)	2.65 (3.48)	2.18 (3.38)	2.97 (3.50)
Female	137 (34.1)	155 (48.0)	58 (44.3)	95 (50.0)
Immunisation	383 (96.0)	307 (95.3)	124 (95.4)	183 (96.3)
Parental Smoking	145 (36.4)	132 (41.1)	42 (31.8)	90 (47.6)
Family size ≥ 5	163 (39.6)	74 (23.2)	33 (25.2)	41 (21.6)
Daycare attendance	121 (30.5)	160 (50.5)	75 (58.1)	86 (45.7)
RTI prior	129 (31.5)	NA	NA	NA
LRTI	NA	64 (20.4)	38 (29.5)	26 (14.1)
Pneumonia	NA	35 (10.9)	27 (20.6)	8 (4.2)
Hospitalisation	NA	41 (13.5)	32 (25.2)	9 (5.1)

Data are mean (SD) or n (%). (RTI Respiratory tract infection, ED Emergency Department, GPC General Practitioner Cooperative, AHR Airway hyperresponsiveness, LRTI Lower respiratory tract infection (including pneumonia), NA Not applicable). Immunisation refers to the national immunisation program in the Netherlands.

second capillary blood sample was collected and information was recorded about the duration of the RTI, treatment and hospitalisation. Children who tested positive for *M. pneumoniae* by PCR were invited to participate in a longitudinal follow-up study, from August 2010 to November 2011. After informed consent was obtained, each child was tested monthly for the presence of *M. pneumoniae* in the URT until the test was negative on two consecutive occasions.

Study procedures

From each child, a pharyngeal swab (BD, BBL™ CultureSwab™ EZ) was taken by gently stroking between the palatine arches superior to the tonsils. The swab was stored in phosphate-buffered saline. Subsequently, two nasopharyngeal specimens were taken. First, a flexible swab (Copan) was inserted into one nostril, guided to the posterior nasopharyngeal wall, removed and stored in Amies transport medium. Second, one millilitre of normal saline was instilled into each nostril and then suctioned by a flexible catheter applied to a container. The catheter was rinsed with two millilitres of normal saline. Specimens were kept at 4°C until further preparation within hours of collection.

To prevent contamination of samples, we took the following precautions. First, the two members of the study-team who performed all procedures tested *M. pneumoniae* PCR-negative throughout the study period. Second, samples were prepared and tested in different subunits of the laboratory. In a 'nucleic acid-free' laboratory, the samples were divided in aliquots used for culture, nucleic acid isolation and storage. In other

laboratory subunits, samples were either cultured or used for nucleic acid isolation. Each step in the PCR procedure, i.e. the preparation of real-time PCR premixes, the addition of purified nucleic acids to these premixes, and the actual PCR reactions, were carried out in different laboratories. Finally, each PCR run contained positive and negative controls.

Detection of *M. pneumoniae* in the URT was performed by real-time PCR and culture on both the pharyngeal swabs and the nasopharyngeal washings. DNA isolation was performed on 200 µl of the original samples using the QIAamp DNA mini kit (QIAGEN). A quantitative real-time (TaqMan®) PCR assay was used to detect and quantify *M. pneumoniae* genomic DNA, as described [16]. Adequate negative control samples were included in each PCR run. Culture was performed using 100 µl of the original sample [17]. Molecular (sub)typing of *M. pneumoniae* was performed on *M. pneumoniae*-positive samples, using a pyrosequencing-based assay [16]. Rest material was stored at -80°C until further use.

Serum was stored at -80°C. Detection of anti-*M. pneumoniae*-specific antibodies was performed using Serion ELISA *classic M. pneumoniae* kits (Clindia Benelux).

The nasopharyngeal swabs were used for the detection of *S. pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*, following standard microbiological procedures [18].

The batch-wise detection of viral nucleic acids was performed after the enrolment was closed. As all samples were stored at -80°C, selected samples were thawed. For the detection of viral nucleic acids, 60 µl of the original sample from a pharyngeal swab was diluted 10-fold with Dulbecco's Modified Eagle Medium to a total of 600 µl. Internally controlled nucleic acid extraction (input volume 200ul, output volume 100ul), subsequent (multiplex) real-time PCRs and its quality control were performed as described before [19]. A cycle threshold below 40 was considered a positive result. These viruses were determined in all *M. pneumoniae* PCR-positive children and in *M. pneumoniae* PCR-negative children matched with respect to month and year of sampling as well as age. Of 30 *M. pneumoniae*-positive participants, material was unavailable and could therefore not be included in this analysis.

Outcome variables and statistical analyses

All data were analysed using software package SPSS version 16.0.1. The protocol-defined objectives were: (1) to determine the distribution of genomic copy loads in both the asymptomatic group and the symptomatic group to differentiate carriage from infection and (2) to assess age distribution of the presence of *M. pneumoniae* in both symptomatic children and asymptomatic children, and to determine the influence of *M. pneumoniae* genotype as well as viral and bacterial co-infections on the severity of *M. pneumoniae* RTIs.

The prevalence of a positive test result for *M. pneumoniae* was calculated in both groups and compared using the χ^2 test. Groups were compared for the distribution of bacterial loads (genomic copy number) using the Mann-Whitney U test. P-values below 0.05 were considered significant. Within the symptomatic group, a child was defined as having a *M. pneumoniae* infection when at least one of the samples from this child was found to be *M. pneumoniae*-positive by serology, culture or PCR. Within the asymptomatic group, *M. pneumoniae* carriage was defined by a *M. pneumoniae*-positive result obtained by either culture or PCR.

The children in the symptomatic group with a *M. pneumoniae* infection were divided into two groups carrying the two *M. pneumoniae* subtypes (i.e. subtype 1 and subtype 2), and into two groups according to the presence of co-infections. Diagnosis (upper or lower RTI) and hospitalisation were used as proxies for severity of disease. Groups were compared for these variables using the χ^2 test.

To identify possible factors that could determine the presence of *M. pneumoniae*, subgroup analyses were done for variables that could reasonably have an effect on *M. pneumoniae* prevalence including age, gender, season and year of enrolment, family size, active or passive smoking and day-care attendance. Additional variables in the symptomatic group were: diagnosis and hospitalisation. Additional variables in the asymptomatic group were: RTI prior to enrolment, or just following enrolment. These variables were entered in multiple logistic regression analysis regardless of their bivariate association. Because these were all exploratory analyses, we did not adjust for multiple comparisons. To test for collinearity we calculated condition indices (CI) for the multivariate analyses. We found a CI of 9.9 for the asymptomatic group and a CI of 12.1 for the symptomatic group. Because both values are below the generally accepted warning signal of 15, and there were no strong correlations between the covariates, we believe that multicollinearity was not a major problem in this study.

To allow the inclusion of a sufficiently high number of *M. pneumoniae*-infected children below the age of five, we used a pre-calculated sample size of 400 children ≤5 years and 100 children >5 years in the symptomatic group based on an estimated prevalence of *M. pneumoniae* of 10%. In the asymptomatic group we took the same number. After enrolment of a total of 412 children in the asymptomatic group, it was apparent that the study question on the existence of carriage could be addressed. This was due to a higher prevalence of *M. pneumoniae* than anticipated in the asymptomatic group, and in the symptomatic group. Because additional enrolments would not have a significant influence on these outcomes of the study, it was deemed unethical to subject additional children to the study and the enrolment was subsequently discontinued.

RESULTS

726 children, aged 3 months to 16 years, were enrolled in this study from July 2008 until November 2011 (Figure 1 and 2). 405 children were enrolled in the asymptomatic group. Enrolment for this group started in January 2009. In the symptomatic group, 321 children were enrolled. On the basis of age, sex or time of enrolment of study participants, there were no differences between the group for which consent was given and the group for which consent was not given (Table S1).

We found that the prevalence of *M. pneumoniae* by real-time PCR did not differ significantly (p = 0.11) between the asymptomatic group (21.2%, 95% CI 17.2% - 25.2%, n=85) and the symptomatic group (16.2%, 95% CI 12.2% - 20.2%, n=51). A significant difference between the groups was also not found by culture; 4 (1.0%, 95% CI 0.03% - 1,97%) of the asymptomatic children and 5 (1.6%, 95% CI 0.23% - 2.97%) of the symptomatic children were *M. pneumoniae*-positive (p = 0.52). In the symptomatic group, the prevalence of *M. pneumoniae* by real-time PCR did not differ significantly (p = 0.85) between the children with a lower RTI (15.6%, 95% CI 11.6% - 19.6%, n=10) and the children with an upper RTI (15.9%, 95% CI 11.9% - 19.9%, n=41).

In the asymptomatic group, multiple logistic regression analysis showed that season and year of enrolment were significantly related to prevalence of *M. pneumoniae* (Table 2). In the symptomatic group, we found the presence of *M. pneumoniae* to be positively associated with enrolment in 2010 and 2011. As shown in table 3 none of the variables were independently related to the prevalence of *M. pneumoniae*. The presence of *M. pneumoniae*

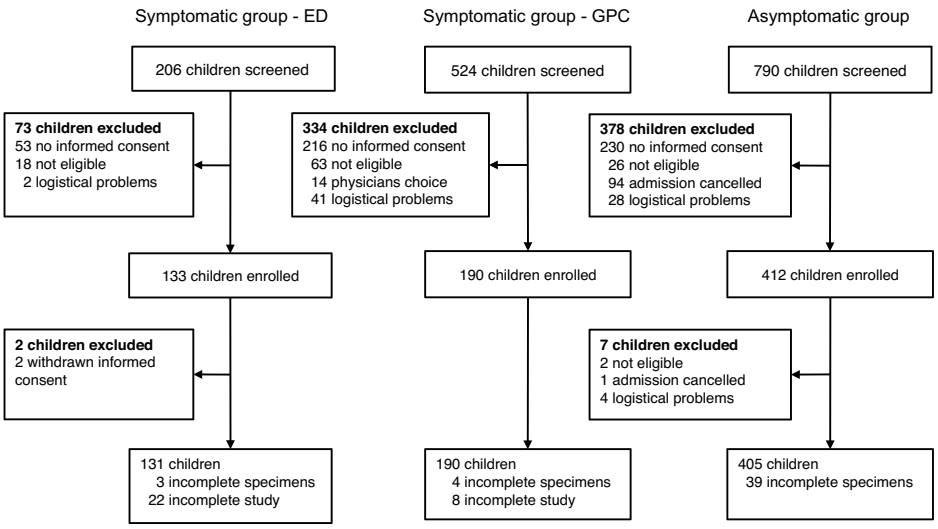

Figure 1. Enrolment flow diagram
Emergency Department (ED). General Practitioners Cooperative (GPC).

was not significantly associated with age or asthma-like symptoms (Tables 2 and 3). The two subtypes of *M. pneumoniae* were equally distributed between the two groups (Table S4).

A similar distribution of *M. pneumoniae* DNA loads was observed among the asymptomatic and symptomatic children (Figure 3A and B). We did not find a significant correlation between bacterial load in nasopharyngeal and pharyngeal samples (Figure 3C). The distribution of bacterial loads was different among children with a lower RTI and children with an upper RTI (Figure 3D and 3E). However, both very high and very low bacterial loads were detected in both subgroups. The study was not powered to perform a statistical subgroup analysis for this item.

To investigate how long *M. pneumoniae* can persist in the respiratory tract of children, a longitudinal follow-up study was performed. In this study, 43 (68%) of the 63 children who were eligible for inclusion participated in the follow-up study. Of these 43 children, 21 children originated from the asymptomatic group and 22 from the symptomatic group.

The most important reason not to participate in the follow-up study was distance from home to the study site (10/20 (50%)). 15 of the 21 (71%) asymptomatic children and 19 of the 22 (86%) symptomatic children in this longitudinal follow-up study tested negative after one month. Six of the asymptomatic children also tested positive at 2 months and 2 children also tested positive at 3 months. Three of the symptomatic children tested positive at 2 months and none tested positive at 3 months (Figure 4). To confirm their negative status, children were tested an additional time after becoming negative.

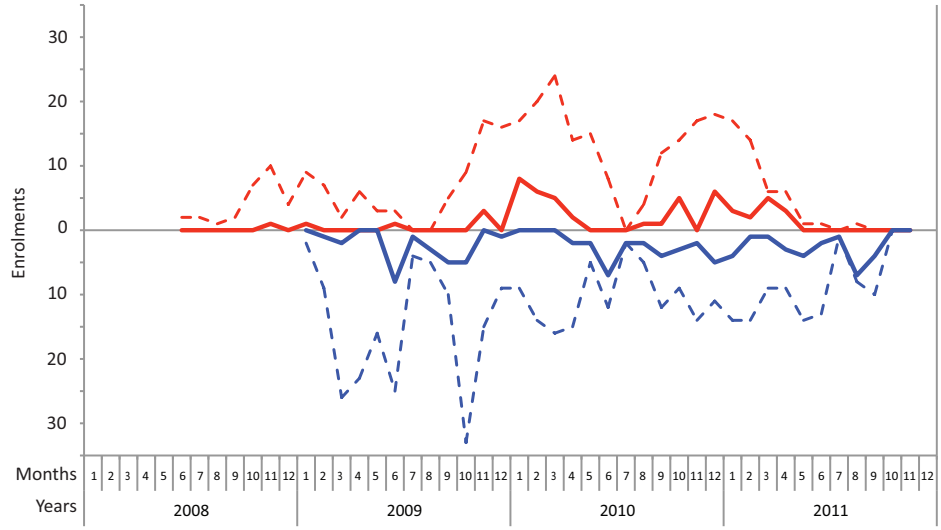

Figure 2. Monthly enrolments during the course of the study
The enrolments for the symptomatic group are represented in the upper panel of the figure by a red dotted line. The enrolments for the asymptomatic group are represented in the lower panel by a blue dotted line. The straight lines in the respective panels represent the absolute number of *M. pneumoniae* positive participants. Enrolment for the asymptomatic group started in January 2009.

Table 2. Results from the multiple logistic regression analysis for a positive *M. pneumoniae* PCR in the asymptomatic group

	Odds ratio (95% CI)	P-value
Age (≥ 5)	0,96 (0,47-1,96)	0,91
Gender (girl)	1,44 (0,81-2,56)	0,22
Season		<0,001 (overall)
Spring vs. Winter	0,81 (0,33-1,97)	0,64
Summer vs. Winter	7,43 (3,09-17,85)	<0,001
Autumn vs. Winter	2,90 (1,26-6,70)	0,01
Year (2009 versus 2010 and 2011)	3,31 (1,75-6,27)	<0,001
Family size (≥ 5 family members)	1,55 (0,77-3,13)	0,22
Smoking (active or passive)	0,70 (0,38-1,26)	0,23
Presence or history of wheezing	2,30 (0,69-7,61)	0,17
Daycare attendance	0,82 (0,38-1,77)	0,62
RTI prior enrolment	0,95 (0,50-1,79)	0,86
RTI post enrolment	0,59 (0,29-1,20)	0,15

RTI Respiratory tract infection. The variable 'Immunisations' was not entered in the regression analysis because the vast majority of the children were immunized (> 95%).

Table 3. Results from the multiple logistic regression analysis for a positive *M. pneumoniae* PCR in the symptomatic group

	Odds ratio (95% CI)	P-value
Age (≥ 5 years)	1,56 (0,604-4,02)	0,36
Gender (girl)	0,93 (0,46-1,87)	0,84
Season		0,87 (overall)
Spring vs. Winter	0,80 (0,35-1,81)	0,59
Summer vs. Winter	0,54 (0,11-2,67)	0,45
Autumn vs. Winter	0,85 (0,39-2,16)	0,74
Year (2009 versus 2010 and 2011)	5,80 (1,94-17,35)	0,002
Family size (≥ 5 family members)	1,63 (0,74-3,61)	0,23
Smoking (active or passive)	0,61 (0,30-1,23)	0,17
Presence or history of wheezing	1,96 (0,93-4,13)	0,08
Daycare attendance	0,84 (0,37-1,89)	0,67
Diagnosis (LRTI)	1,05 (0,46-2,42)	0,91
Hospitalisation	1,54 (0,52-4,60)	0,44

LRTI Lower respiratory tract infection. The variable 'Immunisations' was not entered in the regression analysis because the vast majority of the children were immunized (> 95%). Symptoms and signs were not entered in the regression analysis because these are represented in the variable 'Diagnosis'.

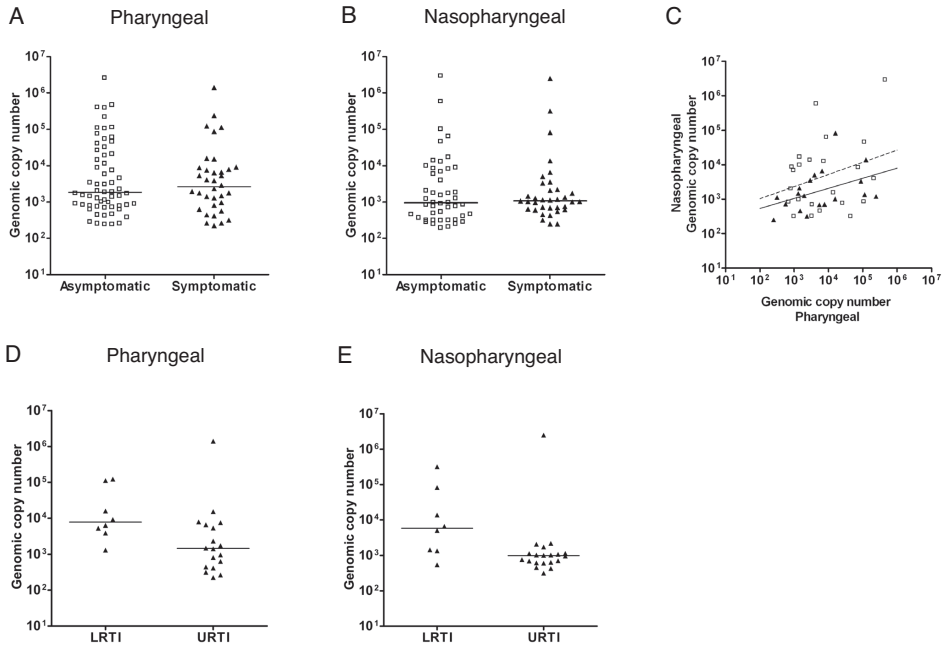

Figure 3. *M. pneumoniae* DNA loads
In panel A the pharyngeal bacterial loads (Genomic copy number per millilitre on the y-axis) of *M. pneumoniae* PCR-positive participants in the asymptomatic group (indicated by open squares) and the symptomatic group (indicated by filled triangles) are represented. In panel B the nasopharyngeal bacterial loads (Genomic copy number per millilitre on the y-axis) of *M. pneumoniae* PCR-positive participants in the asymptomatic group and the symptomatic group are shown. The bacterial load distribution was compared using the Mann-Whitney U test. Panel C shows the comparison of the bacterial loads in pharyngeal samples and nasopharyngeal samples for the participants that were positive in both. Correlation was calculated using the Spearman rank test. In panel D and E, the distribution of bacterial loads is shown for upper and lower RTIs in the pharyngeal and nasopharyngeal samples. The horizontal line in each graph represents the median.

We found that the prevalence of a positive ELISA for anti-*M. pneumoniae* IgM antibodies was not significantly different in the asymptomatic group (12.6%, 95% CI: 9.4% - 15.8%, n=43) and the symptomatic group (9.2%, 95% CI: 6.0% - 12.4%, n=26) (p = 0.23). The prevalence of a positive ELISA for anti-*M. pneumoniae* IgG antibodies differed significantly (p <0.001) between the asymptomatic group (25.1%, 95% CI: 20.9% - 29.3%, n=85) and the symptomatic group (14.2%, 95% CI: 10.4% - 18.0%, n=40). However, when adjusted for age, there was no significant difference in the prevalence of anti-*M. pneumoniae* antibodies between the two groups. The prevalence of a positive ELISA for anti-*M. pneumoniae* IgM and IgG was low among children below the age of five (7.3%, 95% CI: 3.5% - 11.1% and 8.3%, 95% CI: 4.3% - 12.3%) in the asymptomatic group vs. 6.7%, 95% CI: 0.0% - 13.4% and 5.0%, 95% CI: 0.0% - 5.9% in the symptomatic group) and much higher among children above the age of five (20.4%, 95% CI: 14.1% - 26.7% and 50.7%, 95% CI: 42.8% - 58.5%

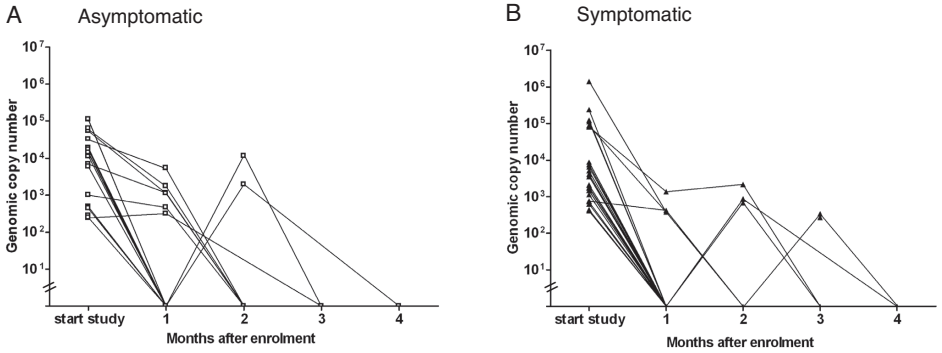

Figure 4. *M. pneumoniae* DNA loads in the longitudinal study.
The bacterial DNA loads in the study participants during the follow-up study. In panel A, each open square represents one visit of one participant and is connected by a line to the square representing the next visit. On the y-axis the bacterial DNA load (Genomic copy number per millilitre) is shown. On the x-axis the consecutive visits are represented. Panel B shows the bacterial DNA loads for the symptomatic group. Each filled triangle represents one participant on one single time-point.

in the asymptomatic group and 11.9%, 95% CI: 7.7% - 16.1% and 47.6%, 95% CI: 41.1% - 54.1% in the symptomatic group).

The median levels of IgM- and IgG-antibodies did not differ significantly between the two groups (Figures 5A and 5B). The prevalence of a positive ELISA for anti-*M. pneumoniae* IgA antibodies was very low in both groups (2.0%, 95% CI 0.6% - 3.4% vs.

Table 4. Agreement between PCR and serology

	Asymptomatic			Symptomatic		
	PCR		Kappa	PCR		Kappa
	Positive	Negative		Positive	Negative	
Serology IgM						
Positive	11	32		7	13	
Negative	55	242	0.06	40	215	0.12
Serology IgA						
Positive	4	3		0	1	
Negative	62	271	0.08	46	224	-0.01
Serology IgG						
Positive	16	68		6	25	
Negative	50	203	-0.01	41	202	0.02
Immunoglobulin class switch						
Positive	NA	NA		7	19	
Negative	NA	NA		31	176	0.10

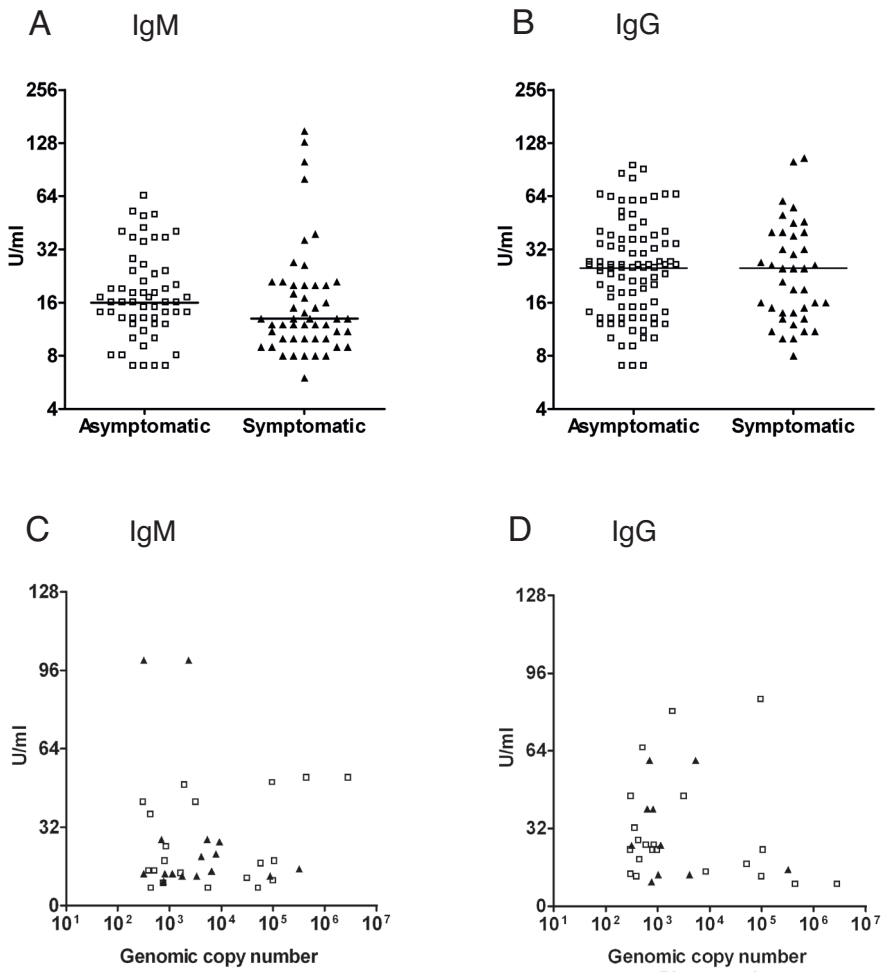

Figure 5. Anti-*M. pneumoniae* serum antibody levels.
Serum IgM (panel A) and IgG (panel B) antibody levels (in U/ml) are compared between the asymptomatic group and the symptomatic group (using the Mann-Whitney U test). In panel C and D, IgM and IgG antibody levels, respectively, are plotted against the bacterial DNA load (Genomic copy number per millilitre) in all samples. Open squares indicate asymptomatic participants. Filled triangles indicate symptomatic participants. The horizontal line in each graph represents the median.

0.4%, 95% CI 0.0% - 1.1%), and none of the children below the age of five tested positive for anti-*M. pneumoniae* IgA. The serological data did not correspond significantly with the PCR results (Figures 5C, 5D and Table 4). Interestingly, a higher percentage of asymptomatic PCR-positive children tested positive for IgG compared to symptomatic PCR-positive children (Table 4: 32% vs. 15%). However, this difference was not found to be statistically significant when adjusted for age.

Because an immunoglobulin class-switch from IgM to IgG is generally accepted as evidence for a recent *M. pneumoniae* infection, a second serum sample was collected from 233 (72.6%) of the symptomatic children. From this group, 26 (11.2 %) children developed an immunoglobulin class switch. The agreement between an immunoglobulin class switch and a positive PCR result was poor (Kappa = 0.10) (Table 4). Besides age, we did not find any determinants of an immunoglobulin class switch or a single positive test for IgM, IgG or IgA.

Bacterial and viral pathogens do co-exist in the respiratory tract and co-infection may influence severity of disease. Therefore, we determined the presence of 4 other bacterial respiratory pathogens in all children and the presence of 15 viral respiratory pathogens in a selection of the children (n = 202). Two or more pathogens were found in 56% (63/112) of the asymptomatic children and in 55.5% (50/90) of the symptomatic children. The prevalence of the four bacterial pathogens is shown in Table 5. As reported previously, the prevalence of these bacterial species was largely age-dependent [20]. We found a significant higher prevalence of *S. aureus* in the asymptomatic group compared to the symptomatic group. However, when adjusted for age this difference in prevalence was not significant. An association between the presence of any of these four bacterial species and *M. pneumoniae* was not detected. Almost all the viruses screened for were detected in both groups (Table 6).

We did not perform a statistical analysis to detect differences in the distribution of Ct-values since our study was not powered for this purpose. Rhinovirus, bocavirus and parainfluenzavirus 4 were detected more frequently in asymptomatic children than in symptomatic children. In contrast, Influenzaviruses A and B, hMPV and RSV were predominantly detected in symptomatic children. The majority of the children (from both groups) tested positive for more than one pathogen (Figure 6). Group-specific combinations of pathogens could not be identified. In addition, none of the viruses were associated with the presence of *M. pneumoniae*. Due to the limited number of *M. pneumoniae*-positive children without bacterial and/or viral co-infection it was not possible to analyse the influence of bacterial and viral co-infection on disease severity.

Table 5. Bacterial results in 714 study participants

	Asymptomatic	Symptomatic	P-value
Bacterium	N = 393	N = 321	
S. pneumoniae	109 (27.7)	87 (27.1)	0.92
S. aureus	84 (21.4)	32 (10.0)	<0.001*
M. cattharalis	71 (18.1)	74 (23.1)	0.12
H. influenzae	57 (14.5)	51 (15.9)	0.68

Data are n (%). The p-values compare the difference in prevalence between the two groups indicated by χ^2-test. $p<0.05$ is considered a significant difference (indicated by *).

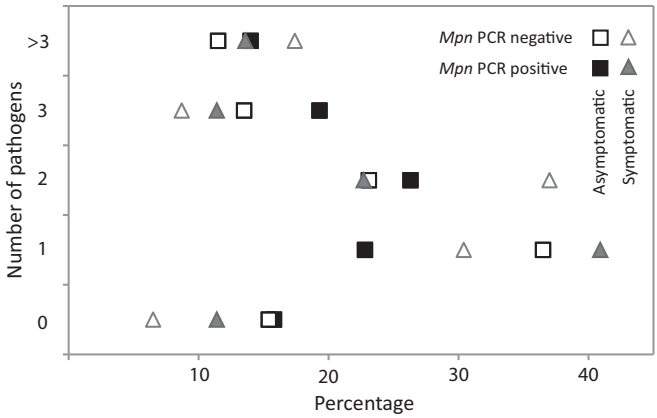

Figure 6. Number of detected viral and bacterial pathogens
The dot plot shows the percentages of participants with 0 to 3, or more than 3 pathogens present in the upper respiratory tract. On the x-axis the percentages are shown, on the y-axis the number of pathogens is shown. The filled and open triangles show respectively the *M. pneumoniae* (*Mpn*) PCR positive symptomatic children (n = 44) and the *M. pneumoniae* PCR negative symptomatic children (n = 46). The filled and open squares show respectively the *M. pneumoniae* PCR positive asymptomatic children (n =57) and the *M. pneumoniae* PCR negative asymptomatic children (n = 52).

Table 6. Virology results in 202 study participants

Virus	Asymptomatic		Symptomatic		
	Ct-value, Median (IQR)	N = 112	Ct-value, Median (IQR)	N = 90	P-value
Influenza A virus	20.1	1 (0.9)	23.0 (18.0-33.8)	6 (6.7)	0.03*
Influenza B virus	Undetectable	0 (0.0)	26.0	1 (1.1)	0.26
hMPV	36.7-38.7	2 (1.8)	24.6 (23.0-30.5)	7 (7.8)	0.10
RSV A	27.9	1 (0.9)	22.0 (20.0-29.6)	11 (12.2)	0.001*
RSV B	35.4	1 (0.9)	22.0 (17.3-29.5)	9 (10.0)	0.003*
Parainfluenzavirus 1	36.3 (22.0-37.6)	7 (6.3)	25.3 (20.0-26.5)	3 (3.3)	0.34
Parainfluenzavirus 2	35.8 (34.6-35.9)	3 (2.7)	31.1	1 (1.1)	0.43
Parainfluenzavirus 3	20.7, 35.8	2 (1.8)	30.6 (25.1-39.6)	3 (3.3)	0.48
Parainfluenzavirus 4	36.5 (31.9-37.4)	10 (8.9)	38.7	1 (1.1)	0.02*
Rhinovirus	26.5 (23.1-31.1)	35 (31.2)	24.7 (22.3-29.4)	17 (18.9)	0.04*
Coronavirus 229E	Undetectable	0 (0.0)	17.6	1 (1.1)	0.26
Coronavirus OC43	35.6 (28.7-37.3)	5 (4.5)	29.0 (24.1-32.9)	5 (5.6)	0.72
Coronavirus NL63	37.5 (34.8-38.6)	6 (5.4)	27.5 (27.0-38.0)	7 (7.8)	0.49
Bocavirus	30.9 (26.5-34.0)	16 (14.3)	32.0 (28.8-34.0)	3 (3.3)	0.008*
Adenovirus	31.9 (27.9-33.8)	17 (15.2)	27.0 (26.2-30.5)	9 (10.0)	0.28

Data are n (%). The p-values compare the difference in prevalence between the two groups indicated by χ^2-test. $p<0.05$ is considered a significant difference (indicated by *). IQR Inter-quartile range, Ct-value Cycle threshold value.

DISCUSSION

Statement principal findings

To our knowledge, our study demonstrates for the first time that *M. pneumoniae* is carried at high rates in the URT of healthy children, and that this asymptomatic carriage cannot be differentiated from symptomatic RTI by serology or quantitative PCR. 21% of 405 healthy children tested positive for *M. pneumoniae* in the URT by PCR. As a result of this high prevalence, the inclusion of children was terminated at an earlier time-point than anticipated at the start of the study. For now we can conclude that *M. pneumoniae* carriage is detectable and its prevalence is higher than expected, but the actual prevalence of carriage is unreliable. Prevalence varied between year and season of sampling from 3% during the spring of 2009 to 58% during the summer of 2010. These data suggested that carriage follows a cyclic epidemic pattern. It is tempting to speculate that this fluctuation in prevalence is related to the known cyclic epidemic pattern of *M. pneumoniae* infections that occurs at 3-7 year intervals, in addition to a background endemic pattern [4, 21]. Longitudinal sampling of *M. pneumoniae*-positive asymptomatic children indicated that *M. pneumoniae* can be present in the URT without causing disease, followed by clearance within several weeks.

While previous studies have demonstrated the presence of *M. pneumoniae* in seemingly healthy individuals, none of these studies could draw clear conclusions concerning the actual existence of carriage of *M. pneumoniae* [8-15]. Instead, some studies explained the presence of *M. pneumoniae* in an asymptomatic individual as a consequence of a recent infection with this bacterium [14, 15]. Other studies were inconclusive due to lack of a specific study design or due to the use of relatively insensitive diagnostic tools such as culture [9-12].

Strengths and weaknesses

Although we have demonstrated the existence of asymptomatic carriage with *M. pneumoniae* in children, there are several limitations to our study. These include the single study site at one city in the Netherlands and a limited sample size. Although the sample size was adequate to address our research questions, it is not large enough to unravel the dynamics of colonisation by *M. pneumoniae*. Furthermore, we performed exploratory analyses that show that *M. pneumoniae* prevalence is determined by season and year of sampling, although these analyses did not form part of the original design of the study. Finally, we have performed a longitudinal follow-up study that shows the persistence of *M. pneumoniae* in a small number of children for up to four months. Although this strongly suggests the existence of carriage, we only enrolled a small number of children in this part of the study. Future studies are needed to confirm our results and should aim at

finding determinants of *M. pneumoniae* carriage. These studies should preferably have a multicenter design.

Furthermore, we aimed to assess the association between severity of disease and *M. pneumoniae* subtype and genomic copy load. Although we did not find this association in the symptomatic group, it should be noted that *M. pneumoniae* subtype 2 positive participants were underrepresented overall (as shown in Table S4). This small number hampered the analysis of this secondary research question. It might also be argued that these results are negatively influenced by a general low severity of disease in our study population and the limited number of participants with lower RTI (20.9 % of all symptomatic children). Indeed, none of our children were admitted to an intensive care unit or required respiratory support by mechanical ventilation. Still, we did find a difference between asymptomatic and symptomatic children for well-established respiratory pathogens such as influenza A and RSV. We feel that this underlines the appropriateness of our study population with regard to severity.

In agreement with recent other studies on *M. pneumoniae* infections, a poor correlation was found between data obtained by PCR and serology [22, 23]. Given the excellent performance of the PCR assay in Quality Control for Molecular Diagnostics (QCMD) panels, and the validation of the commercial ELISA used, we are confident that the data obtained by both assays are reliable. It is possible that the positive serological results simply reflect one or more previous encounters with *M. pneumoniae* and are not necessarily related to a current RTI or carriage of *M. pneumoniae* as determined by PCR. However, we have not collected convalescent serum samples in the asymptomatic group, therefore we can only speculate on the prevalence of immunoglobulin class switch or the levels of convalescent antibodies in this group.

Meaning of the study

As the pathogenicity of *M. pneumoniae* has been well-documented in studies with volunteers in the 1950s [24], one can speculate that asymptomatic carriage of *M. pneumoniae* may in some cases lead to symptomatic infection as is well known for other pathogens [25]. Obviously, the finding of asymptomatic carriage of *M. pneumoniae* has major implications for the interpretation of the diagnosis of *M. pneumoniae* infections and its impact for clinical management, as well as the interpretation of studies on the aetiology of RTD in children. We searched Medline with the terms '*Mycoplasma pneumoniae*', 'respiratory tract infection', 'asymptomatic carriage' and 'diagnosis' and found that many studies and some reviews have addressed the performance and clinical value of different diagnostic methods for *M. pneumoniae*. It is striking, however, that these methods are used interchangeably and almost each positive result is regarded as indicative for a symptomatic *M. pneumoniae* infection. In addition, the occasional presence of *M. pneumoniae* in respiratory secretions of healthy individuals has often been explained either as the

first sign of a developing symptomatic infection or as bacterial persistence following symptomatic infection [3, 11, 26-28]. Current guidelines on community-acquired pneumonia recommend testing for *M. pneumoniae* in patients with a high pre-test probability [1, 2]. As we have shown, the available procedures for diagnosis of *M. pneumoniae* RTIs in children do not discriminate between carriage of *M. pneumoniae* in the respiratory tract and symptomatic *M. pneumoniae* infection. Therefore we may have to re-address the clinical significance of a positive test result.

Our data indicate that the aetiology of RTI in children is complex. The mere presence of one or more putative pathogens in the URT does not seem to be the sole determining factor in the development of a symptomatic RTI. The host immune response, the timing of colonization, the presence of other pathogens and the initial bacterial or viral load may collectively determine whether carriage proceeds to infection or not. Future studies will therefore have to focus on how an RTI can be accurately defined and whether or not it requires treatment.

Conclusion and future studies

The results of this study suggest that *M. pneumoniae* behaves similar to many other bacterial species in the respiratory tract. Future studies at different sites and in different populations are required to confirm our findings. This is important because these findings imply that the daily clinical practice of diagnosing *M. pneumoniae* RTI is not adequate. Specifically, it does not seem appropriate to use the detection of *M. pneumoniae* in the URT by PCR as a method to diagnose symptomatic RTIs caused by this bacterium. Thus, *M. pneumoniae*-induced RTD can not be based exclusively on serology or the detection of *M. pneumoniae* DNA in the URT, and caution should be taken in the interpretation of diagnostic tests for *M. pneumoniae*. Future studies should address this diagnostic challenge and aim at finding diagnostic tools that can differentiate carriage from infection as well as the factors that may determine progression from asymptomatic carriage of *M. pneumoniae* to symptomatic infection.

SUPPORTIVE INFORMATION (ONLINE AVAILABLE ON WWW.PLOSMEDICINE.ORG)

Table S1. This table shows the comparison between the children for which consent was given (enrolled in the study) and the children for which consent was not given.

Table S2. In this table the results are shown from the bivariate analysis in the asymptomatic group. The table shows the prevalence of *M. pneumoniae* as determined by PCR for the variables age, gender, immunisations, season of enrolment, year of enrolment, family

size, smoking, presence or history of wheezing, day-care attendance, respiratory tract infections prior to enrolment and respiratory tract infection in the month after enrolment.

Table S3. In this table the results are shown from the bivariate analysis in the symptomatic group. The table shows the prevalence of *M. pneumoniae* as determined by PCR for the variables age, gender, immunisations, season of enrolment, year of enrolment, family size, smoking, presence or history of wheezing, day-care attendance, symptoms present at the time of enrolment, diagnosis (presence of a lower respiratory tract infection) and hospitalisation.

Table S4. The distribution of the different genotypes (i.e. subtype 1 and 2) in the asymptomatic and symptomatic group is shown.

Text S1. Study protocol, online available.

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ABBREVIATIONS

ED Emergency Department, GPC General Practitioners Office, RTD Respiratory Tract Disease, RTI Respiratory Tract Infection, URT Upper Respiratory Tract.

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Chapter 8

Comparison of *Mycoplasma pneumoniae* genome sequences from strains isolated from symptomatic and asymptomatic patients

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ABSTRACT

Mycoplasma pneumoniae is a common cause of respiratory tract infections (RTI) in children. We recently demonstrated that this bacterium can be carried asymptomatically in the respiratory tract of children. To identify potential genetic differences between *M. pneumoniae* strains that are carried asymptomatically and those that cause symptomatic infections, we performed whole-genome sequence analysis of 20 *M. pneumoniae* strains. The analyzed strains included 3 reference strains, 3 strains isolated from asymptomatic children, 13 strains isolated from clinically well defined patients suffering from an upper (n=4) or lower (n=9) RTI and one strain isolated from a follow-up patient who recently recovered from an RTI. The obtained sequences were each compared to the sequences of the reference strains. To find differences between strains isolated from asymptomatic and symptomatic individuals, a variant comparison was performed between the different groups of strains. Irrespective of the group (asymptomatic versus symptomatic) from which the strains originated, subtype 1 strains formed a separated cluster from subtype 2 strains. We could not identify a specific genotype associated with *M. pneumoniae* virulence. However, we found marked genetic differences between clinical isolates and the reference strains, which indicates that the latter strains may not be regarded as appropriate representatives of circulating *M. pneumoniae* strains.

BACKGROUND

Mycoplasma pneumoniae is a human bacterial pathogen that has been estimated to cause pneumonia in up to 40% of children hospitalized because of community-acquired pneumonia [1]. A recent study has shown that this bacterium can also be carried asymptomatically: *M. pneumoniae* was detected in the upper respiratory tract of 21% of children that did not show any signs of a respiratory tract infection (RTI) [2]. The finding of asymptomatic carriage of a potential bacterial or viral pathogen is a well known, and common phenomenon in the general population. Other pathogens, such as *Streptococcus pneumoniae, Staphylococcus Aureus, and Rhinovirus*, can cause RTIs, but can also be carried asymptomatically. Clearly, it is important to identify the determining factors, both from pathogen and host, involved either in the control of an asympomatic carriage status or in triggering symptomatic infection. In the case of *S. pneumoniae*, specific bacterial gene clusters were found to be associated with invasive disease [3]. In contrast, genes associated with carriage and/or pathogenicity have not yet been identified in *M. pneumoniae*. Moreover, little is known about the genetic variability of clinical isolates of *M. pneumoniae* and the relationship between bacterial genotype and virulence.

Until a few years ago, only a single genome sequence was available for *M. pneumoniae*[4]. This sequence was derived from laboratory strain M129. Since 2013, several other genomic sequences have been published [5-7]. Most of these sequences were obtained in two independent studies. One of these studies reported the comparative genome analysis of 15 *M. pneumoniae* strains isolated form respiratory tract samples and cerebrospinal fluid samples collected from patients between 1940 and 2009 in the USA, China and England [5]. The other study reported the sequences of 23 clinical *M. pneumoniae* strains isolated between 1964 and 2011 in six different countries [6].

Despite the availability of a significant set of *M. pneumoniae* genome sequences, it is currently difficult to determine the association between *M. pneumoniae* genotype and virulence. This is mainly due to the fact that all known *M. pneumoniae* sequences were exclusively obtained from strains isolated from patients with RTI symptoms. Clearly, this precludes determination of the putative genetic differences between strains causing symptomatic infections and strains carried by asymptomatic individuals. The recent isolation of a set of *M. pneumoniae* strains from both asymptomatic children and children suffering from RTI [2], however, allows a direct comparison of the genetic make-up of strains associated with bacterial carriage and those involved in symptomatic infection. We therefore set out to determine the genome sequence of 3 reference strains (M129, FH and R003), 3 strains from asymptomatic individuals, and 13 strains from clinically well-defined patients suffering from either an upper or lower RTI. The analysis and comparison of the obtained sequences did not reveal a specific genotype associated with *M. pneumoniae* virulence. However, we did find striking differences between the genomes of clinical isolates and those of the *M. pneumoniae* reference strains.

METHODS AND MATERIALS

Patient samples

Most of the patient samples were collected as part of a clinical study designed to investigate the existence of *M. pneumoniae* asymptomatic carriage in children [2]. This study was carried out in Rotterdam, the Netherlands between 2008 and 2012. Patient information was collected and documented prospectively as part of the study. All *M. pneumoniae* culture positive samples from the different groups (asymptomatic, symptomatic, and follow-up group) were selected for analysis as part of this study. The other samples from symptomatic patients were collected by the Regional Laboratory of Public Health Kennemerland, Haarlem, The Netherlands, and kindly provided for analysis. These samples were taken from patients with suspected *M. pneumoniae* infection as part of the medical work-up ordered by the treating physician. All samples with a positive culture for *M. pneumoniae* were selected and used in this study. The Medical Ethics Review Board of

the Erasmus MC approved the study on asymptomatic carriage (NL20418.078.08) and written informed consent was obstained in this study from all parents and children above the age of 12 years. The Medical Ethics Review Board of the Erasmus MC approved the use of the samples collected during routine medical work-up in the Regional Laboratory of Public Health Kennemerland (MEC 2013-344).

Culture and DNA isolation

Culture was performed in the laboratory of pediatrics of the Erasmus MC, as previously described [8]. In short, 100 µl original sample and 10-fold dilutions were used for culturing. Culturing was performed in *Mycoplasma* medium containing 1.4% Difco™ PPLO broth (Becton Dickinson), 0.15% Difco™ TC Yeastolate, UF (Becton Dickinson), 1.4% glucose, 20% horse serum, 1,000 U/ml Penicillin G, 500 U/ml Polymyxine B, and 0.02 mg/ml phenol red. The pH of the medium was adjusted to 7.8-8.0 using a solution of 2 N NaOH, followed by filter-sterilization. Strains were grown in 3 ml of medium at 37°C/5% CO_2 in 25 cm^2 tissue culture flasks (Greiner). Cells were harvested upon color change of the medium (from red/orange to yellow). DNA was isolated form the harvested cells as described previously [9].

Sequencing, de novo assembly and scaffold alignment

The sequencing of all strains including re-sequencing of the reference strains was performed at the Center for Biomics of Erasmus MC using next-generation sequencing on a HiSeq2000 platform from Illumina. A paired-end 100-bp sequencing protocol was used. Between 0.9 and 2.5 gigabases of DNA sequence was generated for each of the isolates. Thus, a 1,224- to 3,300-fold genome coverage was obtained.

Reads were purged from the Illumina sequence adapter and renamed according to the standards expected by Abyss (Software Abyss 1.3.4). After the initial processing, Abyss was run for each of the samples individually. This resulted in files containing unitigs, contigs and scaffolds. The unitig files hold the assemblies that were generated without taking the pair information into account. The contigs are the assemblies generated with the pair information taken into account. Scaffolds are the contigs after being merged based on read pairs, and differ from the contigs as they may contain unresolved repeats. For all downstream analysis, the scaffolds with a minimum PHRED score of 30 were used (base call accuracy ≥ 99.9%).

After de novo assembly, potential open reading frames (ORFs) were identified by Glimmer 3.02 software, using standard parameters except for the codon translation table (which was adjusted for the *Mycoplasma*-specific genetic code). The predicted ORFs were aligned to the genes identified in the genome of *M. pneumoniae* reference strain M129. The ORFs that could not be aligned to the known genes were subjected to BLAST analysis using the NCBI non-redundant database.

In addition to the gene-based analysis, MUMmer 3.23 software was used to align the scaffolds to the *M. pneumoniae* M129 genome, to find unique and absent regions as well as structural rearrangements. Results of these analysis were further processed and compared using R 2.15.2

Variant comparisons

To discover differences in strains isolated from asymptomatic and symptomatic individuals, samples were arranged in 4 groups as depicted in Table 1. The three reference strains (Group 1) are *M. pneumoniae* M129 (subtype 1, ATCC 29342), *M. pneumoniae* FH (subtype 2, ATCC 15531) and *M. pneumoniae* R003 (subtype 2). The other groups consisted of strains isolated from asymptomatic children (Group 2), patients with an upper RTI (Group 3) and patients with a lower RTI (Group 4). Reads were mapped to the *M. pneumoniae* M129 reference genome (NCBI accession number NC_000912.1) using BWA 0.5.9.

RESULTS

Selection of strains and isolates of *M. pneumoniae*

We have determined the complete genome sequences of a total of 20 strains or isolates of *M. pneumoniae*. The isolates were obtained either from asymptomatic children (n=3), from patients with an upper RTI (URTI; n=4), or from patients with a lower RTI (LRTI; n=9). The names (ID) and origins of the isolates are listed in Table 1. Two of the isolates from the LRTI group were obtained at the same time from a single patient (C036-1 and C036-2). These two isolates were selected because they differed in colony morphology on agar plates. Two other samples from the LRTI group were also collected from a single patient, one at the moment of RTI (HAP157) and one collected 4 weeks later, after recovery of the clinical symptoms (HAP157FUP). In addition to the clinical isolates, three reference laboratory strains were included in this study, i.e. subtype 1 strain M129, and subtype 2 strains FH and R003.

DNA sequencing and de novo genome sequence assembly

The genomic sequences of the 20 *M. pneumoniae* strains were determined using a paired-end 100-bp sequencing protocol on the HiSeq2000 platform (Illumina). For each of the strains, genome assemblies were generated that ranged between 777 kb (HAP157) and 834 kb (HAP111) in length (Table 1). These genome lengths are similar to the published genome length of reference strain M129 (816 kb) [4]. For strain M129, which was also included in our study, the total amount of sequence contained in scaffolds larger than 200 bp was found to be ~17 kb lower than the previously published

genome size of this strain (Table 1). Thus, approximately 2% of the M129 genome could not be retrieved with the procedures used in this study. The sequences of the other strains could have similar gaps, because all but one of the sequences are smaller than 800 kb. It is likely that these gaps are caused by the significant number of repetitive sequences (RepMP sequences) in the genome of *M. pneumoniae,* which are known to pose problems for short read sequencers such as the Illumina HiSeq 2000.

Table 1. Sample ID, patient information and strain information.

Group	Strain/ patient ID	Diagnose group	Sequence ID	Size (bp) [a]	Subtype
1	M129	Reference	1	798,964	1
	FH	Reference	2	784,762	2
	R003	Reference	3	798,075	2
2	B174	Asymptomatic	11	796,334	1
	B247	Asymptomatic	12	797,815	1
	B406	Asymptomatic	14	788,600	2
3	A016	Upper RTI	4	799,326	2
	A058	Upper RTI	6	790,906	1
	C024	Upper RTI	15	793,805	1
	H030	Upper RTI	19	796,875	1
4	A035	Lower RTI	5	797,591	2
	A103	Lower RTI	7	780,444	1
	HAP111	Lower RTI	10	834,662	2
	HAP157	Lower RTI	9	777,116	1
	H010	Lower RTI	18	797,111	1
	H016	Lower RTI	20	797,503	1
	H026	Lower RTI	13	784,292	2
	C036-1[b]	Lower RTI	16	796,987	1
	C036-2[b]	Lower RTI	17	796,664	1
	HAP157FUP	Follow-up	8	796,422	1

[a] Total amount of sequence contained in scaffolds over 200 bp in length

[b] Morphologic different colonies of the same patient sample. Both a large (C036-1) and a small colony (C036-2) were sequenced.

Sequence differences between strains isolated from asymptomatic and symptomatic individuals

In order to compare the genome sequences of the different *M. pneumoniae* isolates, the sequences were subjected to hierarchical clustering analysis based on Euclidian distance (Figure 1). This analysis clearly distinguished two major families of strains,

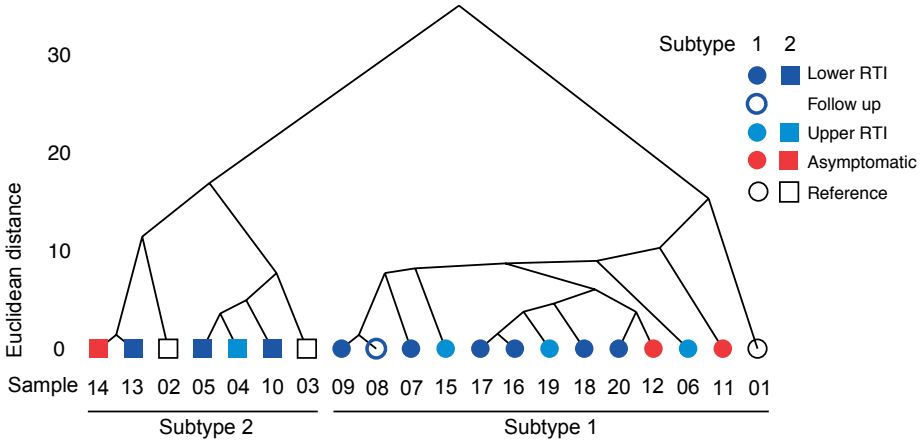

Figure 1. Hierarchical clustering based on Euclidean distance.
The Euclidean distance between variant frequencies (on the y-axis) is plotted against the different strains sequenced in this study. The strains are indicated by their sequence ID listed in Table 1. Only the variants with a frequency above 0.2 were used in this analysis (see the text for details). Hierarchical clustering was applied to the calculated Euclidean distances using the average linkage method (hclust).

which correspond to the known major subtypes of *M. pneumoniae*, i.e. subtype 1 and 2. Notably, the strains were not classified according to the clinical groups of patients they were isolated from (Groups 1-4 in Table 1). Instead, some strains show high degrees of similarity, while being isolated form different groups of patients. For example, strain B406, which was isolated form an asymptomatic child (group 2), shows a high degree of similarity with strain H026, which was isolated from an adult with a LRTI (group 4). The hierarchical clustering does therefore not indicate that the overall genome similarity is greater among the strains within each group than between strains from different groups.

To investigate whether the strains within each of the four groups can still be grouped together on the basis of relatively subtle similarities in their genomes, the genome sequences were compared as follows. Reads were aligned to the reference genome using BWA 0.5.9 and an in-house developed variant caller was used to determine small sequence variants for the sequenced samples. Variants are variant is differences between the sequenced sample and the reference genome that are typically less than 10 bp in length and are typically divided in 2 categories, namely single nucleotide polymorphisms (SNPs) and short insertions and deletions (InDels). The in-house variant caller takes the output from samtools mpileup [10] and counts the number of reference and non-reference bases (including InDels). With these counts, variant frequencies were determined. We used the following criteria in this procedure: (1) variants were compared with a frequency greater than 0.20 (which means that a variant is present in more than 20% of the reads for a single strain), (2) all variants with a low coverage (less than 21 times covered sequence) are filtered in each sample, (3) variants present in the asymptomatic group

are filtered from those in the other groups except the reference group (since variants in the asymptomatic group are unlikely to cause a 'phenotype'), (4) all variants present in the reference group are filtered from those in the other groups except the asymptomatic group, and (5) only variants are analyzed that are present in all samples in one group. Using this procedure, we were unable to identify variants that were shared between all samples from the same group. Although a number of variants were shared between some samples from the same group (Table 2), we did not find specific sequence differences between the strains from the group of symptomatic patients (Group 3 and 4) and those from the other groups (Groups 1 and 2). Variant frequencies for the different comparisons are shown in Tables 2-4.

Table 2. SNV frequencies of the isolates from symptomatic groups

Upper RTI group

SNV	Strain/patient ID				Locus
(NC_000912.1)	05	07	09	10	
111954-111954:C-A	0.28	0.00	0.24	0.20	MPN089
528806-528806:G-GT	0.38	0.42	0.33	0.05	NA
536132-536132:A-ACC	0.67	0.00	0.00	0.71	MPN442
622601-622601:G-GTT	0.38	0.40	0.10	0.33	NA
706408-706408:A-ACC	0.21	0.01	0.00	0.22	NA

Lower RTI group

SNV	Strain/patient ID									Locus
(NC_000912.1)	05	07	09	10	13	16	17	18	20	
41006-41006:G-A	0.00	0.00	0.00	0.00	0.00	0.99	1.00	1.00	0.00	MPN034
140960-140960:C-A	0.00	0.00	0.00	0.00	0.00	1.00	0.99	1.00	0.00	MPN108
171528-171528:A-AG	0.00	0.00	0.00	0.00	0.00	0.67	0.68	0.66	0.00	MPN132
171529-171529:C-CCCAAG	0.00	0.00	0.00	0.00	0.00	0.66	0.67	0.65	0.00	MPN132
405692-405692:A-T	0.00	0.00	0.00	0.00	0.00	0.99	0.99	1.00	0.00	MPN341
428005-428005:A-G	0.00	0.00	0.00	0.00	0.00	1.00	0.99	1.00	0.00	MPN358
536132-536132:A-ACC	0.68	0.00	0.00	0.01	0.00	0.73	0.70	0.71	0.67	MPN442
622601-622601:G-GTT	0.18	0.06	0.40	0.39	0.01	0.04	0.06	0.30	0.33	NA
733651-733651:G-A	0.00	0.00	0.00	0.00	0.00	0.99	0.99	1.00	0.00	MPN612

NA *Not applicable*

Variation in follow-up samples of a single patient

Two of the genome sequences that were determined were obtained from isolates that were obtained at two different time-points from a single patient. The first isolate, HAP157, was obtained when this patient suffered from a lower RTI. The other isolate, HAP157FUP, was obtained 4 weeks later, after resolution of the infection following a

course of antibiotics (azithromycin). Eight significant differences were found between strains HAP157 and HAP157FUP (Table 3). One of these differences was localized to the P1 gene (MPN141); in contrast to strain HAP157, HAP157FUP was found to have a deletion of an AGT triplet at position 182,792-182,794 within the P1 gene. This AGT is part of a tandem repeat in this gene. The significance of this repeat, which has been described before, is yet unknown [11].

Two of the strains (C036-1 and C036-2) were isolated from the same patient at a single time point. These strains were analyzed separately because they displayed different colony morphologies on agar plates. The sequences of these strains were found to be highly similar (Fig. 1); as shown in Table 4, only 5 SNPs were identified between these strains.

Table 3. Variant frequencies of strains HAP157 and HAP157FUP

SNV	Strain/patient ID		Locus
(NC_000912.1)	08	09	
182792-182794:AGT-	0.66	0.01	MPN141
195459-195459:C-CA	0.25	0.04	NA
570767-570767:G-T	0.89	0.62	NA
570769-570769:A-G	0.88	0.61	NA
570770-570770:C-G	0.85	0.61	NA
622601-622601:G-GTT	0.04	0.40	NA
622601-622601:G-GT	0.41	0.14	NA
649041-649041:G-T	0.91	0.00	NA

NA *Not applicable*

Table 4. Variant frequencies of the isolates C036-1 and C036-2 with different colony morphologies

SNV	Strain/patient ID		Locus
(NC_000912.1)	CO36-1	C036-2	
195459-195459:C-CA	0.03	0.25	NA
626378-626378:G-T	0.00	0.35	NA
649044-649044:G-C	0.99	0.00	NA
649047-649047:G-T	0.00	0.99	NA
716850-716850:C-A	0.00	0.38	MPN594

NA *Not applicable*

DISCUSSION

We determined the complete genome sequences of *M. pneumoniae* isolates obtained from asymptomatic *M. pneumoniae* carriers, and patients suffering from an upper or lower RTI caused by *M. pneumoniae*. In addition, we analyzed the genomes of 3 *M. pneumoniae* reference strains. We could not identify a specific genotype that can be associated with *M. pneumoniae* virulence or asymptomatic bacterial carriage.

Our previous findings on sequence variation among *M. pneumoniae* strains, which mainly focussed on the P1 gene and RepMP sequences of *M. pneumoniae*, indicate that sequence variation is very common in *M. pneumoniae* [9, 13]. In the current study, we did not find large rearrangements. These findings are concordant with those of Xiao et al. [5]. In their analysis, the sequences of 15 *M. pneumoniae* strains seem very stable over time and at different locations in the world. Similarly, Lluch-Senar et al. found a low number of non-synonymous SNPs among the genome sequences analysis of 23 strains from 6 different countries. However, they do describe a high rate of variation among repetitive elements in the *M. pneumoniae* genomes [6]. Our study differs from those of Xiao et al. and Lluch-Senar et al. due to the open sequence technique used. Instead of mapping the generated sequences to the reference genomes, as was done by Xiao et al.[5] and Lluch-Senar et al.[6], we analyzed, scaffolds in an unbiased fashion. Consequently, we did not generate artificial circular genomes.

Although, we provided a well-defined group of patients and were able to reach a great coverage in sequencing, the most important drawback of our study is the relatively small sample set. Only in future studies, we aim to obtain more *M. pneumoniae* isolates from asymptomatic patients. This should provide more insight in the physiology of asymptomatic colonization of the human respiratory tract by *M. pneumoniae*. Furthermore, for the analysis in this study we assumed that *M. pneumoniae* was the causative agent in the patients suffering from RTI. This is an assumption because recent studies have indicated that multiple pathogens can be present in the respiratory tract of children and adults with RTI [2, 14-16]. Therefore, other pathogens might have played a role in the symptomatic patients and it is possible that we wrongly attributed the symptomatic 'phenotype', i.e. being the causative agent of an upper or lower RTI, to *M. pneumoniae*.

In conclusion, in this study we have shown that there is no specific genotype that can be associated with *M. pneumoniae* virulence or asymptomatic carriage. Second, we found marked genetic differences between clinical isolates and the reference strains, which indicated that the latter strains may not be regarded as appropriate representatives of circulating *M. pneumoniae* strains.

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Chapter 9

Macrolide-resistance determination and molecular typing of *Mycoplasma pneumoniae* in respiratory specimens collected between 1997 and 2008 in the netherlands

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ABSTRACT

Macrolide (ML) antibiotics play an important role in the treatment regimens for *Mycoplasma pneumoniae* infections. In the past few years, however, a steady increase has been detected in the worldwide prevalence of ML-resistant *M. pneumoniae* strains. It is obvious that this increase necessitates a continuous monitoring of ML resistance (ML^R) and, when detected, modification of antibiotic treatment modalities. Previously, we developed a pyrosequencing-based assay system for the genetic determination of ML^R as well as molecular typing of *M. pneumoniae*. In this study, the system was applied to 114 *M. pneumoniae*-positive specimens that were obtained from a collection of 4390 samples from patients with acute respiratory tract infections. These samples were collected between 1997 and 2008 in The Netherlands. The pyrosequencing system produced reliable data in 86-92% of the specimens that contained >500 *M. pneumoniae* genome copies/ml patient sample. Each of these samples contained DNA of the ML-sensitive genotype. While 43% of the samples were found to harbor the *M. pneumoniae* subtype-1 genotype, 57% contained the subtype-2 genotype. We conclude that the pyrosequencing-based assay system is a useful tool for ML^R determination and molecular typing of *M. pneumoniae* in patient samples. ML^R-associated *M. pneumoniae* genotypes, however, were not found in the current study population.

INTRODUCTION

Mycoplasma pneumoniae is a pathogen of the human respiratory tract and one of the most prevalent causes of community-acquired pneumonia. General treatment options for *M. pneumoniae* infections include macrolides (MLs), tetracyclines and fluoroquinolones. In young children, however, tetracyclines and fluoroquinolones are contra-indicated due to unfavorable effects, such as discoloration of teeth by tetracyclines and putative cartilage damage induced by fluoroquinolones. The antibiotics of choice for this patient group are therefore ML antibiotics, such as clarithromycin and azithromycin. In the past decade, however, increasing numbers of *M. pneumoniae* clinical isolates were found to be resistant against these antibiotics. ML-resistance (ML^R) was first reported in Japan in 2002 [1], and since then it has also been detected in the United States [2, 3], Europe [4-7], Eastern Asia [8] and the Middle East [9]. The prevalence of ML^R varies widely from 1-2% in Denmark [5] to as high as 90% in China [10]. Clearly, the emergence of ML^R poses a significant threat to the use of MLs in the treatment of *M. pneumoniae* infections. It is therefore important that ML^R among *M. pneumoniae* isolates is rapidly and efficiently monitored in order to allow effective antibiotic treatment.

Because *M. pneumoniae* is difficult to culture, the monitoring of ML^R in this species is most efficiently performed using molecular assays. These assays are aimed at the detection of specific point mutations in the bacterial 23S rRNA gene, which determine the ML^R phenotype. Since the first report of ML^R in *M. pneumoniae*, several molecular assays have been developed to detect the ML^R-associated point mutations. In most cases, these assays rely either on time-consuming Sanger sequencing of PCR products or on real-time PCR followed by high-resolution melting analysis, which requires a high level of expertise in interpretation of the results [3, 6].

Previously, we described a pyrosequencing system to assess ML^R and to determine the genotype (i.e. subtype 1 or subtype 2) of cultured *M. pneumoniae* strains [11]. This system consists of four separate assays (two assays to assess ML^R and two assays to determine genotype) and was found to be highly useful when applied to cultures of *M. pneumoniae*. In order to develop a culture-independent pyrosequencing system that is suitable for the determination of both ML^R and the genotype of *M. pneumoniae* in clinical specimens, we modified the original pyrosequencing system by increasing its sensitivity. In the current study, we applied this modified system to *M. pneumoniae*-positive clinical specimens collected during a 12-year surveillance period in the Netherlands.

Here we demonstrate that the pyrosequencing system is highly convenient for molecular characterization of *M. pneumoniae* directly on patient material. The system can be used in parallel with a PCR-based surveillance system and can be applied in the monitoring of epidemics. In addition, the pyrosequencing system can serve as a guide for clinicians in the diagnosis and treatment of acute respiratory infections (ARIs) caused by *M. pneu-*

moniae. Finally, we show that all *M. pneumoniae*-positive specimens from the collection contained bacterial isolates with an ML-sensitive genotype. Thus, ML^R was not detected among *M. pneumoniae* strains circulating in the Netherlands between 1997 and 2008.

MATERIALS AND METHODS

Surveillance network, patients and clinical specimens.

The nationwide 'Continuous Morbidity Registration at the Dutch Sentinel General Practice Network', coordinated by NIVEL (the Netherlands Institute for Health Service Research), continuously monitors consultations for influenza-like illness (ILI) in The Netherlands. In addition, this network aims to monitor the circulation of influenza viruses and other pathogens, including *M. pneumoniae*, among patients with ILI or other ARI. Sixty-one general practitioners (GPs) of 45 general practices participate in this surveillance network. Together they serve about 0.8% of the Dutch population, nationally representative by age, gender, geographic distribution, and population density [12]. The GPs weekly report the number of patients consulting their practice with ILI, and are asked to collect a nose swab and a throat swab on a weekly basis from two patients with ILI. Preferably, one of these patients is younger than 10 years of age. If the GPs do not encounter patients with ILI, they are asked to sample two patients with another ARI [13-15]. From 2001 until 2003, healthy controls were asked to participate in the surveillance as part of a case-control study on environmental risk factors for ARI [13].

In this study, we used the data and all *M. pneumoniae*-positive specimens collected in this surveillance network from 1997 to 2008. *M. pneumoniae* was monitored during each year of the surveillance, except for week 40-52 in 2003, entire 2004, and week 1-36 in 2005. For each patient, a questionnaire was completed, which includes age, gender, time of onset of illness, clinical symptoms, and diagnosis. The nose and the throat swab were combined and transported in 4 ml gelatine-lactoalbumin-yeast (GLY) medium containing 0.1 mg/ml pimaricin and 0.2 mg/ml gentamycin at ambient temperature. The specimens were sent by regular mail to the Virology Department of the Laboratory for Infectious Diseases and Perinatal Screening of the National Institute for Public Health and the Environment for analysis.

M. pneumoniae strains.

All *M. pneumoniae* strains used in this study, including reference strains M129 (ATCC® no. 29342), PI 1428 (ATCC® no. 29085), FH (ATCC® no. 15531), MAC (ATCC® no. 15492) as well as clinical strains R035, P05/132, M688/98 and T79 [11] were cultured in Mycoplasma medium, as described previously[16].

Detection and quantification of *M. pneumoniae* DNA.

Patient specimens were analyzed prospectively for the presence of *M. pneumoniae* DNA by nested PCR, as described by Dorigo-Zetsma et al. [17]. Residual material of the original specimen was stored at -80°C for future purposes. For this study, all stored *M. pneumoniae*-positive specimens were retrieved. DNA was extracted from each original specimen (200 µl) using the QIAamp DNA mini kit (QIAGEN, the Netherlands) according to the manufacturer's recommendations. Phocine herpesvirus was added before isolation as an internal control for real-time PCR, as described before [18]. The DNA was eluted in a final volume of 50 µl. A quantitative real-time (TaqMan®) PCR assay was used to quantify the *M. pneumoniae* DNA load, as previously described [11].

ML^R genotype identification and subtype determination by pyrosequencing.

To identify genotypes (point mutations) associated with either ML^R or ML sensitivity (ML^S), two pyrosequencing assays targeting the *M. pneumoniae* 23S rRNA were used [11]. These assays were originally designed to be performed on cultured bacteria. In this study, we aimed to use these assays directly on DNA isolated from clinical specimens. We therefore designed a nested PCR protocol to improve the detection limit and specificity of the original protocol [11]. PCR and sequence primers for both assays are listed in Table S1. The first (external) PCR was identical for both assays. The PCR mixtures (25 µl) contained 0.4 µM of each primer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.02 U/µl *Pfu* DNA polymerase (Fermentas), 1×*Pfu* buffer (Fermentas) and 5 µl template DNA. The following cycling conditions were used: 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. The (internal) PCRs were performed as previously described [11], using 1 µl of template from the first PCR. A negative control was taken along in each PCR run. The resulting (biotinylated) PCR products from the second PCR were immobilized to Streptavidin Sepharose™ High Performance beads (GE healthcare) and processed to yield high-quality, single-stranded DNA using the PyroMark™ Vacuum Prep Workstation [11]. The pyrosequencing reactions and sequence analysis were performed using the PyroMark™Q96MD sequencer (Qiagen) and accompanying software.

To discriminate between the two major *M. pneumoniae* subtypes (i.e. subtype 1 and 2), we previously developed two different pyrosequencing assays [11]. The first assay targets a subtype-specific nucleotide located in a conserved region near the 3' end of the MPN141 gene, whereas the second targets a subtype-specific nucleotide in the MPN528a gene. Both assays were improved by a nested protocol in a similar fashion as described above for the ML^R/ML^S assays, using similar conditions. The primers used in each assay are listed in Table S1.

Statistical analysis.

The analysis of a possible association between genomic copy load and either patient characteristics or subtype, was performed using the Mann-Whitney U test. To analyze the relationship between subtype and age groups, or the presence of clinical symptoms, we used the Chi-Square test. Significance was set at $P = 0.05$. All statistical analyses were performed using SPSS statistical software version 16.0.1.

RESULTS

Prevalence of *M. pneumoniae* in The Netherlands between 1997 and 2008 among patients with ARI. Of the 4390 specimens that were collected between 1997 and 2008 from patients with ILI or another ARI, 114 (2.6%) tested positive for *M. pneumoniae* using a conventional, nested PCR assay. Of 588 specimens that were collected from healthy individuals, five were positive for *M. pneumoniae*. These specimens were not included in this study. From three of the 114 *M. pneumoniae*-positive specimens (each originating from 1997), original material was no longer available. The *M. pneumoniae* DNA load in the remaining 111 *M. pneumoniae*-positive specimens was determined using a quantitative, real-time PCR assay [11]. In 15 of these specimens, the real-time PCR assay was negative. These specimens were therefore not analyzed further. In the other 96 specimens, the *M. pneumoniae* genomic DNA could be quantified. The genomic copy loads in these specimens ranged from 250 genomic copies/ml up to $1.2'10^6$ copies/ml original patient sample. As shown in Fig. 1, the prevalence of *M. pneumoniae* fluctuated over the years and was highest in 2005 (11.5%). Most *M. pneumoniae* cases were observed in two groups of patients, i.e. patients aged 0-15 years (32.3%) and patients aged 31-45 years (31.3%, Table 1). The symptom that was reported most frequently was coughing. A relation between the genomic copy loads, age and clinical symptoms was not found. There was also no relation between the genomic copy load and the delay between the date of onset of illness and the moment of specimen collection.

Generation and evaluation of a highly sensitive pyrosequencing system. Previously, we described the design and use of four pyrosequencing assays for determination of both ML^R and genotype of *M. pneumoniae* strains [11]. A major drawback of these assays is, however, that they can only be applied to cultured bacterial isolates, as their lower detection limit is relatively high (5000 copies per ml original sample). We therefore set out to improve the detection limit of the four assays, which will be referred to here as the 'pyrosequencing system', such that they can be performed directly on clinical specimens from *M. pneumoniae*-positive patients. To this purpose, the initial PCR reactions of the pyrosequencing system were converted into nested reactions (as described in Materials and Methods).

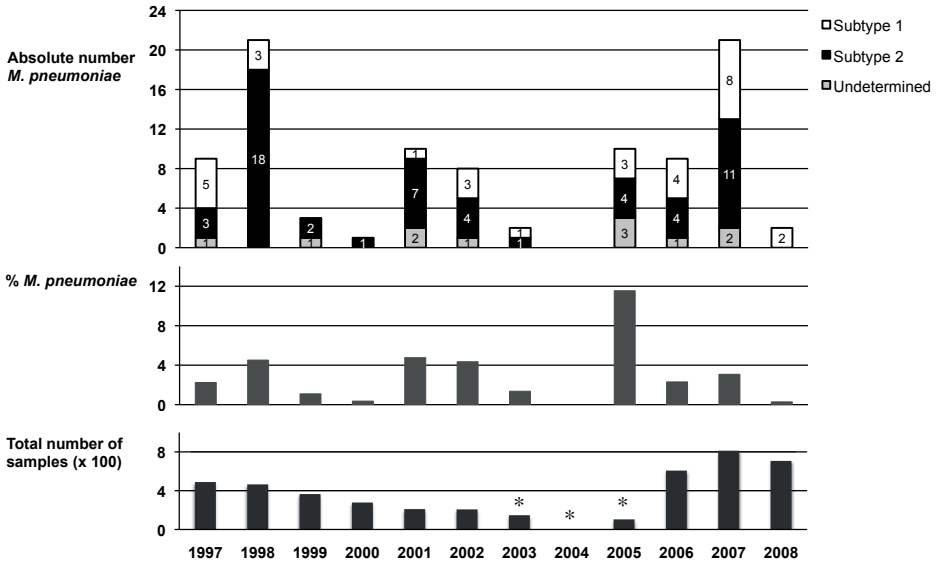

Figure 1. *M. pneumoniae*-positive specimens (96) and *M. pneumoniae* subtype detected per year. Upper panel: absolute numbers of *M. pneumoniae* PCR-positive specimens. The white and black areas in each bar represent the number of subtype 1 and subtype 2 genotypes, respectively. The grey areas depict the number of specimens in which a subtype could not be determined ('Undetermined'). Middle panel: prevalence of *M. pneumoniae* PCR-positive specimens per year in the sentinel surveillance network. Lower panel: the total number of collected specimens each year. No specimens were tested for *M. pneumoniae* in week 40-52 of 2003, entire 2004 and in week 1-36 of 2005 (indicated by *).

To determine the lower detection limit of the system on patient specimens, we performed experiments in which throat swabs from healthy persons were spiked with known quantities of *M. pneumoniae* DNA. The swabs were rinsed in 1 ml phosphate-buffered saline and, after DNA extraction, the *M. pneumoniae* DNA load was measured by real-time PCR. The detection limit for each of the four assays from the pyrosequencing system was found to be ~5×10^2 genomic copies per ml sample (which is ~10-fold more sensitive than the original protocol). At copy numbers below 5×10^2 genomic copies per ml, the assays failed to provide reliable results. In these cases, the amount of generated PCR product was too low to allow the generation of high-quality, reproducible pyrosequencing data.

To test whether the modified pyrosequencing system is able to detect the genotypes associated with either ML resistance (ML^R) or ML sensitivity (ML^S), and to discriminate between the two different subtypes of *M. pneumoniae*, we applied the system to different isolates from our *M. pneumoniae* strain collection. These isolates included four strains with an ML^R genotype (the subtype 1 strains R035 and P05/132, and subtype 2 strains M688/98 and T79) and four strains with an ML^S genotype (subtype 1 strains M129 and PI1428, and subtype 2 strains MAC and FH). Similar to the original pyrosequenc-

Table 1. Characteristics of 96 confirmed *M. pneumoniae* PCR-positive patients

Variable	Total (%) [a]	Distribution per genomic copy load (%) [b]		
		< 500	500 - 5000	> 5000
	N=96	n=12	n=37	n=47
Age (years)				
0-15	31 (32.3)	7 (58.3)	13 (35.1)	11 (23.4)
16-30	16 (16.7)	2 (16.6)	5 (13.5)	9 (19.1)
31-45	30 (31.3)	1 (8.3)	12 (32.4)	17 (36.2)
45-60	15 (15.6)	1 (8.3)	4 (10.8)	10 (21.3)
>60	4 (4.2)	1 (8.3)	3 (8.1)	0 (0.0)
Gender				
Female	55 (57.3)	7 (58.3)	20 (54.1)	28 (59.6)
Male	41 (42.7)	5 (41.7)	17 (45.9)	19 (40.4)
Duration of Illness at the time of sampling				
≤ 7 days	83 (88.3)	9 (75.0)	31 (86.1)	43 (93.5)
> 7 days	11 (11.7)	3 (25.0)	5 (13.9)	3 (6.5)
Symptoms				
Fever	60 (69.8)	8 (72.7)	27 (77.1)	25 (62.5)
Malaise	50 (58.1)	6 (54.5)	22 (62.9)	22 (55.0)
Headache	22 (33.8) [c]	0 (0.0)	10 (38.5)	12 (38.7)
Muscle pains	31 (36.0)	2 (18.2)	13 (37.1)	16 (40.0)
Sore throat	31 (36.0)	6 (54.5)	16 (45.7)	9 (22.5)
Cough	76 (88.4)	9 (81.1)	32 (91.4)	35 (87.5)
Shortness of breath	2 (8.7) [c]	0 (0.0)	2 (25.0)	0 (0.0)
Rhinorrhea	17 (27.0) [c]	2 (22.2)	12 (44.4)	3 (11.1)
Diagnosis				
ARI	43 (44.8)	8 (66.7)	16 (43.2)	19 (40.4)
ILI	53 (55.2)	4 (33.3)	21 (56.8)	28 (59.6)

[a.] Questionnaires were not complete for all patients.
[b.] Genomic copy loads per ml original specimen determined by real-time PCR. None of the patient characteristics were associated with low or high genomic copy load.
[c.] The symptom 'shortness of breath' was only recorded from 1997 through 1999. The symptom 'frontal headache' was not recorded from 2000 through 2003. The symptom 'rhinorrhea' was recorded from 2000 onwards.

ing system, the modified system correctly determined the subtypes as well as ML^R/ML^S genotype of each of the tested strains (data not shown).

ML^R/ML^S and *M. pneumoniae* subtype determination in clinical specimens. The modified pyrosequencing system was applied directly to DNA isolated from the 96 *M. pneumoniae* real-time PCR-positive patient samples. Seventy-seven of these specimens (80.2%) yielded a reliable result in the ML^R/ML^S assays from the pyrosequencing system. As expected from the spiking experiments, the performance of the tests was highly dependent on the genomic load in the original sample. In specimens with a load above $5'10^2$ genomic copies/ml, the ML^R/ML^S assays generated reliable data in 86% of the cases. At genomic copy numbers lower than $5'10^2$ genomic copies/ml, however, results were obtained in only 41% of the cases. All specimens that produced reliable data in the ML^R/ML^S assays were found to carry the ML^S genotype. Thus, the ML^R genotype was not detected in the current sample collection.

The two *M. pneumoniae* subtyping assays from the pyrosequencing system were slightly more sensitive than the ML^R/ML^S assays: in 85 of the 96 *M. pneumoniae* DNA-positive specimens (88.5%), the subtype could be determined. Similar to what was reported for the ML^R/ML^S assays, the success of the subtyping assays was highly dependent on the *M. pneumoniae* genomic load in the original specimens (Fig. 2).

In specimens with a load above $5'10^2$ per ml, the genotype could be reliably determined in 92% of the cases. In specimens with loads of $5'10^2$ genomic copies/ml or lower, the subtype was determined in 67% of the cases. The majority of the specimens obtained during the entire sample period contained *M. pneumoniae* subtype

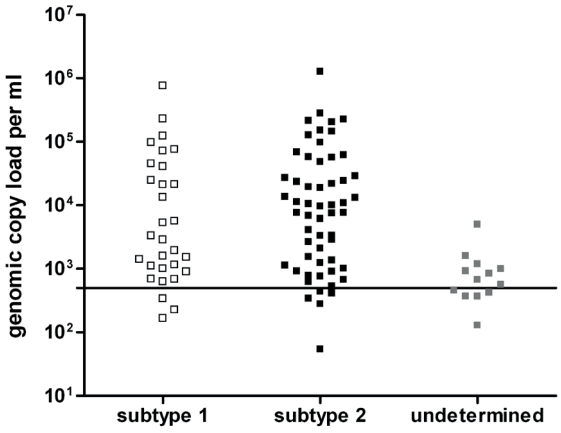

Figure 2. Genomic copy loads of 96 *M. pneumoniae* positive specimens.
The Y-axis shows the genomic copy load per ml original specimen as determined by real-time PCR. Subtype determination was successful in the majority of the specimens containing more than ~500 genomic copies per ml. The horizontal cut-off line represents this lower limit of detection.

2 sequences (57%). This percentage varied over the years and was highest in 1998 and 2001 (Fig. 1). We did not find a relation between subtype, patient age and clinical symptoms, or between subtype and genomic copy load.

DISCUSSION

In this study, a modified pyrosequencing system was used for ML^R determination and molecular typing of *M. pneumoniae* directly in clinical specimens. We found this modified system to generate reliable results in 92% of the samples that carried >5000 *M. pneumoniae* genome copies/ml. The main modification made to the original pyrosequencing protocol (20) was the inclusion of a nested PCR instead of a single PCR reaction. Clearly, the use of a nested PCR protocol may also have drawbacks. First, a higher PCR sensitivity may lead to increased problems related to contamination. However, strict guidelines were followed in the handling and containment of PCR products, i.e. each step of the pyrosequencing system was performed in a separate room. As a consequence, we have not experienced problems with DNA contamination during the course of this study. Second, a nested PCR step may increase the risk of generating PCR errors, which may lead to incorrect sequencing results and incorrect assignment of subtype or ML^R/ML^S genotype. To avoid this problem, a heat-stable DNA polymerase with proofreading activity was used throughout this study, and we have not experienced any (PCR-induced) sequence variation in any of the analyzed amplicons.

Although ML-resistant *M. pneumoniae* isolates have recently been detected in many European countries (Germany, Denmark, France and Italy), we did not detect any genotypes associated with ML^R in the Dutch patient cohort that we studied. This apparent discrepancy may be explained by the nature of our study population, which consists of ambulant patients. It might be that the prevalence of ML^R is higher among (hospitalized) patients that are subjected to antibiotic treatment. It is also possible that ML^R among *M. pneumoniae* strains in The Netherlands emerges more slowly than in other countries due to the restricted use of antibiotics in The Netherlands [19]. Thus, the selection for strains with an ML^R genotype may not be as strong in this country as neighboring countries, in which antibiotics are administered with a higher frequency. However, the easy administration and the favorable pharmacokinetic profile of newer MLs, like clarithromycin and azithromycin, will likely stimulate their prescription. It should therefore be considered that ML^R will also emerge in The Netherlands in the near future.

In our study population, a remarkable fluctuation was observed in the prevalence of *M. pneumoniae* over time. Similar fluctuations have been reported in epidemiological studies from other countries [5, 20, 21]. A possible explanation for this phenomenon is periodic antigenic variation of surface-exposed proteins of *M. pneumoniae*, such as the

P1 protein [22]. Antigenic variation may result in a temporary reduction of the immunity against *M. pneumoniae* in the population [23]. It is also possible that the specific immunological memory against *M. pneumoniae* wanes rapidly, allowing the bacteria to re-infect individuals within one to two years after the previous infection [21].

Another notable epidemiological observation in this study was the relatively low prevalence of *M. pneumoniae* in our patient population. This may, at least in part, be explained by the age distribution of the population and the seasons in which sampling was performed. More specifically, the burden of *M. pneumoniae* infections lies primarily in childhood [24], whereas the specimens in the surveillance network were collected regardless of age. In addition, the primary aim of the surveillance network is to monitor influenza virus infections, which are most prevalent during fall and winter. Most specimens from the collection were therefore obtained during these seasons and not in seasons in which *M. pneumoniae* may be detected more frequently among patients with respiratory tract infections [25].

As a consequence of the relatively low prevalence of *M. pneumoniae* in our study population, it was difficult to analyze the putative relation between the detection of either subtype 1 or subtype 2 isolates. Shifts in the prevalence of these subtypes have been reported to occur, such that the subtype that predominates in the human population is replaced by the other subtype every 8-10 years [21]. In this study, however, such shifts were not observed, which may also be due to the length of the sampling period being too small. Regardless, the clinical implications of the detection of either subtype 1 or subtype 2 strains are still unclear. In this study, we did not find any correlation between the subtype of the infecting *M. pneumoniae* strain, the pathogenesis of infection and bacterial load. As a consequence, molecular (sub)typing of *M. pneumoniae* isolates is currently only useful for epidemiological purposes.

In conclusion, pyrosequencing is a valuable tool for the molecular characterization of *M. pneumoniae*. Using this system, we did not detect any ML^R-associated *M. pneumoniae* genotypes in patient specimens collected between 1997 and 2008 in the Netherlands. However, as ML^R is increasing in neighboring countries, either due to local antibiotic pressure or due to actual spread, it is important that ML^R be monitored not only in countries with a high prevalence of ML^R but also in countries with a low prevalence of antibiotic resistance, such as The Netherlands.

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Chapter 10

Mycoplasma pneumoniae infections – does treatment help?

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J Infect. 2014 Nov;69 Suppl 1:S42-6.

ABSTRACT

M. pneumoniae is a common cause of respiratory tract infections (RTI's), especially in children. While severe *M. pneumoniae* infections are generally treated with antibiotics, the diagnosis as well as treatment of these infections should be reconsidered in the light of recent clinical findings. First, *M. pneumoniae* was found to be carried in the upper respiratory tract of a relatively high percentage of healthy, asymptomatic children. Clearly, this complicates the diagnosis of a suspected *M. pneumoniae* RTI and, thus, the decision when to initiate treatment. Another complication in the treatment of these infections is that data on the efficacy of antibiotic treatment of *M. pneumoniae* RTI's are sparse and derived exclusively from comparative studies. A recent Cochrane review concluded that there is insufficient evidence about the efficacy of antibiotics for *M. pneumoniae* lower respiratory tract infections (LRTI) in children. Due to side effects associated with the use of tetracyclines and quinolones in children, only macrolides can be used to treat *M. pneumoniae* infections in young patients. The general applicability of macrolides, however, is currently threatened by the worldwide increase in macrolide-resistant *M. pneumoniae* strains. Finally, limited evidence is available that corticosteroids might have an additional benefit in the treatment of *M. pneumoniae* infections. In this review, the current issues related to the diagnosis and treatment of *M. pneumoniae* infections will be discussed.

INTRODUCTION

Respiratory tract infections (RTI's) form a major burden of disease worldwide in children. The World Health Organization estimates that there are 150.7 million cases of pneumonia each year in children younger than 5 years of age, with as many as 20 million cases severe enough to warrant hospital admission [1]. A wide range of pathogens may cause respiratory tract infections. One of the most common bacterial causes of both upper and lower RTI is *Mycoplasma pneumoniae*. This bacterium is of particular importance in children, in which it is the second most common bacterial cause of pneumonia after *Streptococcus pneumoniae* [2]. In this review, we will focus on the treatment of RTI's caused by *M. pneumoniae* in children.

Biological characteristics of *M. pneumoniae*.

M. pneumoniae is a human pathogen from the bacterial class of *Mollicutes* (lat. soft skin). The common features shared by the bacteria in this class are: (1) the permanent lack of a rigid cell wall, (2) relatively small cellular dimensions, and (3) a relatively small genome [3-5]. As a consequence of the limited genomic size, *M. pneumoniae* has a limited metabolic capacity; the bacterium is thus dependent on its host for the production of crucial biomolecules, such as purines and pyrimidines [3]. It is therefore imperative that *M. pneumoniae* be in close contact with its host to scavenge for nutrients. To establish a close association with the host respiratory epithelium, *M. pneumoniae* contains a specialized attachment organelle. This organelle consists of a number of adhesion proteins and accessory proteins that are essential for attachment [6-8]. Loss of function of one of these proteins results in a non-virulent bacterium that is unable to attach to the human respiratory epithelium [9, 10].

Diagnosis of *M. pneumoniae*.

The clinical signs and symptoms of an *M. pneumoniae* RTI and available diagnostic assays have recently been reviewed elsewhere [11]. In short, there are no clinical, biochemical or radiological findings that are pathognomonic or specific for an *M. pneumoniae* RTI. Although a clinical syndrome has previously been described that was considered to be characteristic for *M. pneumoniae* infections (i.e. 'walking pneumonia'), a recent Cochrane review did not reveal any *M. pneumoniae*-specific clinical symptoms and signs [12]. The diagnosis of an *M. pneumoniae* RTI therefore has to be supported by microbiological findings. At present, different diagnostic techniques are available, but all of these have drawbacks that should be taken into consideration before starting treatment for a presumed *M. pneumoniae* RTI.

Although culture media have been optimized over the years, bacterial culture is still an insensitive tool to detect *M. pneumoniae* in clinical samples. In addition, it has a long

turnaround time and is both laborious and expensive. The culturing of *M. pneumoniae* is therefore rarely used as a diagnostic method for clinical purposes. The current diagnosis of *M. pneumoniae* RTI relies either on the detection of serum antibodies against *M. pneumoniae* (serology) or on the detection of bacterial DNA in samples from the upper respiratory tract, as recommended in the guidelines published by the British Thoracic Society and the Infectious Disease Society of America [13, 14].

From a clinical point of view, serology is an inconvenient diagnostic tool since it requires a serum sample in the acute phase of the disease and a convalescent serum sample taken 2-4 weeks later to provide reliable results. This inherent retrospective aspect of serology is not helpful for clinicians to make a therapeutic management decision in the acute phase of the infection. As a consequence, clinicians often rely on single-sample IgM and IgG antibody titers in order to diagnose acute *M. pneumoniae* infections. Such a procedure, however, lacks predictive power. This was recently demonstrated in a study performed in the Netherlands, in which a similar range of single-sample *M. pneumoniae* IgM and IgG antibody titers was found in children with and without symptoms of an RTI [15].

In the past two decades, a solution to the drawbacks of culture and serology has seemingly been provided by molecular diagnostic methods (based on nucleic acid amplification techniques, such as PCR), which can provide fast, sensitive, and specific results in the acute phase of an infection. Consequently, molecular methods are increasingly used in clinical practice, as well as in clinical studies for the detection of *M. pneumoniae* DNA. However, the abovementioned observational study on a population of children with and without signs of an RTI detected a similar prevalence of *M. pneumoniae* by real-time PCR in asymptomatic children and symptomatic children with 21.2% [95% CI 17.2%–25.2%] versus 16.2% [95% CI 12.2%–20.2%], respectively [15]. Moreover, a difference in *M. pneumoniae* genomic copy load in respiratory tract samples was not detected between the symptomatic and asymptomatic group. Real-time PCR therefore does not represent an unambiguous method for the diagnosis of symptomatic *M. pneumoniae* infections. Moreover, the diagnostic accuracy did not improve when the PCR results were combined with serological data [15]. Thus, at this moment, it appears that there is no definitive procedure that allows the reliable diagnosis of acute (symptomatic) infections with *M. pneumoniae*. Clearly, this notion has major consequences for the management of children with a symptomatic RTI and calls for novel procedures that allow discrimination between (harmless) carriage of *M. pneumoniae* and symptomatic infections caused by this bacterium.

TREATMENT OF *M. PNEUMONIAE* RESPIRATORY TRACT INFECTIONS

Choice of antibiotics based on biological characteristics.

M. pneumoniae lacks a rigid bacterial cell wall, but is instead protected by a sterol-containing membrane. As a direct result, *M. pneumoniae* has an innate resistance to any antibiotic that is directed at the destruction or disruption of a bacterial cell wall, such as beta-lactams and glycopeptides. In contrast, antibiotics that are directed at the inhibition of DNA metabolism and protein synthesis do have appropriate inhibitory activity against *M. pneumoniae*. The antibiotics with the best minimum inhibitory concentration (MIC) values include macrolides, tetracyclines and fluoroquinolones. Although the latter two have a good *in vitro* inhibitory effect against *M. pneumoniae*, tetracyclines may cause teeth discoloration and fluoroquinolones may affect the developing cartilage in young children [16, 17]. That is why they are not recommended by current guidelines in young children (age limit tetracyclines: ≥8 years; fluoroquinolones: adolescence with skeletal maturity) [13]. However, the occurrence of arthropathy due to fluoroquinolones is uncertain, and all musculoskeletal adverse effects reported in the literature have been reversible following withdrawal of treatment [17]. Macrolide antibiotics have a more favorable side effect profile and are therefore the preferred choice for treatment of children with a *M. pneumoniae* RTI.

Clinical effectiveness of antibiotic treatment.

Although antibiotics are effective against *M. pneumoniae in vitro* [18], there is a lack of evidence on the effectiveness of antibiotics against *M. pneumoniae in vivo*. A recent Cochrane review by Mulholland *et al.* [19] evaluated the effectiveness of antibiotic treatment of *M. pneumoniae* LRTI. The authors selected and evaluated *7* studies on the antibiotic treatment of LRTI. These studies differed significantly concerning: (1) the diagnostic criteria for an *M. pneumoniae* infection, (2) the type and duration of antibiotic treatment, (3) inclusion criteria, and (4) outcome measures. Only one of the included studies was designed as a randomized clinical trial (RCT). In this study, efficacy was shown in a group of children with a LRTI due to either *M. pneumoniae* or *Chlamydophila pneumoniae*, but these children were not evaluated separately. Consequently, this Cochrane review did not result in a conclusive answer concerning the effectiveness of antibiotic treatment of *M. pneumoniae* LRTI. Therefore, the efficacy of treatment with antibiotics for *M. pneumoniae* infection has yet to be established. Mulholland *et al.* underline the need for well-designed, randomized and controlled trials on this topic, and conclude that there is insufficient evidence about the efficacy of antibiotics for *M. pneumoniae* LRTI in children [19].

Antibiotic resistance of *M. pneumoniae*.

As mentioned above, the preferred choice of antibiotics in children are protein synthesis inhibitors of the macrolide class. However, due to the widespread use of these antibiotics, the past decade has witnessed a worldwide increase in the incidence of macrolide-resistant *M. pneumoniae* (MRMP) strains, primarily in children. MRMP strains were first reported in Asia (in ~2000) and later in Europe (~2005) and the USA (~2007). The most recent literature from 2013 reports MRMP incidences of 50%-90% in Japan [20, 21], 63%-97% in China [22-24], 23% in Taiwan [25], 8.7%-62,9% in South Korea [26, 27], 12.1% in Canada [28], 3.6% in Germany [29] and 8.3% in France [30]. Recently, MRMP isolates were also reported in Scotland [31], but the actual incidence was not determined.

Macrolide resistance of *M. pneumoniae* is the result of specific point mutations in the peptidyl transferase loop of the V domain of the bacterial ribosome that causes ineffective attachment of the macrolide molecules. The mutations with the highest incidence and a high level of resistance are A2063G, A2063C and A2064G [18]. A low level of resistance is induced by the mutations A2067G, C2617G and C2617A [32, 33], which were first found after *in vitro* challenge of *M. pneumoniae* strains with erythromycin and other macrolides and now in low prevalence *in vivo*. Because specific mutations correlate well with susceptibility testing results (i.e. MIC values for various antibiotics [18]), the detection of specific mutations (e.g. by real-time PCR) is generally sufficient for clinical purposes. Moreover, due to the slow growth of *M. pneumoniae*, susceptibility testing for an individual patient will not provide reliable results in a realistic time frame and will therefore not be helpful for clinical decision-making.

It is generally accepted that pressure selection *in vivo* is the main reason for the rapid emergence of resistance in the past decade. Different findings support this notion. First, the prevalence of resistance seems to be highest in areas where *M. pneumoniae* infections are highly prevalent and, thus, macrolides are prescribed on a regular basis [18]. Second, *in vitro* selection of MRMP strains has been described long before the actual isolation of MRMP strains from clinical samples [34]. Third, the emergence of MRMP strains during macrolide treatment has been documented in children [35-37]. And fourth, recent studies could not find a clear correlation between MLVA typing and macrolide resistance, which supports polyclonal expansion of MRMP and the absence of a single resistant clone [30, 38]. Because there are no appropriate antibiotic alternatives in young children, a reduction of macrolide antibiotic usage may be the only solution to control the further emergence of MRMP.

Signs or symptoms of patients infected with an MRMP strain do not seem to differ from those in patients infected with a macrolide-susceptible strain, on initial presentation. However, it has been shown that disease progresses more severe in patients infected with MRMP and treated with macrolide antibiotics than in those infected with macrolide-susceptible *M. pneumoniae*. This is reflected in a prolonged period of fever

and coughing in the first patient group, in combination with more prominent chest X-ray changes and a prolonged hospitalization [39-42]. Recent studies reported that a switch in treatment from macrolides to a different class of antibiotics was beneficial to the recovery of MRMP-infected patients [43-45].

There has been debate about the relevance of MRMP, since *M. pneumoniae* generally causes mild disease, and some patients benefit from macrolide antibiotics even in the presence of resistance [46]. Indeed, Kawai et al. observed defeverescence in patients infected with MRMP within 48 hours after the initiation of macrolide antibiotics in 41 - 48% [44]. There are at least two possible explanations for this latter phenomenon. First, as discussed above, the accurate diagnosis of *M. pneumoniae* RTI is difficult to perform. A proportion of the cases referred to as symptomatic *M. pneumoniae* infections in the literature may therefore not have been diagnosed correctly. Clearly, this may confound the analysis of the patient data. Second, there might be an important role for the known anti-inflammatory effects of macrolides in recovery of patients with an RTI caused by MRMP. Indeed, there is substantial evidence that the immune response plays an important role in the development of severe *M. pneumoniae* RTI [5, 11] and it is possible that macrolides suppress this response[47].

CONCLUSION

At present, there is no clear evidence that antibiotic treatment of presumed *M. pneumoniae* RTI is effective. This lack of evidence, however, may be caused by (1) the lack of appropriate diagnostic tools to diagnose symptomatic *M. pneumoniae* infections, and (2) the lack of well designed randomized controlled trials on antibiotic treatment for *M. pneumoniae* RTI. Studies on treatment results in children infected with MRMP demonstrate a significant reduction of duration of disease when treated with tetracyclines or fluoroquinolones compared to treatment with macrolides, suggesting that antibiotic treatment may be beneficial in children infected with *M. pneumoniae*. The worldwide increase in MRMP infections that is associated with increase in the use of macrolides, however, warrants deliberate use of macrolides. The lack of specific signs and symptoms and the lack of diagnostic tools that differentiate asymptomatic carriage from symptomatic infection with *M. pneumoniae* underlines the importance of following the latest guidelines on community acquired pneumonia and start macrolide antibiotics at any age if there is no response to first-line empirical treatment or in very severe disease.

CONFLICT OF INTEREST

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Chapter 11

Discussion

THE POSITION OF THESIS IN THE FIELD OF PEDIATRIC INFECTIOUS DISEASES

"Without urgent, coordinated action (…), the world is headed for a post-antibiotic era, in which common infections (…) which have been treatable for decades can once again kill."

(Dr. Keiji Fukuda, WHO Assistant Director-General for Health Security)

This warning in the WHO report on antibiotic resistance (April 2014), points to the serious threat of global antibiotic resistance due to the overuse of broad-spectrum antibiotics [6]. To handle this threat, WHO suggests that health care workers should only prescribe *"antibiotics when they are truly needed"* and *"the right antibiotic(s) to treat the illness"* [6]. Although these suggestions seem obvious, they surpass the fact that the trigger to prescribe antibiotics is more often than not merely the suspicion of a, possibly severe, bacterial infection.

In children, bacterial pneumonia, is a common and serious infection [8, 9]. Approximately 1.1 million children under the age of five years die worldwide every year as a result of pneumonia [14]. It is therefore not surprising that in the field of pediatrics the suspicion of bacterial pneumonia is the most frequent reason to prescribe antibiotics [15].

Based on recent studies [2-4], including our own [16], it is save to state that multiple pathogens co-inhabit the respiratory tract of both healthy children and children suffering from pneumonia. It is known that true pneumonia can be caused by pneumococci, staphylococci and *Mycoplasma pneumoniae*. However, considering the fact that multiple pathogens may be part of the upper respiratory tract microbiome (panel), it remains difficult to identify a single responsible pathogen in a child that suffers from pneumonia. Moreover, it is possible that a combination of multiple pathogens may cause disease, with each pathogen having its own role in breaching parts of the human immune system at specific stages of disease. At this point, however, this notion is only based on speculation. Novel diagnostics modalities to address this issue are needed. Nonetheless, in understanding of the cause and pathogenesis of respiratory tract infections, the main question should not be *'who is the culprit?'* (adapted quote by Prof. R. Dagan, ESPID meeting 2012) but '*who are the culprits, and at which stage of the disease are they important pathogens?'* In this regard, the WHO's aim to prescribe the right antibiotics *'when they are truly needed'* seems an insurmountable task.

The main subject of the studies described in this thesis is the pathogen *M. pneumoniae*. This bacterium is responsible for responsible for up to 40% of children hospitalized because of community-acquired pneumonia (CAP)[17] and is even becoming a more important pathogen in the post-pneumococcal vaccination era [18]. Two main aspects

of this pathogen were addressed, i.e. (1) genetic variation of *M. pneumoniae*, and (2) colonization of the respiratory tract of children by this pathogen. The overall aim of these studies was to increase the understanding of the pathophysiology of *M. pneumoniae* infections and the value of current methods available to diagnose *M. pneumoniae* disease. In the discussion of the consequences of our results, we will first focus on *M. pneumoniae* colonization of the respiratory tract.

IMPLICATIONS OF THE RESULTS OF THIS THESIS

M. pneumoniae colonization or disease

In the MymIC study, we found asymptomatic colonization by *M. pneumoniae* in children in a single-population, single-center study. This study is the first to describe asymptomatic colonization by *M. pneumoniae* in a systematic fashion. Furthermore, we showed that it is impossible to distinguish the carrier state from actual acute infection using state-of-the-art diagnostic tests. This finding is of major importance for clinical practice because these tests are used in virtually every clinical microbiological laboratory to detect either *M. pneumoniae* DNA in respiratory tract samples or to detect antibodies against *M. pneumoniae* in serum of children (or adults) with a suspected RTI. In light of our findings, it is likely that *M. pneumoniae*-positive children with an RTI are administered antibiotics for an alleged *M. pneumoniae* RTI, while there is a significant chance that in at least part of these children, the symptoms are caused by pathogens other than *M. pneumoniae*.

Apart from the clinical consequences, our findings are highly relevant for past, present and future research of *M. pneumoniae*. As described in chapter 6, we searched the literature for papers that described and evaluated current diagnostic tests. We found that diagnostic tests were used interchangeably and positive results are easily interpreted as indicative for infection. However, this does no longer suffice. Instead, a more detailed interpretation of results for each individual patient is warranted. A good first theoretical start for such an interpretation can be based on a recent proposition of different states of *M. pneumoniae* disease[19].

Five states of *M. pneumoniae* disease

In a recent review, our group proposed different pathogenic states of *M. pneumoniae* starting at the site of the respiratory tract[19]. The key to *M. pneumoniae* disease might be the first colonizing step. First contact of *M. pneumoniae* with the respiratory tract may then either lead to a primary colonizing state or acute infection with local cytotoxic effects. Dependent on the host pathogen interaction, both states may lead to either clearance of *M. pneumoniae*, antigenic variation resulting in a chronic infection, direct

invasiveness of *M. pneumoniae* cells or an inflammatory response. The latter two may lead to dissemination of *M. pneumoniae* cells or antibodies and may result in different extra-pulmonary disease states [19]. All these different *M. pneumoniae* disease states are shown in Fig. 1. It is important to notice that PCR techniques are mainly suitable to detect the presence of *M. pneumoniae* at a certain site in the human body. Used on respiratory secretions this might merely reflect colonization. However, used on blood or secretions from within the deeper pulmonary system, a positive PCR might reflect direct invasiveness or dissemination. The detection of antibodies in serum could reflect a number of pathogenic states, e.g. acute infection with local inflammatory effects, chronic infection or a systemic inflammatory reaction. Fig. 1 also clarifies that the current detection techniques to identify *M. pneumoniae* are insufficient to discern the different disease states in an individual patient.

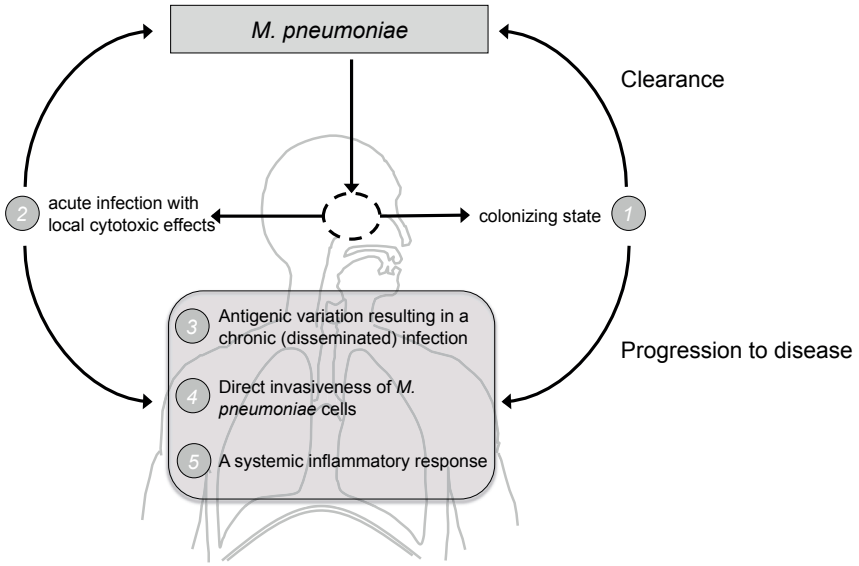

Figure 1. Different potential disease states with *M. pneumoniae*

Genetic variation of *M. pneumoniae*

Our studies on molecular variation of *M. pneumoniae* had two major outcomes. First, each *M. pneumoniae* strain can be classified as either a subtype-1 strain or a subtype-2 strain based on sequence differences present throughout the genome. This finding suggests that the two subtypes represent genetically distinct lineages. Based on the findings

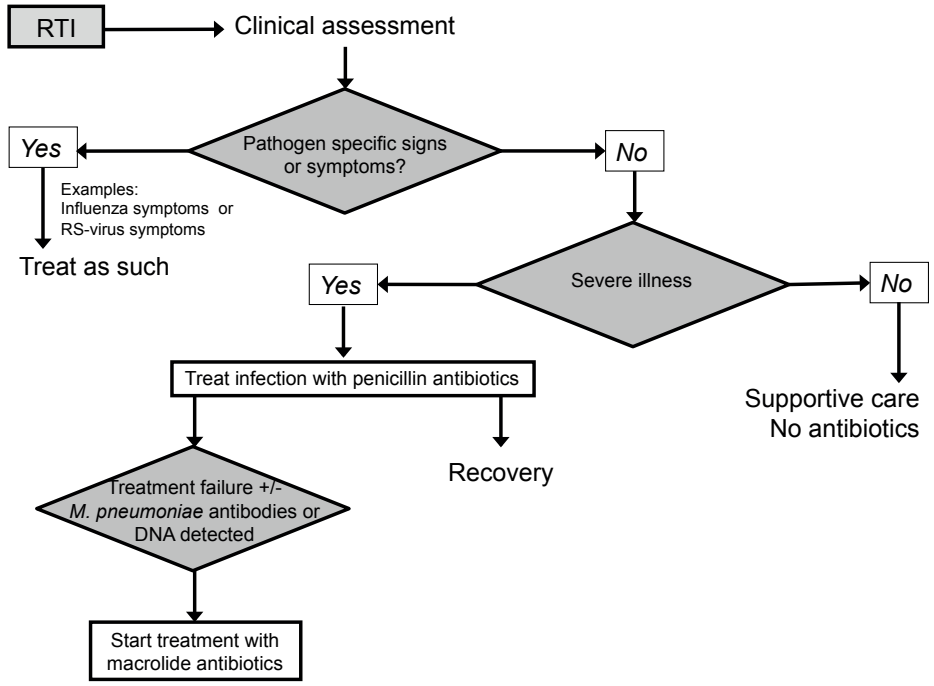

Figure 2. Schematic proposal for treatment decisions in RTI.

presented in chapter 2 and 3, it is unlikely that combined subtype 1 and 2 sequences will be found in the future.

Second, we found a high potential for variation in the genes encoding the adhesion proteins P1 and P40/90 at the surface of the attachment organelle of *M. pneumoniae*. The genes encoding these proteins, MPN141 and MPN142, respectively, contain repetitive sequence elements (RepMP elements) of which numerous variants can be found throughout the *M. pneumoniae* genome. We detected novel putative recombination events between RepMP variants, as well as previously unknown variations in MPN142. Furthermore, we were able to explain the origins of all MPN141 variants that were previously detected, and we found a genetic variation and in the genomes of *M. pneumoniae* strains recently isolated form patients (chapter 8, manuscript in preparation).

To investigate whether inter-RepMP recombination events also take place *in vitro*, we performed a multiple passage study, in which a small number of strains were serially cultured for about one year. After each passage, a sample was taken to study recombination events. Because we were unable to detect any new recombination events (unpublished), we hypothesize that these events are relatively infrequent in the absence of immunological pressure.

The importance of genetic variation of *M. pneumoniae* for individual human hosts and the population

We studied *M. pneumoniae* from a microbial and clinical viewpoint. This translational set-up enabled us to combine research questions from both perspectives and make a first attempt to determine the clinical impact of genetic variation of *M. pneumoniae*. A significant outcome of our studies was that we were unable to link colonization or severity of disease to either of the two major subtypes of *M. pneumoniae*. The occurrence of these subtypes in the population, however, does seem to be related to the long-term epidemiological pattern of *M. pneumoniae* infections. Kenri et al. observed this in a study published in 2008[20]. Subtype-specific epidemics occur every 3-7 years on top of an endemic background and in each epidemic wave a change in dominant subtype occurs. Thus, during a period of approximately 10 years, subtype-1 strains are highly prevalent and subtype-2 strains are endemically present. Such a period is then followed by a period in which subtype-2 strains become highly prevalent and subtype-1 strains gradually decline [20].

Although there is no direct benefit for an individual patient, it is important to monitor the prevalence *M. pneumoniae* in a certain population for several reasons. First, monitoring the prevalence of *M. pneumoniae* creates awareness. At this point the prevalence is highly variable spanning 7-10 years[20, 21]. Knowing that the prevalence is increasing at a certain time is valuable because this might guide create awareness for clinicians. Second, it is important to monitor genetic variation. We showed that subtype 2 strains have higher levels of variation. Consequently, these strains may have more potential for antigenic variation. This is important for future studies on severity of disease and possibly for future vaccine development. Third, it is important to monitor the prevalence of macrolide-resistance *M. pneumoniae*. This might guide antibiotic usage and eventually may result in a more directed therapy. The first choice antibiotics for *M. pneumoniae* infections in children are ML antibiotics. Current treatment strategies for community-acquired pneumonia are based on the notion that the incidence and prevalence of *M. pneumoniae* infection is higher above the age of five and that *M. pneumoniae* infections are almost non-existent below the age of five. Therefore, to treat suspected pneumonia, it is recommended to prescribe beta-lactam antibiotics as a first choice below the age of five and ML-antibiotics as a first choice above the age of five [8]. Based on our results, these treatment strategies seem no longer valid, as the presence of *M. pneumoniae* in the upper respiratory tract did not differ between the different age groups.

Importantly, as described in chapter 7 and 8, ML-resistance has become a widespread problem throughout the world, which has a direct link with the use of ML. Morozumi et al. demonstrated in 2005 that a higher degree of ML usage results in a higher degree of ML-resistance [22]. This has clinical consequences as outlined in chapter 8. In the Netherlands, we have so far not found any *M. pneumoniae* strains that harbored a

known ML-resistant genotype. However, because ML are easy to administer to children and have a favorable pharmacokinetic profile, these antibiotics are frequently prescribed to children. Moreover, a recent study showed that early administration of ML to children with upper RTI could help to prevent lower RTI[23]. If these results are incorporated in clinical practice or in guidelines, it will be a matter of time before the first ML-resistant *M. pneumoniae* strains will emerge in the Netherlands. It is therefore important to keep testing *M. pneumoniae*-positive samples for ML-resistant genotypes. The pyrosequencing technique developed and used in our studies can serve as the basis for the detection of the most common ML-resistant genotypes in *M. pneumoniae*.

Should we treat a presumed *M. pneumoniae* infection?

First of all, it is difficult to determine whether an RTI is caused by *M. pneumoniae*, because there are no specific symptoms or signs of *M. pneumoniae* RTI. In addition, results from ancillary laboratory or radiologic investigations are not helpful in the diagnosis of *M. pneumoniae* RTI[24]. Nevertheless, historically, a clear delineated clinical course, described as a 'walking pneumonia', is well known for *M. pneumoniae* infections. This clinical course tends to be more severe (prolonged period of fever and coughing) in patients infected with ML-resistant *M. pneumoniae* strains versus patients infected with ML-susceptible strains. And, patients infected with ML-resistant strains recover more quickly when treated with doxycycline or quinolones [25-27].

Based on the results in this thesis we should at least be very cautious to prescribe antibiotics to a child with a positive PCR for *M. pneumoniae*. Such a positive test might just as well reflect bacterial colonization. There are no studies that show clear benefit from a ML-antibiotic for a presumed *M. pneumoniae* infection.

Without a reliable microbiological test and a clear phenotype to lean on, we are left without a well-defined recommendation whether or not to treat *M. pneumoniae* RTI. However, there are pathogens that do cause a clear RTI phenotype, such as *S. pneumoniae, S. aureus,* influenza virus, RS virus and human metapneumovirus. *S. pneumoniae* generally causes a much more severe infection and can be treated effectively with penicillin. In addition, in high-risk patients (e.g. with impaired mucociliary clearance), it might be beneficial to prescribe antiviral agents early during influenza virus infections.

Thus, without good alternatives, a reasonable path to follow while managing a child with an RTI is to prescribe an antibiotic of the penicillin class in order to treat the most serious possible infection. When this treatment fails, and either *M. pneumoniae* or anti-*M. pneumoniae* antibodies are detected, ML antibiotics should be prescribed. A possible scheme to follow for an RTI in children is presented in Fig. 2

FUTURE PROSPECTS

Future studies on *M. pneumoniae* should focus on the following aspects.

1. Epidemiology

It is important to study the prevalence of *M. pneumoniae* in different countries and in different seasons and years, because this will indicate whether or not *M. pneumoniae* should be considered as a likely etiological agent of RTI.

Monitoring of *M. pneumoniae* should also focus on antigenic variation and the prevalence of ML resistant *M. pneumoniae* strains. First this is important to create a better understanding of *M. pneumoniae* variation and in time it will be important to adjust antimicrobial therapy and also provide data for possible vaccine development in the future.

2. Immunology and improved diagnostic tools

Future studies should aim to increase the understanding of the pathophysiological processes that occur after potential pathogens have entered in the human respiratory tract. The focus of these studies should lie on the triggered immune responses. Consecutively the studies should be aimed at unraveling the posed different disease states (as shown in Fig. 1). This should give more insight in the triggered immune responses and maintained immune response that serve in protection from future encounters with *M. pneumoniae*. A similar strategy could be performed for different respiratory tract infection pathogens.

Currently, diagnostic studies are purely based on the presence of a pathogen or specific antibodies and not *per se* on the (local) host immune response and consequences of the presence of a microorganism. Thus, the future studies should aim at diagnosing the immune response that has been triggered by a specific microorganism in a specific disease state instead of merely identifying the microorganism in a specific disease state. This should aid in new, targeted therapies for RTI and also aid clinicians to prescribe antibiotics when truly needed.

In short, future studies should focus on the development of a fast and minimally invasive diagnostic tool that identifies the bacterial pathogen causing pneumonia and that defines its role as invasive infection-causing microorganism.

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Chapter 12

English summary
Nederlandse samenvatting

SUMMARY

This thesis focuses on bacterial genetic variation and colonization of the respiratory tract of children by *Mycoplasma pneumoniae*. *M. pneumoniae* is an endemic cause of RTI unbound to a specific season and is regarded as a common cause of community acquired pneumonia. Besides RTIs, *M. pneumoniae* has been associated with a number of extrapulmonary manifestations. This bacterium has some notable biological characteristics. It is one of the smallest free living micro-organisms, but needs to be in close human contact to scavenge for important nutrients, due to its small size and genome and its limited metabolic capacities. In order to accomplish this, *M. pneumoniae* has an attachment organelle, which consists of multiple attachment proteins. These proteins are encoded by genes of which there are multiple copies throughout the genome. Rearrangement of these genes may result immune evasion by antigenic variation of the attachment organelle.

The current diagnosis of *M. pneumoniae* RTIs relies on the detection of either serum antibodies against *M. pneumoniae* or bacterial DNA in samples of the upper respiratory tract (URT). Due to drawbacks of serology, detection of bacterial DNA with PCR is mostly used. However, if *M. pneumoniae* would be commonly carried asymptomatically in the URT of children, the detection (of DNA) of this bacterial species may not indicate a symptomatic infection.

M. pneumoniae has an innate resistance to any antibiotic that is directed to the destruction or disruption of a bacterial cell wall. In general, antimicrobials that are directed at the DNA metabolism and protein synthesis have a good inhibitory activity against *M. pneumoniae*. In the past decades a growing number of reports show an increase of antimicrobial resistance against macrolide (ML) antibiotics, especially in Asia.

We posed the hypothesis that *M. pneumoniae* is able to colonize the human respiratory tract in an asymptomatic fashion and, assuming that our hypothesis could be confirmed, we also set out to study whether possible asymptomatic carriage could be distinguished from infection. Specifically, we aimed to: 1. identify asymptomatic colonization by *M. pneumoniae* using current available microbiological tools, 2. find host factors associated with asymptomatic carriage versus symptomatic disease, 3. classify genetic variation in *M. pneumoniae* in a concise understandable manner and 4. to find specific genetic pathogen variation that is associated with disease and disease severity. In addition we aimed to investigate the upcoming ML-resistance in the Netherlands.

A summary of the most important findings of **PART 1:** genetic variation of *Mycoplasma pneumoniae*

- *M. pneumoniae* subtype-1 and subtype-2 represent genetically distinct lineages and are not associated with severity of *M. pneumoniae* respiratory tract infection.
- Besides the subtype division, a high potential for variation is present in the genomes of different *M. pneumoniae* strains **(Chapters 3, 4 and 5)**.

- Gene conversion, a form of homologous DNA recombination, of RepMP elements results in antigenic variation of major adhesion proteins. It is probably immunologically governed **(Chapter 3 and 4)**.
- Macrolide-resistance is governed by mutations in the ribosome of *M. pneumoniae* which can be identified using pyrosequencing **(Chapter 6)**.

A summary of the most important findings of **PART 2:** clinical aspects of *Mycoplasma pneumoniae*

- Asymptomatic colonization by *M. pneumoniae* of the respiratory tract in children seems to exist **(Chapter 7)**.
- The prevalence of *M. pneumoniae* detected by PCR is just as high in asymptomatic children as in symptomatic children but changes over time and season **(Chapter 7)**.
- The strains found in asymptomatic and symptomatic children do not differ genetically (based on subtype-1 and subtype-2 sequencing and whole genome sequencing) **(Chapter 7 and 8)**.
- *M. pneumoniae* macrolide resistance strains are prevalent throughout the world, but have not been identified in the Netherlands **(Chapter 9)**.
- It is questionable whether treatment should be given to children with mild infections supposedly caused by *M. pneumoniae*. Future studies are needed on this subject **(Chapter 10)**.
- The choice which antibiotic should be prescribed for an alleged *M. pneumoniae* infection in a child is limited. Macrolides are still the first choice **(Chapter 10)**.

This thesis ends with a general discussion on our findings on *M. pneumoniae* colonization, and discuss that diagnostic tests for an alleged *M. pneumoniae* infection should be interpreted with caution and should take in consideration the recently proposed different states of *M. pneumoniae* disease. The key to *M. pneumoniae* disease might be the first colonizing step.

Concerning the genetic variability we discuss and conclude that there is no direct benefit for an individual patient. However, it is important to monitor the prevalence *M. pneumoniae* to create clinical awareness, to monitor genetic variation and to monitor the prevalence of macrolide-resistance *M. pneumoniae*. This might guide antibiotic usage and eventually may result in a more directed therapy.

Finally we discuss whether a presumed *M. pneumoniae* infection should always result in antibiotic treatment. We end this discussion concluding that without good alternatives, a reasonable path to follow while managing a child with an RTI is to prescribe an antibiotic of the penicillin class in order to treat the most serious possible infection. When this treatment fails, and either *M. pneumoniae* or anti-*M. pneumoniae* antibodies are detected, ML antibiotics should be prescribed.

We conclude the general discussion with some future prospects which should include studies on epidemiology and genetic variation to monitor ML resistance in the Netherlands and world-wide and immunological studies to increase the understanding of the pathophysiological processes that occur after potential pathogens have entered in the human respiratory tract.

SAMENVATTING

Het onderwerp van dit proefschrift is bacteriële genetische variatie en kolonisatie van de luchtwegen van kinderen door *Mycoplasma pneumoniae*.

M. pneumoniae is een endemisch oorzaak van luchtweginfecties en wordt beschouwd als een veel voorkomende oorzaak van longontsteking. Deze bacterie heeft een aantal opmerkelijke biologische kenmerken. Het is één van de kleinste vrij levende micro-organismen, maar moet in nauw contact staan met zijn menselijke gastheer om belangrijke voedingsstoffen te vergaren, vanwege zijn kleine formaat en kleine genoom en daarmee beperkte metabole capaciteit. Om dit te bereiken, heeft *M. pneumoniae* een aanhechtingsorganel, bestaande uit meerdere oppervlakte eiwitten. Deze eiwitten worden gecodeerd door genen waarvan meerdere kopieën gelegen zijn in het genoom. Verandering van deze genen door recombinatie kan leiden tot een verandering van oppervlakte eiwitten en daardoor antigene variatie het aanhechtingsorganel.

De diagnose van *M. pneumoniae* luchtweginfecties is gebaseerd op de detectie van serum- antilichamen tegen *M. pneumoniae* of bacterieel DNA in samples vanuit de bovenste luchtwegen. Vanwege nadelen van serologie (meerdere bloedafnames nodig en daarna een retrospectieve diagnose) wordt de detectie van het bacteriële DNA met PCR tegenwoordig steeds vaker gebruikt. Indien *M. pneumoniae* ook bij asymptomatische kinderen in de bovenste luchtwegen voorkomt, zou een positieve PCR op materiaal vanuit de bovenste luchtwegen ook kunnen duiden op dragerschap en dus niet op een symptomatische infectie.

M. pneumoniae is intrinsiek resistent tegen antibiotica die zijn gericht op de vernietiging of verstoring van een bacteriële celwand. In het algemeen hebben antibiotica die gericht zijn op het DNA en de eiwitsynthese een goede remmende activiteit tegen *M. pneumoniae*. In de afgelopen decennia blijkt een toename van antimicrobiële resistentie tegen macrolide (ML) antibiotica, vooral in Azië.

In dit proefschrift wordt de hypothese gesteld dat *M. pneumoniae* in staat is de menselijke luchtwegen te koloniseren op een asymptomatische wijze en ervan uitgaande dat onze hypothese kan worden bevestigd, stellen we ook dat asymptomatisch dragerschap kan worden onderscheiden van infectie. Specifieke doelen waren 1. identificeren asymptomatische kolonisatie door *M. pneumoniae* met de huidige beschikbare microbiologische testen, 2. Het vinden van gastheer factoren die samenhangen met asymptomatisch dragerschap versus symptomatische ziekte, 3. Classificeren van genetische variatie in *M. pneumoniae* in een beknopte begrijpelijke manier en 4. specifieke pathogeen genetische variatie die is geassocieerd met de luchtweginfectie te vinden. Daarnaast was het doel om de komende ML-resistentie in Nederland te onderzoeken.

Een samenvatting van de belangrijkste bevindingen van **Part 1**: the genetic variation of *Mycoplasma pneumoniae*

- *M. pneumoniae* subtype-1 en subtype-2 vertegenwoordigen genetisch verschillende lijnen en zijn niet geassocieerd met de ernst van *M. pneumoniae* luchtweginfectie.
- Naast het subtype onderscheid, is een sterk vermogen tot variatie aanwezig in de genomen van verschillende *M. pneumoniae* stammen **(hoofdstuk 3, 4 en 5)**.
- genconversie, een vorm van homologe recombinatie DNA van RepMP elementen leidt tot antigene variatie van grote adhesie-eiwitten. Het is waarschijnlijk immunologisch bepaald **(hoofdstuk 3 en 4)**.
- macrolide-weerstand wordt geregeld door mutaties in het ribosoom *M. pneumoniae* die kunnen worden geïdentificeerd met behulp pyrosequencing **(hoofdstuk 6)**.

Een samenvatting van de belangrijkste bevindingen van **Part 2**: clinical aspects of *Mycoplasma pneumoniae*

- asymptomatische kolonisatie door *M. pneumoniae* luchtwegen bij kinderen lijkt te bestaan **(hoofdstuk 7)**.
- De prevalentie van *M. pneumoniae* gedetecteerd door PCR is net zo hoog bij asymptomatische kinderen als bij symptomatische kinderen, maar varieert in de tijd en per seizoen **(hoofdstuk 7)**.
- De stammen gevonden bij asymptomatische en symptomatische kinderen zijn niet genetisch verschillend (gebaseerd op subtype-1 en subtype-2 sequencing en whole genome sequencing) **(hoofdstuk 7 en 8)**.
- *M. pneumoniae* macrolideresistentie stammen zijn niet geïdentificeerd in Nederland **(hoofdstuk 9)**.
- Het is de vraag of de behandeling moet worden gegeven aan kinderen met milde infecties vermoedelijk veroorzaakt door *M. pneumoniae*. Toekomstige studies zijn nodig over dit onderwerp **(hoofdstuk 10)**.
- De keuze in antibiotica die kunnen worden voorgeschreven aan kinderen voor een vermeende *M. pneumoniae* infectie is beperkt. Macroliden zijn nog steeds de eerste keuze **(hoofdstuk 10)**.

Dit proefschrift eindigt met een algemene discussie over onze bevindingen op *M. pneumoniae* kolonisatie, en bespreekt dat diagnostische tests voor een vermeende *M. pneumoniae* infectie moet met voorzichtigheid dienen te worden geïnterpreteerd. Er moet rekening gehouden worden met de onlangs voorgestelde verschillende stadia van de ziekte van *M. pneumoniae*. Als sleutel tot ziekte voor *M. pneumoniae* zou de eerste stap kolonisatie (dragerschap) kunnen zijn.

Betreffende de genetische variabiliteit worden de conclusies besproken dat er geen rechtstreeks consequentie lijkt te zijn voor een individuele patiënt. Het is echter van belang om de prevalentie *M. pneumoniae* te blijven volgen voor klinische bewustwording, om genetische variatie volgen en de prevalentie van macrolide-resistentie *M. pneumoniae*

controleren. Dit zou het gebruik van antibiotica kunnen leiden en uiteindelijk resulteren in een meer gerichte therapie.

Tot slot bespreken we de vraag of een veronderstelde *M. pneumoniae* infectie altijd zou moeten leiden tot een behandeling met antibiotica. We eindigen deze discussie concluderend dat zonder goede alternatieven, bij ernstige luchtweginfecties als eerste een penicilline antibioticum zou moeten worden gebruikt. Wanneer deze behandeling niet leidt tot verbetering, en of *M. pneumoniae* of anti-*M. pneumoniae* antilichamen worden gedetecteerd, kan ML antibiotica worden voorgeschreven.

We sluiten de algemene discussie met enkele toekomstperspectieven. Dit betreft met name studies over de epidemiologie en genetische variatie van *M. pneumoniae*. Daarnaast is het belangrijk om ML resistentie te blijven controleren in Nederland en wereldwijd en immunologische studies uit te voeren om het begrip van de pathofysiologische processen die optreden na contact met potentiële ziekteverwekkers te vergroten.

Appendices

Supplementary Tables

List of Publications

PhD Portfolio

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLES CHAPTER 2

Table S1. Names and genome locations of the RepMP2/3 and RepMP4 elements and sequences of primers used for amplification of the elements.

Element[a]	Genome position[b]	Element length (bp)	Primer 1 (5′-3′)	Primer 2 (5′-3′)
RepMP2/3-a	127355-128821	1467	TTCCGTGCTAACTTAGGTTC	AAATGGAGGTAGAGC-GAATTG
RepMP2/3-b	168051-168681	631	TCTCTACATAAAACTAGCATTG	CCCACTAACTTTCCCATAAG
RepMP2/3-c	169920-171370	1451	GGGAATTGGTTTGACCG-GAG	GCACTTGAAAGAGTTAGTT-GAT
RepMP2/3-d	182860-184769	1910	GTCCAATCCAAAATGTGGTTG	ACTACTGAGGTTACCAC-TACTCG
RepMP2/3-e	190154-191782	1629	TAAGAGAGAAGTGATGCAGC	CACCACTAAGCACACTGC-CAAT
RepMP2/3-f	245562-247243	1701	GAAATTAGAAAGTCT-CAAATCTC	GGTGACAGTTGATTGGTGGT
RepMP2/3-g	341759-343166	1408	GAACCCGCATCCACTTTTTC	CGTCCCAAATACCGGTGTGA
RepMP2/3-h	441236-442915	1680	GGCACTTGAAAGAGTTAGT-TAAC	TGCCAATGTTACCACTATTCG
RepMP2/3-i	492620-494206	1587	TTGAACAACAATTAGGTCAAG	GTGTCGTTGTTTTGAGCGAC
RepMP2/3-j	606031-607756	1726	CCTAGTGTGGCGAAAATCAG	TTGACCTGAGCCTGAAGAAC
RepMP4-a	130355-131994	1640	TCCCACTAAATAAATTGAGTTG	ATCAGTGGTGGTATTGGAATC
RepMP4-b	165770-167046	1277	ACAACTATTAGTAG-TAGAAGAC	CAACTTACTGTTTAAAAGTTGC
RepMP4-c	180820-182441	1622	ATTGCCAAAAGCAAATTGCTG	ATAGCGCACCCTAAAGACAC
RepMP4-d	248448-249818	1371	TCCCACTAAATAAATTGAGTTG	CCTAAAAGTGACTGCCTTTAC
RepMP4-e	443048-444372	1325	TTAAAAGTGTGCAAGACACTC	ACACGGTAAACAAATCTAA-CAG
RepMP4-f	496473-497560	1088	AAAACCGCCAAAAAGAT-TAAAG	ATCCAGCCCCCCATTGAT
RepMP4-g	565050-566094	1045	CCTAACGACAAGAATAAG-TAC	GTGAGGTAATTAATC-CATAGAC
RepMP4-h	609094-610639	1564	TCCCACTAAATAAATTGAGTTG	GCGCCACCAACTTGGTATCC

[a] The RepMP2/3 and RepMP4 elements were individually named by the suffixes -a to -j and -a to -h, respectively.

[b] The genome positions and sequences were derived from GenBank file U00089.

Table S2. GenBank accession numbers of the sequences generated in this study.

Strain	RepMP2/3 elements									
	a	b	c	d	e	f	g	h	i	j
M129	FJ603695	FJ603696	FJ603697	FJ603698	FJ603699	FJ603700	FJ603701	FJ603702	FJ603703	FJ603704
MAC	FJ603713	FJ603714	FJ603715	FJ603716	FJ603717	FJ603718	FJ603719	FJ603720	FJ603721	FJ603722
P1 1428	FJ604091	FJ604092	FJ604093	FJ604094	FJ604095	FJ604096	FJ604097	FJ604098	FJ604099	FJ604100
Mp5	FJ603731	FJ603732	FJ603733	FJ603734	FJ603735	FJ603736	FJ603737	FJ603738	FJ603739	FJ603740
Mp22	FJ603749	FJ603750	FJ603751	FJ603752	FJ603753	FJ603754	FJ603755	FJ603756	FJ603757	FJ603758
Mp72	FJ603767	FJ603768	FJ603769	FJ603770	FJ603771	FJ603772	FJ603773	FJ603774	FJ603775	FJ603776
Mp1042	FJ603785	FJ603786	FJ603787	FJ603788	FJ603789	FJ603790	FJ603791	FJ603792	FJ603793	FJ603794
Mp1116	FJ603803	FJ603804	FJ603805	FJ603806	FJ603807	FJ603808	FJ603809	FJ603810	FJ603811	FJ603812
Mp1286	FJ603821	FJ603822	FJ603823	FJ603824	FJ603825	FJ603826	FJ603827	FJ603828	FJ603829	FJ603830
Mp1397	FJ603839	FJ603840	FJ603841	FJ603842	FJ603843	FJ603844	FJ603845	FJ603846	FJ603847	FJ603848
Mp1842	FJ603857	FJ603858	FJ603859	FJ603860	FJ603861	FJ603862	FJ603863	FJ603864	FJ603865	FJ603866
Mp2004	FJ603875	FJ603876	FJ603877	FJ603878	FJ603879	FJ603880	FJ603881	FJ603882	FJ603883	FJ603884
Mp2018	FJ603893	FJ603894	FJ603895	FJ603896	FJ603897	FJ603898	FJ603899	FJ603900	FJ603901	FJ603902
Mp2157	FJ603911	FJ603912	FJ603913	FJ603914	FJ603915	FJ603916	FJ603917	FJ603918	FJ603919	FJ603920
Mp4599	FJ603929	FJ603930	FJ603931	FJ603932	FJ603933	FJ603934	FJ603935	FJ603936	FJ603937	FJ603938
Mp4817	FJ603947	FJ603948	FJ603949	FJ603950	FJ603951	FJ603952	FJ603953	FJ603954	FJ603955	FJ603956
Mp5181	FJ603965	FJ603966	FJ603967	FJ603968	FJ603969	FJ603970	FJ603971	FJ603972	FJ603973	FJ603974
Mp5191	FJ603983	FJ603984	FJ603985	FJ603986	FJ603987	FJ603988	FJ603989	FJ603990	FJ603991	FJ603992
Mp5192	FJ604001	FJ604002	FJ604003	FJ604004	FJ604005	FJ604006	FJ604007	FJ604008	FJ604009	FJ604010
Mp5194	FJ604019	FJ604020	FJ604021	FJ604022	FJ604023	FJ604024	FJ604025	FJ604026	FJ604027	FJ604028
Mp5196	FJ604037	FJ604038	FJ604039	FJ604040	FJ604041	FJ604042	FJ604043	FJ604044	FJ604045	FJ604046
Mp5245	FJ604055	FJ604056	FJ604057	FJ604058	FJ604059	FJ604060	FJ604061	FJ604062	FJ604063	FJ604064
Ofo	FJ604073	FJ604074	FJ604075	FJ604076	FJ604077	FJ604078	FJ604079	FJ604080	FJ604081	FJ604082

Table S2. GenBank accession numbers of the sequences generated in this study.

Strain	RepMP4 elements							
	a	b	c	d	e	f	g	h
M129	FJ603705	FJ603706	FJ603707	FJ603708	FJ603709	FJ603710	FJ603711	FJ603712
MAC	FJ603723	FJ603724	FJ603725	FJ603726	FJ603727	FJ603728	FJ603729	FJ603730
P1 1428	FJ604101	FJ604102	FJ604103	FJ604104	FJ604105	FJ604106	FJ604107	FJ604108
Mp5	FJ603741	FJ603742	FJ603743	FJ603744	FJ603745	FJ603746	FJ603747	FJ603748
Mp22	FJ603759	FJ603760	FJ603761	FJ603762	FJ603763	FJ603764	FJ603765	FJ603766
Mp72	FJ603777	FJ603778	FJ603779	FJ603780	FJ603781	FJ603782	FJ603783	FJ603784
Mp1042	FJ603795	FJ603796	FJ603797	FJ603798	FJ603799	FJ603800	FJ603801	FJ603802
Mp1116	FJ603813	FJ603814	FJ603815	FJ603816	FJ603817	FJ603818	FJ603819	FJ603820
Mp1286	FJ603831	FJ603832	FJ603833	FJ603834	FJ603835	FJ603836	FJ603837	FJ603838
Mp1397	FJ603849	FJ603850	FJ603851	FJ603852	FJ603853	FJ603854	FJ603855	FJ603856
Mp1842	FJ603867	FJ603868	FJ603869	FJ603870	FJ603871	FJ603872	FJ603873	FJ603874
Mp2004	FJ603885	FJ603886	FJ603887	FJ603888	FJ603889	FJ603890	FJ603891	FJ603892
Mp2018	FJ603903	FJ603904	FJ603905	FJ603906	FJ603907	FJ603908	FJ603909	FJ603910
Mp2157	FJ603921	FJ603922	FJ603923	FJ603924	FJ603925	FJ603926	FJ603927	FJ603928
Mp4599	FJ603939	FJ603940	FJ603941	FJ603942	FJ603943	FJ603944	FJ603945	FJ603946
Mp4817	FJ603957	FJ603958	FJ603959	FJ603960	FJ603961	FJ603962	FJ603963	FJ603964
Mp5181	FJ603975	FJ603976	FJ603977	FJ603978	FJ603979	FJ603980	FJ603981	FJ603982
Mp5191	FJ603993	FJ603994	FJ603995	FJ603996	FJ603997	FJ603998	FJ603999	FJ604000
Mp5192	FJ604011	FJ604012	FJ604013	FJ604014	FJ604015	FJ604016	FJ604017	FJ604018
Mp5194	FJ604029	FJ604030	FJ604031	FJ604032	FJ604033	FJ604034	FJ604035	FJ604036
Mp5196	FJ604047	FJ604048	FJ604049	FJ604050	FJ604051	FJ604052	FJ604053	FJ604054
Mp5245	FJ604065	FJ604066	FJ604067	FJ604068	FJ604069	FJ604070	FJ604071	FJ604072
Ofo	FJ604083	FJ604084	FJ604085	FJ604086	FJ604087	FJ604088	FJ604089	FJ604090

SUPPLEMENTARY TABLES CHAPTER 3

Table S1. Primer sequences and genomic positions and lengths of the RepMP5 elements within strains M129 and FH

Element	Genome position[a]	Primer 1 (5′ - 3′)	Primer 2 (5′ - 3′)	Amplicon length (bp)		Element Length (bp)[b]	
				M129	FH	M129	FH
RepMP5-a	113676-116092	tga tgg cat taa tta cta ttg c	cta cta ata gtt aga tgg agt	2417	2415	2417 (2050)	2415
RepMP5-b	131974-133863	gat tcc aat acc acc act gat	ttc aaa agt gaa aac cct act	1891	1891	1891 (1787)	1891

RepMP5-c	185648-188602	cag tgc ggc taa acc agg a	tgt gag gtc gtc ctg act g	2955	2754	2701 (2701)	2500
RepMP5-d	196236-199306	aca cat cag ggt act cat cag	gaa cac aat ttg aac tag ctg	3071	3071	3071 (725)	3071
RepMP5-e	435836-439626	tga gag gtt taa tgc ctc tca	tcg cct tgg ctt tga agt gt	3791	3792	3360 (1997)	3361
RepMP5-f	497466-499820	ggt gtt tgg gat ttg ttt gtc	ttc ttt tta aca cta cta gcg aa	2373	2373	2373 (2035)	2373
RepMP5-g	566089-568240	gtc tat gga tta att acc tca c	ctc tac atc aaa cta acg gct	2168	2168	2168 (2067)	2168
RepMP5-h	609094-612464	ccc act aaa taa att gag ttg	att tag tgg gag cag aca g	3371	3371	1825 (1764)	1825

[a]Genomic positions are derived from GenBank file U00089.2.
[b]Except for RepMP5-c, the lengths of all RepMP5 elements were redefined as opposed to the originally published sequences (in parentheses) from *M. pneumoniae* M129 U00089.2.

Table S2. Sequence identity of RepMP5 elements

M. pneumoniae strain M129								
RepMP5	a	b	c	d	e	f	g	h
a	100							
b	73	100						
c	73	64	100					
d	64	78	48	100				
e	73	77	65	56	100			
f	75	62	70	59	59	100		
g	87	61	85	70	72	91	100	
h	76	74	70	83	80	85	81	100

M. pneumoniae strain FH								
RepMP5	a	b	c	d	e	f	g	h
a	100							
b	72	100						
c	72	63	100					
d	65	78	54	100				
e	73	77	56	63	100			
f	74	62	76	59	59	100		
g	87	61	90	70	72	91	100	
h	76	74	82	83	80	85	81	100

SUPPLEMENTARY TABLES CHAPTER 6

Table S1. Prevalence of age, gender and season at enrolment for the group for which consent was given versus the group for which consent was not given

Category	Subcategory	Asymptomatic % (n/N)			Symptomatic % (n/N)		
		Consent	No consent [a,b]	p-value	Consent	No consent [c,d]	p-value
Age	< 5	50.1 (230/459)	49.9 (229/459)	0.15	46.4 (260/560)	53.6 (300/560)	0.14
	≥ 5	55.3 (182/329)	44.7 (147/329)		40.1 (61/152)	59.9 (91/152)	
Gender	F	52.8 (272/515)	47.2 (243/515)	0.31	47.8 (153/320)	52.2 (167/320)	0.88
	M	51.3 (140/273)	48.7 (133/273)		48.0 (168/350)	52.0 (182/350)	

Season	Winter	46.4 (84/181)	53.6 (97/181)	0.32	42.5 (122/287)	57.5 (165/287)	0.10
	Spring	55.3 (135/244)	44.7 (109/244)		38.0 (84/221)	62.0 (137/221)	
	Summer	51.6 (79/153)	48.4 (74/153)		41.5 (22/53)	58.5 (31/53)	
	Autumn	53.8 (114/212)	46.2 (98/212)		55.0 (93/169)	45.0 (76/169)	

[a.] Of 2 children in the asymptomatic no consent group, age was not recorded. [b.] Of 2 children in the asymptomatic no consent group, gender was not recorded. [c.] Of 18 children in the symptmatic no consent group, age was not recorded. [d.] Of 60 children in the symptomatic no consent group, gender was not recorded.

Table S2. Prevalences of *M. pneumoniae* in the asymptomatic group

Category	Subcategory	***M. pneumoniae*** PCR positive % (n/N)
Age	< 5	21.4 (48/224)
	≥ 5	20.8 (37/172)
Gender	F	19.2 (51/265)
	M	24.8 (34/137)
Immunizations	Complete	21.6 (83/384)
	Incomplete or none	0.0 (0/5)
Season	Winter	14.6 (12/82)
	Spring	10.5 (14/133)
	Summer	43.8 (32/73)
	Autumn	23.9 (27/113)
Year of enrolment	2009	14.7 (26/177)
	2010	23.4 (29/124)
	2011	30.0 (30/100)
Family size	< 5	18.8 (61/325)
	≥ 5	30.7 (23/75)
Smoking	No	22.8 (56/246)
	Active or passive	18.5 (28/151)
Presence or history of wheezing	None	20.8 (78/375)
	Yes	30.4 (7/23)
Daycare attendance	No	21.7 (59/272)
	Yes	20.2 (25/124)
RTI prior enrolment	No	22.3 (61/274)
	Yes	18.4 (23/125)
RTI post enrolment	No	26.4 (68/258)
	Yes	18.6 (16/86)

RTI respiratory tract infections

Table S3. Prevalences of *M. pneumoniae* in the symptomatic group

Category	Subcategory	***M. pneumoniae*** PCR positive % (n/N)
Age	< 5	14.6 (37/253)
	≥ 5	23.0 (14/61)
Gender	F	17.2 (26/151
	M	15.3 (25/163)
Immunizations	Complete	16.7 (50/300)
	Incomplete or none	0.0 (0/3)
Season	Winter	19.7 (24/122)
	Spring	19.5 (15/77)
	Summer	9.1 (2/22)
	Autumn	10.8 (10/93)
Year of enrolment	2008	3.6 (1/28)
	2009	6.5 (5/77)
	2010	20.9 (34/163)
	2011	23.9 (11/46)
Family size	< 5	15.0 (36/240)
	≥ 5	20.8 (15/72)
Smoking	No	16.2 (29/179)
	Active or passive	15.5 (20/129)
Presence or history of wheezing	None	15.5 (32/207)
	Yes	19.0 (19/100)
Daycare attendance	No	20.0 (30/150)
	Yes	12.2 (19/156)
Rhinorrhea	No	23.2 (26/112)
	Yes	12.4 (25/202)
Sore throat	No	16.5 (43/261)
	Yes	17.0 (8/47)
Earache	No	14.5 (37/256)
	Yes	24.6 (14/57)
Headache	No	16.3 (49/300)
	Yes	14.3 (2/14)
Myalgia	No	16.0 (50/312)
	Yes	50.0 (1/2)
Cough	No	16.9 (14/83)
	Yes	16.0 (37/231)
Fever	No	15.8 (15/95)
	Yes	16.4 (36/219)
Lower respiratory tract infection	No	15.6 (10/64)

	Yes	16.6 (40/241)
Hospitalisation	No	17.1 (7/41)
	Yes	16.5 (41/248)

Table S4. *M. pneumoniae* subtypes in asymptomatic and symptomatic children

		Asymptomatic	Symptomatic
M. pneumoniae subtype	1	31 (75.6)	25 (83.3)
	2	10 (24.4)	5 (16.7)

Table S1. Primers

Assay/Targets	Primers		Sequence (5'®3') and labels	Amplicon size (bp)
MPN141	External	Forward primer	GAA ACA CCT CCT CCA CCA AC	151
		Reverse primer	CCA TCT AAC AGT TCA GCG AG	
	Internal	Forward primer	GAA TGA TGT GGT GGG GGT TG	84
		Reverse primer	*Biotin*-GGG GTG CGT ACA ATA CCA TCA A	
	Sequence primer		CAA CGC CGC AAA GAT	
MPN528a	External	Forward primer	TAC GAC TAG GAG ATC ATC CAG	232
		Reverse primer	TTA AGG TGT TTG AGT TGG TGT	
	Internal	Forward primer	ATC TAC CGA TTC AAC CAA CTG CT	166
		Reverse primer	*Biotin* -GCT AAC TGC GCT AGA GCA AAA T	
	Sequence primer		AGA AAT CGA AAA CTG ACT AT	
23S rRNA (assay 1)	External	Forward primer	ACC ATC TCT TGA CTG TCT CG	183
		Reverse primer	CAT CAA CAA GTC CTA GCG AAC	
	Internal	Forward primer	TCG GTG AAA TCC AGG TAC G	130
		Reverse primer	*Biotin* -CAT CGA TTG CTC CTA CCT ATT CTC	
	Sequence primer		AGG CGC AAC GGG ACG	
23S rRNA (assay 2)	External	Forward primer	GAT TAA AGA GAT ACG TGA GTT G	175
		Reverse primer	ATA GAC ACG TTA CTA CCC AG	
	Internal	Forward primer	*Biotin* -TTC AAA CCG TCG TGA GAC AG	113
		Reverse primer	AAC TGG AGC ATA AGA GGT GTC CT	
	Sequence primer		CTA CGG GCA CAA TAG AT	

LIST OF PUBLICATIONS

Published, reviewed:

1. *Mycoplasma pneumoniae* infections–does treatment help? Spuesens EB, Meyer Sauteur PM, Vink C, van Rossum AM.
   J Infect. 2014 Nov;69 Suppl 1:S42-6. doi: 10.1016/j.jinf.2014.07.017. Epub 2014 Sep 26. Review.

2. Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study.
   Spuesens EB, Fraaij PL, Visser EG, Hoogenboezem T, Hop WC, van Adrichem LN, Weber F, Moll HA, Broekman B, Berger MY, van Rijsoort-Vos T, van Belkum A, Schutten M, Pas SD, Osterhaus AD, Hartwig NG, Vink C, van Rossum AM.
   PLoS Med. 2013;10(5):e1001444. doi: 10.1371/journal.pmed.1001444. Epub 2013 May 14.

3. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* in respiratory specimens collected between 1997 and 2008 in The Netherlands.
   Spuesens EB, Meijer A, Bierschenk D, Hoogenboezem T, Donker GA, Hartwig NG, Koopmans MP, Vink C, van Rossum AM.
   J Clin Microbiol. 2012 Jun;50(6):1999-2004. doi: 10.1128/JCM.00400-12. Epub 2012 Apr 11.

4. Variation in a surface-exposed region of the *Mycoplasma pneumoniae* P40 protein as a consequence of homologous DNA recombination between RepMP5 elements.
   Spuesens EB, van de Kreeke N, Estevão S, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C.
   Microbiology. 2011 Feb;157(Pt 2):473-83. doi: 10.1099/mic.0.045591-0. Epub 2010 Oct 21.

5. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* by pyrosequencing.
   Spuesens EB, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C.
   J Microbiol Methods. 2010 Sep;82(3):214-22. doi: 10.1016/j.mimet.2010.06.004. Epub 2010 Jun 11.

6. Review: Moleculaire typering van *Mycoplasma pneumoniae*
   E.B.M. Spuesens, N.G. Hartwig, A.M.C. van Rossum, C. Vink.

Ned. Tijdschr. Med. Microbiol. 2012;20:1

Letters to the editor (reviewed)

7. Sequence variation within the P1 gene of *Mycoplasma pneumoniae*.
   Spuesens EB, Hartwig NG, van Rossum AM, Vink C.
   J Clin Microbiol. 2011 Oct;49(10):3723; author reply 3723-4. doi: 10.1128/JCM.01318-11. No abstract available.

8. Identification and classification of P1 variants of *Mycoplasma pneumoniae*.
   Spuesens EB, Hartwig NG, van Rossum AM, Vink C.
   J Clin Microbiol. 2010 Feb;48(2):680; author reply 680. doi: 10.1128/JCM.02078-09. No abstract available.
   PMID: 20118382 Free PMC Article

Adaptation from

9. Sequence variations in RepMP2/3 and RepMP4 elements reveal intragenomic homologous DNA recombination events in *Mycoplasma pneumoniae*.
   Spuesens EB, Oduber M, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C.
   Microbiology. 2009 Jul;155(Pt 7):2182-96. doi: 10.1099/mic.0.028506-0.

Unpublished

10. Comparison of *Mycoplasma pneumoniae* genome sequences from strains isolated from symptomatic and asymptomatic patients. In preparation for publication in Frontiers of Microbiology.

PHD PORTFOLIO

Persoonsgegevens			
Name PhD student	E.B.M. Spuesens	PhD period	01-01-2008 – 13-9-2016
		Promotor	Prof. H.A. Moll
Erasmus MC department	Pediatrics		Dr. A.M.C. van Rossum
Research school	MolMed	Co-promotoren	Dr. C. Vink

1. PhD training

	Year	Workload (ECTS)
General academic skills		
- TULIPS PhD curriculum	2011-2013	2.0
- Grant Writing Weekend TULIPS	2011	0.9
- Biomedical English Writing and communication	2011	4.0
- TULIPS workshop 'Mentorship'	2010	0.3
Research Skills		
- Working visit. Subject: Targeted isolation of transposon insertion mutant strains of *Mycoplasma pneumoniae*. Department Microbiology, Georg August Universität, Göttingen, Germany. Head: Prof. Dr. J. Stülke	2010	4.3
- ESPID Research Master Class (29ste European Society of Pediatric Infectious Diseases (ESPID) conference)	2011	1.0
In Depth Courses		
- Medical training (Opleiding kindergeneeskunde) Cluster Rotterdam, Erasmus MC – Sophia. Einddatum 01-01-2016	2012-2016	
Presentations		
- Asymptomatic carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of children. **Poster.** European Society for Pediatric Infectious Diseases	2012	0.4
- Detection of *Mycoplasma pneumoniae* in healthy children by real time PCR – preliminary data from the MymIC study. **Voordracht.** European Society for Pediatric Infectious Diseases.	2011	0.9
- Evidence for antigenic variation of a virulence factor in *Mycoplasma pneumoniae*. **Voordracht,** E-poster presentatie. European Society for Pediatric Infectious Diseases	2011	0.9
- Macrolide resistance determination and molecular typing of *M. pneumoniae* by pryosequencing on clinical samples. **Poster** presentatie. European Society for Pediatric Infectious Diseases	2011	0.4
- Macrolide resistance determination and molecular typing of *M. pneumoniae* by pryosequencing on clinical samples. **Voordracht.** Nederlandse vereniging voor Microbiologie, Papendal, Arnhem,	2011	0.9
- Detection of *Mycoplasma pneumoniae* in healthy children by real time PCR – preliminary data from the MymIC study. **Poster** presentatie. Nederlandse vereniging voor Microbiologie	2011	0.4

- Variation in a surface-exposed region of the *Mycoplasma pneumoniae* P40 protein as a consequence of homologous DNA recombination between RepMP5 elements. **Poster** presentatie. Nederlandse vereniging voor Microbiologie, Papendal	2011	0.4
- Macrolide resistance determination and molecular typing of *M. pneumoniae* by pryosequencing. **Voordracht.** Molecular Medicine (MolMed) day	2011	0.9
- Detectie van *Mycoplasma pneumoniae* met real time PCR in gezonde kinderen – preliminaire data uit de MymIC-studie. **Poster** presentatie. NVK congres, Veldhoven	2010	0.4
- Macrolide resistance determination and molecular typing of *M. pneumoniae* by pryosequencing. **Voordracht.** International Organization for Mycoplasmology Congress	2010	0.9
- Rapid antibitiotic resistance determination and genotyping of *Mycoplasma pneumoniae* by pyrosequencing, **Poster** presentatie. European Society for Pediatric Infectious Diseases	2010	0.4
- Variatie in repeterende DNA elementen in *Mycoplasma pneumoniae*: een bron voor antigene variatie. **Poster** presentatie. NVK congres, Veldhoven, november 2009.	2009	0.4
- Repetitive elements in the respiratory pathogen *Mycoplasma pneumoniae*: a pool for antigenic variation. **Voordracht.** Onderzoeksdag kindergeneeskunde, Erasmus MC – Sophia	2009	0.9
- Sequence Variations in RepMP2/3 and RepMP4 Elements Reveal Intragenomic Homologous DNA Recombination Events in *Mycoplasma pneumoniae*. **Poster** presentatie. European Society for Pediatric Infectious Diseases	2009	0.4
- Sequence Variations Reveal Intragenomic Homologous DNA Recombination Events in *Mycoplasma pneumoniae*. **Voordracht.** NVK, Sectie Pediatrische infectieziekten en immunologie	2009	0.9
International conferences		
- ESPID (European Society for Pediatric Infectious Diseases) Annual Conference attendance 2009, 2010, 2011, 2012		*5.7*
- IOM (International Organization for Mycoplasmology) Biannual Conference attendance 2008, 2010		2.9
Workshops and seminars		
- Mycoplasma Techniques Workshop, IOM Conference	2008	1.0
Didactic skills		
Other		
- **Article review for** Journal of Medical Microbiology, Respiratory Journal, and Nederlands tijdschrift voor geneeskunde	2008-2012	
2. Teaching activities		
Supervising master theses		
- D. Bierschenk, HLO student	2010-2011	

Project 1: Macrolide resistance determination and molecular characterization of *Mycoplasma pneumoniae* strains isolated in the Netherlands in a 12 years period.	
Project 2: Characterization of two putative helicases of *Mycoplasma pneumoniae*.	
- N. van de Kreeke, MLO student (november 2009 – juli 2010)	2009-2010
Genetische variatie van het ORF6 gen *Mycoplasma pneumoniae* als gevolg van homologe DNA recombinatie tussen RepMP5 elementen.	
Supervisising Research students MymIC study	
- L. Spadon	2009-2012
- I. Gondrie	

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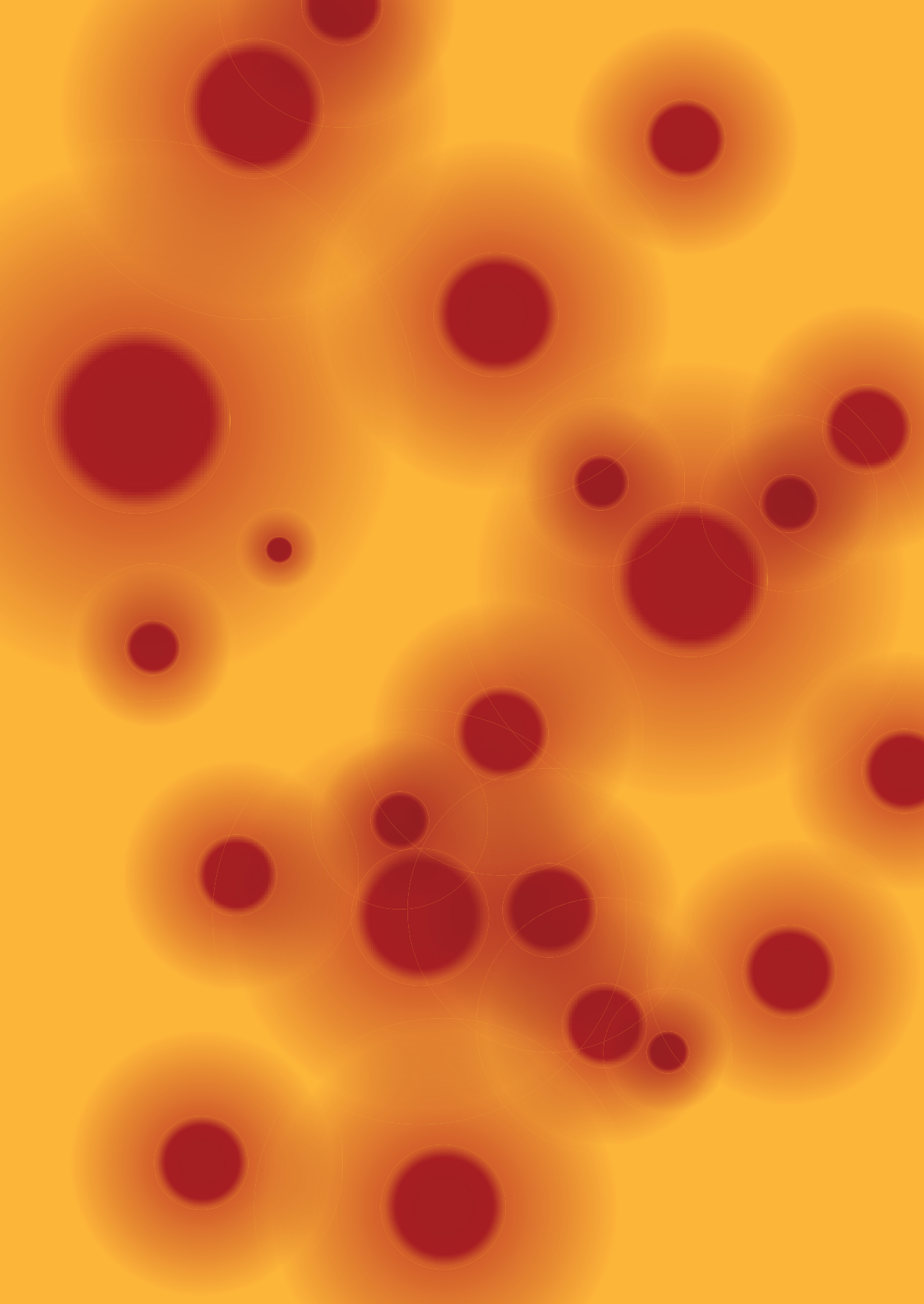
Emiel

ABOUT THE AUTHOR

Emiel Spuesens was born on December 2nd 1978 in Oostburg, West Zeeuws Vlaanderen, the Netherlands. In 1998 he completed secondary school at 't Zwin College in Oostburg. He started studying medicine at the University of Leiden in 1998 and he became a medical doctor in 2005, after which he started working as ANIOS first in IKAZIA hospital in Rotterdam in 2006 and later in the Albert Schweitzer Hospital in Dordrecht. In 2007 he started working in Erasmus MC Sophia and in 2008 he started his PhD project (project leader Dr. A.M.C. van Rossum) at the department of Pediatric Infectious Diseases and Immunology (head Dr. N.G. Hartwig, promotor Prof.dr. H.A. Moll). As part of his PhD training he visited the Georg August Universität, Department Microbiology, Göttingen, Germany in 2010 (Head Prof.dr. J. Stülke) to study transposon insertion mutant strains of *M. pneumoniae*. From 2011-2013 he attended the TULIPS (Training Upcoming Leaders in Pediatric Science) program.

In 2012 he started his training to become a pediatrician at Erasmus MC Sophia. Meanwhile he continued working on his PhD thesis and published his final articles. In 2016 he became a pediatrician and started working at the department of Pediatrics at LUMC in Leiden. In June 2016 he returned to Erasmus MC Sophia to work as general pediatrician at the deparment of general pediatrics (head Prof.dr. H.A. Moll).

Emiel is married to Ilana Spuesens - Hoefnagel and together they live with their daughter Isabella in Rotterdam.



Emiel B.M. Spuesens

Mycoplasma pneumoniae

Bacterial genetic variation and colonization of the respiratory tract of children

M. pneumoniae is regarded as a common cause of community acquired pneumonia. We posed the hypothesis that *M. pneumoniae* is able to colonize the human respiratory tract and, assuming that our hypothesis could be confirmed, we also set out to study whether possible asymptomatic carriage could be distinguished from infection. In the first part the findings of bacterial genetic variation are described. We describe that *M. pneumoniae* subtype-1 and subtype-2 represent genetically distinct lineages with a high potential for variation by homologous DNA recombination. The second part describes the prevalence of *M. pneumoniae* detected by PCR, which is just as high in asymptomatic children as in symptomatic children but changes over time and season. The strains found in asymptomatic and symptomatic children do not seem to differ genetically. *M. pneumoniae* macrolide resistant strains have not been identified in the Netherlands. Finally we discuss the consequences of our findings on *M. pneumoniae* colonization and bacterial genetic variation. We discuss that diagnostic tests for an alleged *M. pneumoniae* infection should be interpreted with caution and we question whether a presumed *M. pneumoniae* infection should always result in antibiotic treatment.