Molecular detection and epidemiological studies of atypical bacteria causing respiratory tract infections

KAROLINA GULLSBY
Respiratory infections are common causes of morbidity and mortality. *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Bordetella pertussis* cause respiratory infection, often with similar symptoms. Molecular diagnostic methods are preferred since these bacteria are difficult to culture. The aim of this thesis was to evaluate and improve the diagnostics and knowledge of the epidemiology of these bacteria.

A real-time polymerase chain reaction (PCR) method targeting the IS481 element present in the genome of *B. pertussis* was compared to culture and serology results, and a duplex real-time PCR method was constructed for detecting *C. pneumoniae* and *M. pneumoniae*, which was compared to two endpoint PCR methods. Both real-time PCR methods showed high sensitivity and specificity.

Typing of 624 *M. pneumoniae* samples, collected from 1996 to 2017 from four counties, was performed by P1 typing and multiple-locus variable number tandem repeat analysis (MLVA). A polyclonal distribution of strains was seen over all epidemic periods, but strains of P1 type 2/variant 2 and MLVA types 3-5-6-2 and 4-5-7-2 predominated in 2010–2013. A shift from type 2 strains to different variant 2 strains was seen and a new variant, 2e, was detected in 2016–2017. An A2063G mutation associated with macrolide resistance was detected by a fluorescence resonance energy transfer (FRET) PCR method in one (0.16%) of 608 *M. pneumoniae* strains.

Molecular characterisation using whole-genome sequencing of 93 *B. pertussis* isolates, collected between 1986 and 2016 from three counties showed that there were polyclonal strains in the county of Dalarna, Gävleborg and Uppsala in the years 2014–2016. Changes in virulence-related genes were detected: a shift from isolates harbouring the ptxP3 allele in favour of ptxP1 was seen, and almost all isolates had a disrupted *prn* gene. No detection of macrolide resistance in *B. pertussis* was detected.

In conclusion, the validated real-time PCR methods for detection of *B. pertussis*, *C. pneumoniae* and *M. pneumoniae* have led to improved diagnostic methods for use in clinical laboratories. The molecular characterisation of *M. pneumoniae* and *B. pertussis* strains has contributed to the wider understanding of the genetic changes that has occurred over the epidemic periods, but further studies is needed.

**Keywords:** Chlamydia pneumoniae, Mycoplasma pneumoniae, Bordetella pertussis, real-time PCR, P1 typing, MLVA, whole-genome sequencing, macrolide resistance, molecular diagnostics, molecular epidemiology

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To my family ♥
Staffan, Stina, Molly and Signe
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<td>Acellular vaccine</td>
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<td>Base-pair</td>
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<td>Community-acquired pneumonia</td>
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<td>NIP</td>
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Introduction

The use of sensitive and specific diagnostic methods, which can detect microbial pathogens and reveal the presence of resistance towards antibiotics or other kinds of antimicrobial treatments, are helpful tools to guide the clinician on how to best care for the patient and for limiting the risk of spreading diseases. Microbiological diagnostics contribute to optimisation of the treatment strategy so treatment failure, prolonged illness, a spread of infections and suffering for the patient can be reduced. It is important that unnecessary uses of antibiotics are minimised since increased usage is a driving force for the emergence of antibiotic resistance, which is a serious problem worldwide (1).

Diagnostics can also improve the knowledge of the epidemiology of circulating microorganisms. Monitoring the spread of microorganisms, by studying the variability of strains found within the population over time, may lead to a better understanding of the mechanisms behind the upcoming of epidemics, increased incidences and vaccine efficacy.

Respiratory tract infections

Respiratory tract infections are one of the most common causes of morbidity and mortality (2, 3). Various bacteria and viruses can cause respiratory infections, and symptoms can be diverse. Upper respiratory tract infections are often milder and should usually not be treated with antibiotics, while lower respiratory tract infections, such as pneumonia, can be very serious and life-threatening. Among children below the age of five years, lower respiratory tract infections are the leading causes of death worldwide (4).

Some of the common causes of respiratory infections are *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydia pneumoniae, Bordetella pertussis, Legionella pneumophila*, influenza viruses, respiratory syncytial virus, human metapneumovirus, rhinovirus and parainfluenza viruses (5-11). Coinfections of several respiratory viruses, bacteria, or combinations of viruses and bacteria are common (5-11). The described prevalence and clinical significance of each pathogen depends on the study population, symptom and season when the sampling is taken but also on the diagnostic methods that are used.
Recently, multiplex molecular assays have been developed where several respiratory pathogens can be detected at the same time (5, 12, 13). The use of multiplex methods leads to a higher diagnostic yield which may improve the handling of the patient (14).

Atypical bacteria causing respiratory infections

C. pneumoniae and M. pneumoniae are traditionally classified as atypical bacteria that cause respiratory infections and are important causes of community-acquired pneumonia (CAP) (15). Common characteristics of these atypical bacteria, as opposed to the typical respiratory bacteria (i.e. S. pneumoniae), are that they do not have an ordinary cell wall, they are intracellular and they are difficult to culture, which is the traditional microbiological diagnostic method.

B. pertussis has also been named an atypical respiratory bacteria, which may also be referred to that it has often been overlooked especially when presented with atypical symptoms (8, 16, 17).

C. pneumoniae, M. pneumoniae and B. pertussis cause respiratory infections, often with similar symptoms (8). Molecular diagnostic methods are preferred since culture-based detection of these bacteria is difficult due to specific nutritional needs, has lower sensitivity and is time-consuming (17, 18).

Chlamydia pneumoniae

General characteristics

C. pneumoniae is an obligate intracellular bacterium with a genome-size of 1.2 Mbp. It has a unique biphasic developmental cycle, where it changes between a smaller extracellular infectious form (elementary body), and a larger intracellular metabolically active form (reticulate body) (19).

The bacterium belongs to the family Chlamydiaceae, and it is under the order Chlamydiales and class Chlamydiae. It was first detected in 1965 in the eye of a child in Taiwan but was not associated with respiratory infections until 1985. It was by then thought to be a different strain of Chlamydia psittaci (20, 21). At first, the bacteria was called TWAR, a name based on the first isolated strains, TW-183 and AR-39, and in 1989 it was designated as a new species Chlamydia pneumoniae (22). In 1999 the genus Chlamydia was separated into Chlamydia and Chlamydophila, mainly based on differences in the 16S rRNA gene, and the bacterium was thus called Chlamydophila pneumoniae (19). Recently the two genera have been proposed to be
reunified into one genus, *Chlamydia*, based on further genomic and proteomic studies (23, 24).

**Other species of Chlamydia**

Other well-known human pathogens of the same genus as *C. pneumoniae* are *C. psittaci* and *C. trachomatis*. *C. psittaci* causes respiratory infections predominantly and is primarily transferred from birds to humans (25). *C. trachomatis* is an important cause of urogenital infections which are sexually transmitted, but it may also cause eye infections, trachoma, which lead to blindness if untreated (26, 27).

**Clinical manifestations and prevalence**

*C. pneumoniae* has been isolated in a wide variety of animals such as reptiles, mammals and birds (28). In humans it is primarily a respiratory pathogen but has also been associated with, for example, acute asthma exacerbations and atherosclerosis (29, 30).

*C. pneumoniae* infections can affect all ages, and the incubation period is about three weeks. *C. pneumoniae* is an important cause of community-acquired pneumonia (CAP). Previous studies indicate it is the cause of about 10–15% of the cases, although findings in recent studies are much lower, 0–3.5% (6, 9, 11, 31-35).

Serological studies have shown that 30–50% of children and adolescents of 10–18 years and around 70% of adults carry antibodies against *C. pneumoniae*, which indicates that infections are common (36-39). Infections may present as asymptomatic or with mild symptoms, and in rare cases the infection is persistent (40, 41). Outbreaks in closed settings, such as military compounds, due to *C. pneumoniae*, have also been described (42, 43).

Polymerase chain reaction (PCR) methods for the detection of *C. pneumoniae* have been used since the mid-1990s at Gävle County Hospital. Diagnostic data collected at the clinical microbiological laboratory at the hospital showed a large decrease in the proportion of PCR-positive samples, where very few positives were detected annually from the year 2004 and forward (Figure 1).


Mycoplasma pneumoniae

General characteristics

*M. pneumoniae* is one of the smallest bacteria known with a genome-size of only 0.8 Mbp, as compared to other bacterial genomes ranging from 0.6 to 14 Mbp. It lacks a cell wall and has a flask-like shape with a tip structure, which is important for adhesion of the bacteria to epithelial cells (44).

The bacterium belongs to the family *Mycoplasmataceae*, and it is under the order *Mycoplasmatales* and class *Mollicutes*. It was first isolated from a patient with pneumonia in 1944, and it was called the Eaton agent, after its discoverer (44). Due to the lack of a cell wall and the small size, it was first believed to be a virus. Laboratory testing on volunteer prisoners in the 1960s verified that the agent was a cause of respiratory infections in humans (45). In 1963 it was cultured on a cell-free medium for the first time, and it was designated *Mycoplasma pneumoniae* (46).

Other species of *Mycoplasma*

Examples of other species of *Mycoplasma* that cause infections in humans are *M. genitalium* and *M. hominis*, which can both be found in the urogenital tract. *M. genitalium* is known to cause urogenital infections that are sexually
transmitted (47, 48). The clinical relevance of *M. hominis* as a cause of genital infections is uncertain, but it has been associated with bacterial vaginosis (49, 50).

Clinical manifestations and prevalence

*M. pneumoniae* is exclusively a human pathogen that primarily causes respiratory infections but can sometimes lead to extrapulmonary manifestations such as various autoimmune responses, central nervous infections and hemolytic anaemia (51-54).

Similar to *C. pneumoniae*, the incubation period of *M. pneumoniae* infections is about three weeks. *M. pneumoniae* is an important cause of CAP, with 5–20% of the cases (6, 9, 11, 31, 34). Infections of *M. pneumoniae* are more common during late summer and fall, and there are cyclic epidemics that occur every 3–7 years (44, 55). In the period 2010–2011, an extraordinary epidemic peak was seen in many countries (55-60). Why these epidemics occur is not fully understood, but shifts in circulating strains, together with the natural decrease in the immunity of the population, may be contributing factors (61, 62).

Molecular methods for detection of *M. pneumoniae* have been used since the mid-1990s at the clinical microbiological laboratory at Gävle County Hospital. Diagnostic data from the hospital show a cyclic fluctuation with a rise in the proportion of PCR-positive samples every 4–6 years (Figure 1).

Treatment of *M. pneumoniae* and macrolide resistance

Since *M. pneumoniae* lacks an ordinary cell wall, β-lactam antibiotics, such as penicillin, have no effect. Tetracycline, macrolides and fluoroquinolones can be used to treat *M. pneumoniae* infections (63). The only class of antibiotics that is appropriate to use for the treatment of infections in children is macrolides, due to the adverse effects of tetracycline and fluoroquinolones (63).

In 2000 the first report of macrolide-resistant *M. pneumoniae* came from Japan (64). Since then, there has been a dramatic increase in macrolide resistance in Asia, with about 80–90% resistant strains in China and Japan (65, 66). Other countries such as Australia, Denmark, France, Germany, Israel and the United Kingdom have described findings of macrolide resistance with lower and diverse incidence, from 1 to 30% (67-72). The resistance mechanism is connected to single mutations in the peptidyltransferase loop of domain V of the 23S rRNA gene (Figure 2) (63, 64).
Figure 2. Secondary structure of the peptidyltransferase loop of domain V of the 23S rRNA of *M. pneumoniae*. Nucleotides found in macrolide-resistant *M. pneumoniae* (Escherichia coli numbering in brackets). Modified, with permission to reproduce from Matsuoka et al. (64).

The P1 gene and repetitive sequences

The tip structure of *M. pneumoniae* is responsible for the gliding motility and attachment of the bacterium to ciliated epithelial cells, which are critical for infection (73, 74). One of the primary proteins in the tip structure is the P1 cytadhesion protein, which is coded by the P1 gene (MPN141), which serves as an important immunogen (75-77). The P1 gene is a commonly used target for molecular detection of *M. pneumoniae* (78). Sequence variations of the P1 gene are also used in typing *M. pneumoniae* (79-81). The gene includes two repetitive sequences, RepMP4 and RepMP2/3. In total, there are 8 and 10 copy versions of these repetitive sequences, respectively (denoted as RepMP4 a-h respectively RepMP2/3 a-j), dispersed in the genome (Figure 3) (82, 83). Intragenomic recombination events between copy versions outside the gene and within the gene are suggested as the mechanism behind the occurrence of new P1 variants (83).
**Figure 3.** Structure of the *M. pneumoniae* P1 gene (MPN141) and distribution of RepMP2/3 and RepMP4 elements within the *M. pneumoniae* genome. The location and orientation of the 10 RepMP2/3 and 8 RepMP4 elements within the *M. pneumoniae* M129 genome are indicated by the grey and black boxes, respectively. The elements are labelled ‘a’ to ‘j’ for RepMP2/3 and ‘a’ to ‘h’ for RepMP4. With permission to reproduce from Spuesens et al. (83).

**Bordetella pertussis**

**General characteristics**

*B. pertussis* is a small gram-negative bacterium with a genome size of 4.1 Mbp. It is the cause of pertussis, also called whooping cough.

The bacterium belongs to the family *Alcaligenaceae*, and it is under the order *Burkholderiales* and the class *ß*-proteobacteria. In Chinese medical literature from the seventh century, there is a description of “the cough of 100 days”, which is believed to be the first description of a *B. pertussis* infection (16). Outbreaks of pertussis-like illnesses have also been described in Europe since the 16th century, but the bacterium was not isolated until 1906 by the scientists Jules Bordet and Octave Gengou (16). The bacterium was named *Bordetella pertussis*, and in 1920 Jules Bordet received the Nobel Prize in part for his work in identifying the organism.

**Other species of Bordetella**

There are eight other species belonging to the genus *Bordetella*: *B. parapertussis, B. bronchiseptica, B. holmesii, B. avium, B. hinzii, B. petrii, B. trema-
tum and B. anisorpii. B. parapertussis, B. holmesii, and in rare cases B. bronchiseptica, can cause pertussis-like illnesses but which is often milder (16, 84, 85). Genetic data indicate that B. pertussis and B. parapertussis are closely related to and are each derived from a B. bronchiseptica-like ancestor (86).

Clinical manifestations and prevalence

B. pertussis is a strictly human pathogen which gives rise to whooping cough. In previously immunised or infected individuals, the symptoms are often mild and nonspecific but infants are at high risk of being afflicted by severe and life-threatening disease. WHO estimated that pertussis caused 60,000 deaths in 2013 worldwide (87). B. pertussis infections in the adult and adolescent population are probably underdiagnosed due to the milder and less specific symptoms (16, 88).

The incubation period of B. pertussis is about 1−2 weeks. The classical symptoms of a B. pertussis infection can be divided into three phases which starts with the catarrhal phase, 1−2 weeks, after infection, where the symptoms resemble ordinary cold symptoms with mild cough (16). In the paroxysmal phase, 1−6 weeks after infection, the cough is more violent with paroxysms and inspiratory whooping episodes, and in infants it can lead to hypoxia and death. In the convalescent phase the coughing subsides, but can still last for several weeks.

At the clinical microbiological laboratory at Gävle County Hospital, culturing of B. pertussis was the diagnostic method performed until 2006 when it was complemented by real-time PCR as the primary method. Diagnostic data of PCR-positive samples show a generally high positivity rate, with an average of 11.7% (Figure 1).

Treatment of B. pertussis and macrolide resistance

When the pertussis infection has reached the paroxysmal phase antibiotic treatments have no effect on the symptoms. Macrolides, such as erythromycin, are sometimes given to patients to reduce infectivity and also given as prophylaxis to infants who have been exposed. Macrolide-resistant strains have been detected in the United States, China and other countries in Asia, but they are uncommon in European countries (89-93). Macrolide-resistant strains harbour an A to G mutation at the 2058 position (E. coli numbering) of the 23S rRNA gene, equal as described for M. pneumoniae (Figure 2) (89).

Reemergence of B. pertussis infections

Despite a high vaccine coverage, B. pertussis remains an endemic infection, and lately, there have been large outbreaks and an increased incidence in many countries such as Australia, Japan, the United Kingdom, the United
States and Spain (94-99). Waning vaccine-induced immunity and genetic changes of the circulating strains could be reasons for the increased incidence (100).

Since 2014 the incidence of B. pertussis has risen in Sweden (Figure 4). In 2014 the counties of Dalarna and Gävleborg had among the highest incidences in Sweden (17.9 and 14.6 cases per 100,000 inhabitants, respectively), and in 2016 the county of Uppsala had the highest incidence (16.3 cases per 100,000 inhabitants) (101).

Figure 4. Incidence of B. pertussis in Sweden 1997-2018 (101).

Vaccination

Vaccination with a whole-cell vaccine (WCV) against B. pertussis started in the 1940s and was introduced in 1953 in Sweden. In 1979, due to the adverse effects and decreased efficacy of WCV, the vaccine was withdrawn from the National Immunisation Program (NIP) in Sweden (102). There was no vaccination against B. pertussis included in the NIP until 1996, when acellular vaccines (ACVs) were introduced in Sweden. Booster doses have been added to the vaccination program, which now consists of three primary doses within the first year (at 3, 5 and 12 months), a booster dose at the age of five and one additional dose at around the age of 14–16 years (102). The vaccine coverage is 85% worldwide and > 97% in Sweden (102, 103). Introduction of B. pertussis vaccines has reduced the incidence of infections, although the immunity after vaccination is not life-long (101, 104-106). Studies have shown that household contacts are usually the cause of spreading the infection to infants too young to be fully vaccinated (107). To better
protect the unvaccinated infant, maternal immunisation programs have started in several countries with good results (108).

**Virulence factors and virulence-related genes of *B. pertussis***

*B. pertussis* has several virulence factors that play important roles in the pathogenesis. The pertussis toxin (PT), which is specific to *B. pertussis*, is the most known and is one of the components present in ACVs. The pertussis toxin gene (*ptxA*) is also present in *B. parapertussis* and *B. bronchiseptica* but is not expressed due to differences in the promoter region (86).

Other virulence factors that are common components in some of the ACVs are the adhesion proteins: filamentous heamagglutinin (FHA), fimbriae (Fim) and pertactin (PRN). There are two serotypes of fimbriae, Fim2 and Fim3. In Sweden the vaccine distributed to infants is either a two-component, containing PT and FHA, or a three-component vaccine containing PT, FHA and PRN (102). The three-component vaccine has been used in the counties of Dalarna, Gävleborg and Uppsala since 2013.

A reason for the increased incidence of *B. pertussis* is suggested to be due to genetic differences between circulating strains and components included in the vaccine. There has been a shift in the *B. pertussis* population where strains harbouring the specific pertussis toxin gene promoter allele, *ptxP3*, which induces a higher expression of the pertussis toxin, have replaced strains with *ptxP1* (109, 110). There have also been findings of *B. pertussis* isolates that do not express the antigens: PT, PRN and FHA, which may influence the fitness of the bacteria (111-113).

**Insertion sequences**

Insertion sequences (IS) are small mobile genetic elements which are flanked by an inverted repeat sequence (IR) and a shorter direct target DNA repeat sequence (DR) (Figure 5). They harbour a transposase gene which codes for the enzyme responsible for the mobilisation of the element. There are different types of IS elements which are classified into families primarily based on sequence similarities of the transposase gene (114).

The genome of *B. pertussis* contains many IS elements: around 240 copies of IS481, 4–6 copies of IS1002 and 16–17 copies of IS1663 elements (86, 115-117). Genomic data indicate that IS expansion in *B. pertussis* stands for the majority of rearrangements and has led to a reduction of the genome size (86, 117). Rearrangements of IS elements have caused disruption of virulence-related genes and thus altered the antigenicity and even pathophysiology of *B. pertussis* (98, 115).

Due to the many copies of IS481, it is a sensitive target often used for molecular detection (118). The specificity of the target has been discussed
since it is also present in the genome of *B. holmesii* and some strains of *B. bronchiseptica* (119-122).

| DR | IR | Transposase gene | IR | DR |

*Figure 5. Schematic figure of an IS element. It encodes a transposase gene which is flanked by an inverted repeat sequence (IR) and direct repeat sequence (DR).*

### Diagnostic methods for the detection of bacterial infections

In routine clinical laboratories, the most common diagnostic methods for the detection of bacteria are culture, which is often referred to as the gold standard, serology and molecular methods. Each has its advantage, but it is important that the sample is taken at an appropriate time and in an optimal way to secure the proper performance of the methods.

#### Culture

The advantages of culturing are that bacterial isolates can be obtained, enabling phenotypic antimicrobial-resistance testing and facilitating typing of the bacteria, and it has a high specificity. However, culturing of *C. pneumoniae, M. pneumoniae* and *B. pertussis*, which are all slow-growing bacteria with special growth conditions and nutritional needs, is tedious and takes about 6−10 days for *C. pneumoniae* and *B. pertussis* and up to 21 days for *M. pneumoniae* (18, 123). Culturing also has a lower sensitivity compared to serology and molecular methods (18, 54, 123). Today, no Swedish clinical microbiological laboratory performs routine culturing of *C. pneumoniae* or *M. pneumoniae*. The culturing of *B. pertussis* is performed at few laboratories, usually as a secondary method after molecular detection.

#### Serology

Serological methods have a high diagnostic sensitivity but usually require that both an acute and convalescent serum sample is drawn (123-125). The antibody response usually takes a few weeks to develop, which limits the use of serology for acute diagnostics (125). However, in the later course of an infection, serology may be the only suitable method when viable bacteria or bacterial DNA has been cleared (123). Antibody response can persist for a long time after infection but in some cases an antibody response is absent even if the pathogen is detected (123-125).
Molecular methods

Molecular methods are generally very sensitive and can detect low amounts of bacterial nucleic acid. They can also detect nucleic acid from dead bacteria after, for example, the initiation of antibiotic treatment. PCR is currently the most common molecular method used in clinical laboratories. Advantages of molecular methods include that they are fast and they can be multiplexed, where several agents can be detected from one sample.

PCR

To perform PCR, the bacterial DNA is extracted and separated from other cellular components and ingredients in the sample. PCR is performed by mixing short DNA strings (primers) that are complementary to the target DNA, deoxynucleotide triphosphates (dNTPs) and a thermostable enzyme (Taq polymerase) with the extracted DNA (template). The target DNA, which can be a specific gene or sequence of the bacteria, is exponentially amplified during repeated heat and cold cycles. Detection of the endpoint PCR is performed after the amplification of the DNA, whereas, for newer molecular methods, such as real-time PCR, the detection is performed in real-time, during amplification with fluorescent-marked DNA strings (probes). Real-time PCR offers the possibility of quantification and multiplexing and is faster than endpoint PCR. The performance of a PCR method depends on multiple factors. The choice of the target DNA needs to be specific and conservative, and all steps in the process, including the DNA extraction, need to be optimised and carefully validated.

Detection methods for endpoint PCR

Gel electrophoresis

Gel electrophoresis is a common method for the detection of PCR products in endpoint PCR protocols. The detection is performed after the amplification of the DNA is done. Amplified DNA is pipetted into small wells on an agarose gel. The PCR product is subjected to an electric field, and since the DNA is negatively charged, it will migrate towards the positively charged side of the gel at different speeds, depending on the size of the amplified DNA. The migrated DNA is visualised using intercalating fluorescent dyes specific for double-stranded DNA.

Sequencing of PCR product

The type of sequencing method used in study I, III and IV is Sanger sequencing. Each strings of the amplified PCR products is again amplified with the help of one of the primers and modified di-deoxynucleotide triphosphates (ddNTPs). Each of the ddNTPs (A, T, C, G) are labelled with a different fluorophore that emits light at a specific wavelength when it is incorporated in the PCR product, and a chromogram is produced. The re-
trieved sequence can then be compared to known DNA sequences in genome databases.

**Detection methods for real-time PCR**

In real-time PCR, detection is performed within the same step as the amplification process. No further processing of the amplified product is needed, which minimises the risk of contamination.

The detection is performed with fluorescent-labelled probes, which are short DNA strings that are complementary to the target DNA. When the fluorophore molecule is excited by a light source in the machine, it emits light at a certain wavelength which is detected by the instrument. There are different types of probes.

*TaqMan probe*

The TaqMan probe has a fluorophore and a quencher molecule at each end, where the quencher absorbs the emitted light while in close proximity to the fluorophore. When the TaqMan probe is bound to the target DNA, it is cleaved by the Taq polymerase during the elongation process, and then the fluorescence can be detected by the instrument.

*Molecular beacon*

The molecular beacon probe has a different structure than the TaqMan probe, where only the middle part of the DNA string of the probe, the loop, is complementary to the target DNA. The ends of the probe are complementary to each other – forming a stem structure and bringing the fluorescent molecule and the quencher in close contact, preventing fluorescence to be emitted. When target DNA is present, the loop structure of the probe binds to the target DNA, requiring that the hybridisation of the probe and the target DNA is more stable than the stem structure of the probe. Thus, the fluorescent molecule and the quencher are separated and fluorescence can be detected by the instrument.

*Fluorescence resonance energy transfer probes*

Fluorescence resonance energy transfer (FRET) probes are composed of two short DNA strings, the first has a donor molecule at the end while the second probe has an acceptor molecule at the beginning of the probe. The two probes (anchor and sensor probes) are designed to bind to the target DNA in close contact with each other. When the target DNA is present and the probes bind to their targets, fluorescence energy is transferred from the donor molecule to the acceptor molecule which emits fluorescence of the specific wavelength detected by the instrument. FRET probes can be used to detect single nucleotide polymorphisms (SNPs) by the use of the melting curve analysis. If there is a mismatch in the probe region the probe binds the target with less strength. The lower avidity of the FRET probe can then be
detected showing a maximum off-rate at a lower temperature, displayed in the melting curve analysis.

**Molecular typing**

There are many methods with different discriminatory powers used for typing bacteria (126). Regardless of the method used, the result needs to be put in a context, using references and, if accessible, clinical and phylogenetic information.

**Sequence-based typing**

In sequence-based typing, a target DNA of a specific gene is amplified and sequenced. Differences in the sequences are used to categorise the agent into different types or variants. The P1 gene is traditionally used for typing *M. pneumoniae* (79-81). Sequence variations of the P1 gene divide the strains into two main type strains (type 1 and type 2), which can be further divided into a few additional variants (variants 1, 2a, 2b, 2c, 2d) (80, 127-132).

**Multiple-locus variable number tandem repeat analysis**

In multiple-locus variable number tandem repeat analysis (MLVA), the number of repetitive sequences at different sites (loci) dispersed over the genome are measured. Forward and reverse primers, where one of them is labelled with a fluorescent molecule, for each of the targets are used to amplify the sequences. The lengths of the sequences are measured through capillary electrophoresis, where they are allowed to travel through an electric field together with DNA markers with known sizes.

**Whole-genome sequencing**

Whole-genome sequencing (WGS) is also called massive parallel sequencing. Instead of a specific target DNA, the whole genome is sequenced. The genomic DNA is fragmentised into shorter strings and labelled (library construction). Libraries are pooled and amplified, and then the fragments are sequenced in parallel, producing DNA copy strings (reads) of the fragments. Using a software program, the reads that overlap are aligned and assembled together into longer continuous sequences, contigs (*de novo* assembly), or mapped to a reference genome.
Aims

The overall aim of this thesis was to evaluate and improve the diagnostics of *B. pertussis*, *C. pneumoniae* and *M. pneumoniae*. It was also to improve the knowledge about the molecular epidemiology of *M. pneumoniae* and *B. pertussis* and investigate if the strains in Sweden carried mutations associated with macrolide resistance.

Specific aims

I To evaluate the diagnostic specificity and sensitivity of the real-time PCR targeting the IS481 gene fragment of *B. pertussis* in comparison to culture and serology. The aim was also to investigate if the choice of extraction method could influence the performance of the diagnostic PCR method.

II To develop and evaluate the diagnostic performance of a duplex real-time PCR for the detection of *C. pneumoniae* and *M. pneumoniae*. Of special interest was the investigation of how, and if, the duplex format affected the sensitivity of the method.

III To determine the prevalence of macrolide resistance of *M. pneumoniae* within a large number of Swedish patient samples and also provide a baseline for currently used treatment guidelines in Sweden.

IV To investigate if fluctuations in the prevalence of types and variants of *M. pneumoniae* strains could be detected in context to epidemic periods by genetically characterising strains using two different typing methods.

V To investigate if changes in virulence-related genes of *B. pertussis* can be detected, which could explain the resurgence of *B. pertussis*. The aim was also to investigate if the increased incidence was due to a clonal outbreak and if macrolide resistance could be detected.
Material and methods

Clinical specimens

Study I

The 276 nasopharyngeal aspirates used in this study were collected in the context of a vaccine trial study, Stockholm vaccine trial I 1992–1995, performed previously (133). The samples were collected, irrespective of symptoms, from household contacts of culture-confirmed *B. pertussis*-infected children. Cultures of *B. pertussis* had been performed at the time of an earlier study on all 276 nasopharyngeal aspirates (134). Serology results, against *B. pertussis* IgG and IgA of PT and FHA, of acute and convalescent serum samples from 194 of the 276 household contacts were also acquired from the previous study (134). The aspirates were stored at −20°C and had been freeze-thawed, at least one time, before the start of this study.

For the comparison of the two DNA extraction methods, 34 anonymised nasopharyngeal swab samples dissolved in a 2SP-buffer were used. The samples consisted of routine samples from patients with suspicion of respiratory tract infection and were collected in 2004 at Gävle County Hospital. All 34 samples were spiked with equal amounts of *B. pertussis* (CCUG 33616) to a final concentration of 10³ CFU/ml.

Study II

To evaluate the sensitivity and specificity of the developed duplex real-time PCR method, a selection of 120 clinical samples consisting of nasopharyngeal or throat swabs were used. The samples came from patients with respiratory tract infections, and had previously been tested for *C. pneumoniae* and/or *M. pneumoniae* with endpoint PCR methods (135, 136). However, there was a suspicion of false-positive results with respect of *C. pneumoniae*, and therefore these retrospective PCR results were not taken into account in the evaluation. The samples collected in 1997, 1998, 2001 and 2002 at Gävle County Hospital were transported in 2 ml 2SP-buffer and stored at −20°C until used in this study.

To evaluate the rate and extent of PCR inhibition, 200 consecutively collected nasopharyngeal swab samples that were negative with the duplex real-time PCR were used. The samples were collected in 2003 at Gävle County...
Hospital. Half of the samples were spiked with \textit{C. pneumoniae} and the other half were spiked with \textit{M. pneumoniae}.

All included samples were anonymised, and no information about the patient or connection between the sample and patient could be identified.

Studies III and IV

The 624 positive \textit{M. pneumoniae} samples used for the investigation of the prevalence of macrolide resistance (studies III and IV) and the molecular characterisation of the strains (study IV) were collected from four counties during the period 1996−2017. The samples came from patients with respiratory symptoms and consisted of routine samples previously analysed for \textit{M. pneumoniae} at the clinical microbiology laboratories in Falun, Gävle, Karlstad and Uppsala. The majority of the samples, 423 (67.8\%), were collected during the epidemic period of 2010−2013, including the epidemic peak of 2011. Four-hundred and thirty-nine (70.4\%) samples came from the county of Gävleborg and 185 (29.6\%) came from the other three counties, collected in 2012−2013. The samples were anonymised and only information about the patient’s age and sex, at what year and county the samples were taken and if the sample was taken at a polyclinic or a hospital were connected to the samples.

**Definition of epidemic periods of \textit{M. pneumoniae}**

To investigate if fluctuations of different types of \textit{M. pneumoniae} occurred over time, the strains were grouped into epidemic periods. The epidemic periods were defined as the year before a peak year until the beginning of the next peak, based on the proportion of PCR-positive results (Figure 1). The first two epidemic periods were combined into one period (1996−2004) since very few strains were available from these periods.

Study V

A total of 93 \textit{B. pertussis} patient isolates collected between 1986 and 2016 from the counties of Dalarna (n = 34), Gävleborg (n = 21) and Uppsala (n = 38) were used for molecular characterisation with whole-genome sequencing. Four of the included isolates were collected between 1986 and 1987 when no \textit{B. pertussis} vaccination was present in Sweden, and nine isolates were collected in 1998 when ACVs against \textit{B. pertussis} had recently been introduced. Three isolates came from 2004, which was a year with a high incidence of \textit{B. pertussis}, and 15 isolates came from the years 2009−2013, years with a low incidence of \textit{B. pertussis}. The majority of the isolates, 62 (66.7\%), came from 2014−2016, which includes years with an increase in the incidence of \textit{B. pertussis}. 

Before anonymisation, information about the patient’s age and sex, at what year and county the isolate came from and the vaccination status of the patient were connected to the isolates.

**Bacterial control strains**

Bacterial strains were used in each of the studies as controls to verify the specificity, sensitivity and accuracy of the methods. A summarised listing of all included strains and in which study they were used are shown in Table 1.
Table 1. *Bacterial strains used as control strains and for sensitivity and specificity testing.*

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>Strain or source</th>
<th>Study</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>CCUG 33616</td>
<td>I, II</td>
<td></td>
</tr>
<tr>
<td><em>Bordetella parapertussis</em></td>
<td>CCUG 413</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>CCUG 32723</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>Bu434</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Chlamydia psittaci</em></td>
<td>Clinical isolate</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>IOL 207</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>CCUG 1429</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>CCUG 542</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 25922</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>CCUG 23946</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Haemophilus parainfluenzae</em></td>
<td>CCUG 12836</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>CCUG 9568</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>CCUG 18283</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma genitalium</em></td>
<td>ATCC 33530</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>ATCC 15492</td>
<td>IV</td>
<td>Strain Mac, P1 type 2</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>ATCC 15531</td>
<td>II</td>
<td>Strain FH, P1 type 2</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>ATCC 29342</td>
<td>III, IV</td>
<td>Strain M129, P1 type 1</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>Clinical isolate</td>
<td>III, IV</td>
<td>Macrolide-resistant, mutation 23S rRNA A2063C</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>Clinical isolate</td>
<td>III, IV</td>
<td>Macrolide-resistant, mutation 23S rRNA A2063G</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>Clinical isolate</td>
<td>III, IV</td>
<td>Macrolide-resistant, mutation 23S rRNA A2064G</td>
</tr>
<tr>
<td><em>Neisseria meningitides</em></td>
<td>CCUG 3269</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 29213</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>CCUG 18000</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus maltophilia</em></td>
<td>CCUG 5866</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus constellatus</em></td>
<td>CCUG 24889</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>CCUG 33638</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>CCUG 4207</td>
<td>II</td>
<td></td>
</tr>
</tbody>
</table>

a) Clinical isolates were provided by Professor Cécile Bébéar, University of Bordeaux, France.

Quantified *C. pneumoniae* (CDC-CWL-011 strain) and *M. pneumoniae* (ATCC 15531) DNA controls used in study II were obtained from Advanced Biotechnologies Inc. (Columbia, USA). These preparations had the original concentration of $3.7 \times 10^4$ and $5.5 \times 10^4$ copies/ml, respectively.
Culturing and prepreparation of \textit{B. pertussis} isolates

The \textit{B. pertussis} isolates (study V) were cultured on charcoal agar (Oxoid, Hampshire, UK) supplemented with 10\% horse blood and cephalalexin (40 mg/l). To improve the DNA yield in the DNA extraction a prepreparation step was performed, where the bacterial suspension was premixed 1:1 with MagNa Pure Bacteria Lysis Buffer (Roche Diagnostics, Basel, Switzerland), containing 0.5 µg/µl Bovine Serum albumin (Thermo Fisher Scientific, Wal- tham, MA, USA).

DNA extraction

In study I a comparison was made between the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and Amplicor® Respiratory Specimen Preparation kit (Roche Diagnostic, Basel, Switzerland). The Amplicor® Respiratory Specimen Preparation kit was used to extract the bacterial DNA from the clinical specimens and control strains in study I and II. The majority of the samples in study III and IV were extracted using MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostic, Basel, Switzerland) with the pathogen universal programme. Thirty-eight of the samples in study III and IV were extracted with MagNA Pure Compact (Roche Diagnostics, Basel, Switzerland) using an external lysis programme with proteinase K. In study V the isolates were extracted using MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostic, Basel, Switzerland) with the pathogen universal programme.

Molecular detection

Detection of \textit{B. pertussis} (study I)

Real-time PCR targeting the multicopied IS481 fragment was performed to detect \textit{B. pertussis} in the clinical specimens (137). To verify PCR-positive samples that had been culture-negative and came from serology-negative patients, a nested PCR targeting \textit{recA} was used where the PCR product was sequenced (138). The target sequence \textit{recA} included a polymorphic region which differentiates between \textit{B. pertussis} and \textit{B. holmesii}. An SNP differentiates between \textit{B. pertussis}, \textit{B. holmesii} and \textit{B. bronchiseptica}. Sequencing was performed with ABI PRISM 3700 automated DNA sequencer (Perkin-Elmer, Applied Biosystem, Foster City, USA).
The IS481 real-time PCR method was also performed to detect *B. pertussis* from the spiked samples in the comparison of the two extraction methods (137).

**Detection of *M. pneumoniae* and *C. pneumoniae* (study II)**

A real-time duplex PCR was developed targeting the *ompA* gene of *C. pneumoniae* and the P1 adhesion gene of *M. pneumoniae* using molecular beacons for detection (139). The real-time duplex PCR was compared with two endpoint PCRs, a nested PCR targeting the *ompA* gene of *C. pneumoniae* and a PCR targeting the 16S rRNA gene of *M. pneumoniae*, where detection was achieved by gel electrophoresis (140, 141). Discrepant results were analysed with a third method for each agent, at an independent external laboratory (142, 143).

A coamplification test was performed, where detection of *C. pneumoniae* and *M. pneumoniae* was performed in the background of different concentrations of the other target, respectively.

The constructed duplex real-time PCR was used to verify the presence of *M. pneumoniae* after the samples had been stored and transported, before the start of studies III and IV.

**Detection of macrolide-resistance in *M. pneumoniae* (studies III and IV)**

Detection of mutations associated with macrolide resistance was performed with a duplex real-time PCR with FRET probes, targeting two areas of the 23S rRNA gene of *M. pneumoniae* covering the positions of 2063–2067 and 2617 (study III and IV) (144). The melting temperatures of the strains were compared to the control and reference strains.

**Molecular characterisation**

**P1 typing of *M. pneumoniae* (study IV)**

Sequence-based typing of the P1 adhesion gene of *M. pneumoniae* was performed by amplifying part of the P1 gene with a nested PCR (a PCR performed in two steps) (80). The PCR product was sequenced with ABI 3730 DNA analyser (Applied Biosystem, Foster City, USA).
Multiple-locus variable number tandem repeat analysis of *M. pneumoniae* (study IV)

An MLVA method which included five loci (Mpn1, Mpn13, Mpn14, Mpn15 and Mpn16) was used for typing of *M. pneumoniae* (145). The amplification was performed in two mixes: a triplex mix targeting Mpn1, Mpn14 and Mpn16 and a duplex mix targeting Mpn13 and Mpn15. Fragment size separations through capillary electrophoresis was performed with an ABI 3730 DNA analyser (Applied Biosystem, Foster City, USA). Analysis of the fragment sizes was performed using Peak Scanner 2.0 software (Applied Biosystem, Foster City, USA), according to the guidelines by Chalker et al. (146).

Whole-genome sequencing of *B. pertussis* (study V)

The extracted DNA from the *B. pertussis* isolates was fragmentised and libraries were constructed using the Ion Xpress Plus Fragment Library Kit and AB Library Builder System (Thermo Fisher Scientific, Waltham, USA). Selected libraries of the approximate size of 400 bp were pooled and amplified using the Ion Chef System (Thermo Fisher Scientific, Waltham, USA), and sequencing was performed on the Ion Torrent Ion S5 XL System (Thermo Fisher Scientific, Waltham, USA).

The CLC genomics Workbench v. 11.0 software (Qiagen, Hilden, Germany) was used for quality control, assembly and analysis. The contigs were *de novo* assembled and mapped towards the reference genome of Tohama I and towards reference sequences of virulence-related genes (i.e., *ptxA*, *ptxP*, *fim2*, *fim3* and *prn*). Mutations in the 23S rRNA gene were studied, and the number of IS elements of IS481, IS1002 and IS1663 was estimated. An approximate maximum likelihood tree was constructed based on concatenated alignments of variable sites, using Fasttree v. 2.1 and visualised in Figtree v. 1.4.2.

Statistics and calculations

The 95% probability of detection with the 95% confidence interval was calculated in study II using Probit analysis.

A 95% confidence interval for the rate of detection of macrolide resistance was calculated in study III with a modified Wald interval.

In study IV the diversity index was calculated for the P1 and MLVA typing methods using the Hunter-Gaston diversity index (HGDI). The Poisson probability was calculated to assess if a certain type or variant predominated, where a p-value of < 0.05 was considered statistically significant.
Ethics

Ethical approval was not required for studies I and II since all samples used were anonymised and could not be connected to patient identity. For studies III and IV, an ethical application was sent to the Regional Ethical Review Board in Uppsala, which responded by confirming that no ethical approval was required (Dnr 2014/292). Study V was approved by the Regional Ethical Board in Uppsala (Dnr 2017/189).
Results and discussions

Diagnostic evaluation of IS481 real-time PCR for detection of *B. pertussis* (study I)

Comparison of DNA extraction methods

Comparison of the two DNA extraction methods showed a difference, where the Amplicor® Respiratory Specimen Preparation kit (Roche Diagnostics, Basel, Switzerland) resulted in an average of 5.7 Ct-values lower than the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) for all 34 samples (Figure 6). The difference in Ct-values indicates an approximately 50-fold higher DNA yield was achieved using the Amplicor® Respiratory Specimen Preparation kit (Roche Diagnostics, Basel, Switzerland) compared to the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany).

*Figure 6. Ct-values of the 34 spiked nasopharyngeal swab samples after extraction with two different methods, the QIAamp® DNA Mini Kit and the Amplicor® Respiratory Specimen Preparation kit. Each extracted sample was tested in duplicates in the real-time PCR.*
The choice of DNA extraction method is vital when constructing and optimising a PCR method, and it can affect the analytical sensitivity of the method. The Amplicor® Respiratory Specimen Preparation kit shows good performance for the respiratory samples, although it is a manual method which includes hands-on pipetting steps that are elements of risks. In recent years the development within the clinical microbiology laboratories has led to more automatisations. In studies III-V, the samples were extracted with an automatic method, which enabled more standardised handling of the samples and is more suited for an increased sample throughput.

Analytical performance of the IS481 real-time PCR and the recA PCR

After optimisation of the IS481 real-time PCR method, the linearity of the assay was 0.99 and the PCR efficiency was 95.8%. The IS481 real-time PCR could detect down to 0.05 CFU/reaction; whereas, the developed recA PCR showed a lower analytical sensitivity and could detect 5 CFU/reaction. The high analytical sensitivity of the IS481 real-time PCR is hard to overcome using other DNA targets for the PCR since the IS481 fragment is present in multiple copies at the B. pertussis genome (> 240 copies) (86, 115, 116).

Diagnostic sensitivity and specificity of the IS481 real-time PCR

**IS481 real-time PCR compared to culture**

The IS481 real-time PCR was evaluated on well-defined samples collected in a previous vaccine trial (133). The samples came from household contacts of children with culture-confirmed B. pertussis infection. Of the 276 nasopharyngeal aspirates analysed with culture and real-time PCR, 72 samples were positive while 177 samples were negative with both methods (Table 2). Compared to culture, the sensitivity of the IS481 real-time PCR was 87.8%.

<table>
<thead>
<tr>
<th>Culture result</th>
<th>Real-time PCR result</th>
<th>Serology result&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
<td>177</td>
</tr>
</tbody>
</table>

<sup>a</sup> Serology results were only available from the 194 patients with culture negative results.

<sup>b</sup> Eleven of the 99 aspirates that came from serology positive patients were positive with real-time PCR.

<sup>c</sup> Six of the 95 aspirates that came from serology positive patients were positive with the real-time PCR.
Ten samples that had been culture-positive for B. pertussis were negative with the real-time PCR. This may have been because the samples had been stored in the freezer for several years and freeze-thawed up to four times before use in this study, which can affect the stability of the DNA and thus the outcome of the PCR. Findings of culture-positive B. pertussis samples that were PCR-negative have also been described in other studies (17, 123). Inhibitory substances may also give rise to false-negative PCR results. Our method did not include an internal control but the 10 PCR-negative samples were spiked with small amounts of B. pertussis DNA which showed no presence of inhibitory substances (data not shown).

**IS481 real-time PCR compared to serology**

Serology results against B. pertussis IgG and IgA of PT and FHA of acute and convalescent serum samples were obtained from 194 of the patients (Table 2). A positive serology result was defined as a significant increase in units from acute to convalescent sample, as described by Reizenstein et al. (134). Eleven aspirates from the 99 serology-positive patients were positive with the IS481 real-time PCR. Additional six samples were positive with the IS481 real-time PCR but came from serology-negative patients. It has previously been described that the timing of sampling for receiving a positive serology, positive culture and PCR result differ, where a higher rate of culture and PCR are detected earlier in the infection phase; whereas, serology positive results are obtained later in the infection phase (123). Culture and PCR-confirmed cases where serology responses are not detected have also been described elsewhere and may be due to deficient immune responses (17, 123, 134).

**Verification of the PCR-positive and culture-negative samples**

It is known that the IS481 fragment is present in about 8−10 copies in the genome of B. holmesii, which can cause pertussis-like illness and may therefore be misinterpreted as B. pertussis (119, 147, 148). The IS481 element has also been found in some strains of B. bronchiseptica where a study by Tizolova et al. (119), which screen 120 human isolates of B. bronchiseptica showed that only two (1.7%) of the strains carried the IS481 fragment. B. bronchiseptica has also been described to cause respiratory infections in humans, but this seems rare and mostly involves immunocompromised patients (85, 120, 149). Thus, when using the IS481 as a target for PCR, a possible cross-reaction between B. pertussis and B. holmesii, and some strains of B. bronchiseptica, needs to be taken into consideration.

Eleven of the 17 PCR-positive samples that were culture-negative were confirmed by serology and were therefore regarded as true positives for B. pertussis. To verify the remaining six samples, the samples were analysed using the newly constructed nested recA PCR. The recA PCR was designed to include several SNPs that can separate B. pertussis and B. holmesii. It also
included an SNP that separates *B. pertussis*, *B. holmesii* and *B. bronchiseptica*. Unfortunately, only two of the six samples were positive with the *recA* PCR, probably due to the lower sensitivity of the *recA* PCR compared to the IS481 PCR and the samples contained a low amount of DNA. The sequences of the two successfully analysed samples were homologous to *B. pertussis*. *B. holmesii* or *B. bronchiseptica* was not detected in any of the samples.

Several PCR protocols have been developed, where multiple targets, including the IS481 fragment, are used to detect and distinguish between *B. pertussis*, *B. parapertussis*, *B. holmesii* and in some cases even *B. bronchiseptica* (122, 150-153). Most of the samples could be differentiated using these methods although low positive samples, with Ct-values > 35 in the IS481 PCR, are rarely verified and may therefore be regarded as positive for *Bordetella* spp. (150-153).

**IS481 real-time PCR compared to defined true cases**

A true positive case was defined as either culture-positive or IS481 real-time PCR positive that has been confirmed with serology or the *recA* PCR. Comparing the IS481 real-time PCR result with true cases, the sensitivity and specificity were 89.5% and 97.8%, respectively (Table 3).

<table>
<thead>
<tr>
<th>True cases</th>
<th>IS481 real-time PCR result</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>177</td>
</tr>
</tbody>
</table>

**Performance of the duplex real-time PCR for the detection of *C. pneumoniae* and *M. pneumoniae* (study II)**

Analytical performance of the developed duplex real-time PCR

No false-positive reaction was discovered when other bacterial species were tested in the specificity control of the PCR. The 95% probability of detection was determined to 4.3 Geq/reaction (3.0–10.1) and 2.8 Geq/reaction (2.0–9.5) for *C. pneumoniae* and *M. pneumoniae*, respectively, which is comparable to the sensitivity described in other studies (78, 154-156).

**Duplex detection**

Multiplex analysis enables a more symptom-related diagnostic, where multiple pathogens that can cause similar symptoms can be tested at the same
time. *C. pneumoniae* and *M. pneumoniae* infections are clinically hard to distinguish, and thus the duplex-format of this method is advantageous since it can detect both pathogens in the same reaction and tube. It is important that, in case of dual infection, both pathogens can be detected and there are no competition between the simultaneous amplifications (157). Our study showed that the duplex target amplification did not reduce the sensitivity of the PCR since both pathogens could be detected showing equal Ct-values when detected as a single pathogen or when coamplified with the respective pathogen (Table 4 and 5).

Table 4. *Ct*-values of *C. pneumoniae* at two concentrations when coamplified with different concentrations of *M. pneumoniae* DNA.

<table>
<thead>
<tr>
<th>Coamplification of</th>
<th>Detection of <em>C. pneumoniae</em> (Ct-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 copies</td>
</tr>
<tr>
<td>None</td>
<td>33.9 ± 0.7</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> (10 copies)</td>
<td>33.9 ± 0.7</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> (10⁴ copies)</td>
<td>32.2 ± 0.5</td>
</tr>
</tbody>
</table>

Table 5. *Ct*-values of *M. pneumoniae* at two concentrations when coamplified with different concentrations of *C. pneumoniae* DNA.

<table>
<thead>
<tr>
<th>Coamplification of</th>
<th>Detection of <em>M. pneumoniae</em> (Ct-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 copies</td>
</tr>
<tr>
<td>None</td>
<td>30.2 ± 0.3</td>
</tr>
<tr>
<td><em>C. pneumoniae</em> (10 copies)</td>
<td>30.7 ± 0.2</td>
</tr>
<tr>
<td><em>C. pneumoniae</em> (10⁴ copies)</td>
<td>29.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Inhibition**

Inhibition of a PCR reaction can cause a false-negative test result. This method does not include an internal control that monitors inhibition in each of the samples, but when 200 spiked nasopharyngeal and swab samples were tested, none of the samples showed any signs of total inhibition. One of the samples spiked with *C. pneumoniae* and four of the samples spiked with *M. pneumoniae* showed partial inhibition, defined as having a Ct-value that exceeds more than two standard deviations from the mean Ct-value of all the samples. Partial inhibition was no longer detected when the samples were diluted 20-fold. It is important to know that the type of sample material and choice of DNA extraction method can influence the PCR sensitivity and rate of inhibition (158).
Diagnostic performance of the developed duplex real-time PCR

When the diagnostic performance of the real-time PCR method was compared with the conventional PCR, there was an overall agreement of 98.8% between the methods. The sensitivity and specificity, using the conventional PCR as a reference, was 92.9% and 100% for \textit{C. pneumoniae}, respectively, and 100% and 98% for \textit{M. pneumoniae}, respectively (Table 6).

One \textit{C. pneumoniae} positive sample detected by the nested endpoint PCR was not detected with the real-time PCR. This sample could not be verified by a third method performed by an outside laboratory. Nested PCR are known to be very sensitive but carry a higher risk of false-positive reactions due to amplicon or genomic DNA contamination (157).

Two more samples were positive for \textit{M. pneumoniae} with the real-time PCR, compared to the conventional PCR where one of these samples was verified when analysed by a third method. This suggests that the real-time PCR is a more sensitive method.

Table 6. The diagnostic performance of the newly constructed real-time PCR compared to the result from two conventional reference PCRs.

<table>
<thead>
<tr>
<th>Conventional PCR result</th>
<th>Real-time PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detection of \textit{C. pneumoniae}</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a) The discrepant result could not be verified as positive with a third method performed at an external laboratory.}

\textsuperscript{b) One of the discrepant results was verified as positive by a third method performed at an external laboratory.}

Epidemiological characterisation of \textit{M. pneumoniae} (studies III and IV)

Detection of macrolide resistance in \textit{M. pneumoniae}

In total, 624 patient samples collected from 1996–2017, were analysed with a FRET PCR for detection of mutations associated with macrolide resistance (63, 64, 144). Only one (0.16%) of the 608 samples that could be fully analysed showed a melting temperature concordant with the control strains harbouring either the A2063C or A2063G mutation. Further verification by sequencing showed that the strain had an A2063G mutation. The resistant strain came from a 16–year old male patient taken at an outpatient facility. Unfortunately, the treatment status of the patient was unknown.
Screenings of macrolide resistance among *M. pneumoniae*−positive samples are not routinely performed in Sweden. To our awareness, only one previous case of macrolide resistance, which was induced after treatment with erythromycin, has been described (159). Recently, another clinical isolate with macrolide resistance has been detected at the clinical microbiological laboratory at Gävle County Hospital (unpublished data). Thus, only sporadic cases of macrolide-resistant *M. pneumoniae* have been detected in Sweden so far.

Sweden has strict guidelines for the treatment of respiratory infections within outpatient care where, for example, bronchitis is not an indication for treatment (160). Doxycycline is the treatment of choice for outpatient care−treated pneumonia caused by *M. pneumoniae*, whereas doxycycline or erythromycin is the treatment of choice according to guidelines for hospital treated *M. pneumoniae* infection (160, 161). Sweden has a generally low consumption level of macrolides when compared to other European countries (162). Whether the low prevalence of macrolide-resistant strains in this study reflects the prudent use of macrolides in Sweden remains to be investigated.

The closely related species *M. genitalium* has the same mechanism for macrolide resistance as *M. pneumoniae*. Swedish studies show an incidence of 11.9−21% macrolide resistance among the *M. genitalium* isolates (163-166). According to Swedish guidelines, infections of *M. genitalium* should be treated with antibiotics when detected, and the treatment of choice is azithromycin (167). The previous recommendation of a single-dose treatment with 1 g azithromycin has been associated with the selection of macrolide-resistant *M. genitalium* strains, which perhaps has led to the progress of macrolide resistance in *M. genitalium* as opposed to *M. pneumoniae* (166).

**Molecular typing of *M. pneumoniae***

**P1 typing**

A polyclonal distribution of P1 types was observed during all epidemic periods, but there was a predominance of type 2/variant 2 strains during the epidemic period of 2010−2013 (62.0%, p = 0.0007) (Figure 7a). Although many countries experienced the extraordinary epidemic peak of *M. pneumoniae* in the period 2010−2011, the type distribution pattern differs: the result from our study is consistent with the results from Slovenia but contradicts the findings in France, the United Kingdom, Japan and China, which had a predominance of type 1 strains during these epidemic years (60, 65, 70, 168, 169).

A shift of the predominance in type 2/variant 2 strains could be seen where variants of type 2 strains were more prevalent than type 2 strains from 2005 and forward (Figure 7b). Variant 2a predominated during the epidemic
period of 2005–2009 (39.6%, p = 0.0297) while variant 2c predominated during the epidemic periods of 2010–2013 (57.3%, p < 0.0001) and 2016–2017 (54.2%, p = 0.0014).

Detection of a new P1 variant
In one strain from 2016 and eight strains from 2017, a new sequence of the RepMP2/3 element within the P1 gene was detected. Compared to previously described P1 variants, it resembled variant 2b, with the exception of a 12-nucleotide deletion leading to a loss of four amino acids. The same deletion is present within strains of variant 2a, and the recurrence of this specific deletion may provide a selective advantage. The new variant, designated variant 2e, is likely to be a product of an intragenomic recombination event where a part of the RepMP2/3-a has been inserted into the RepMP2/3-d element within the P1 gene, a mechanism equal as suggested for the upturn of other variants (Figure 8) (83).

MLVA
Using the five loci MLVA method described by Dégrange et al. (145), 38 MLVA types were identified. Since one of the loci, Mpn1, has shown to be unstable, new guidelines suggest it should be excluded, leaving only 12 MLVA types (59, 146). MLVA types 3-5-6-2 (n = 245), 4-5-7-2 (n = 216) and 3-6-6-2 (n = 86) comprised 94.6% of the strains. Each of the other nine MLVA types was detected sporadically and comprised one or two strains at the most per year. Two new MLVA types were detected: 3-7-6-2 and 4-4-6-2. To improve the diversity of the MLVA method, the addition of another locus can be considered, although it needs to be validated so it adds discriminatory power without being unstable (170).

MLVA type 3-6-6-2 predominated during the epidemic period of 1996–2004 (55.2%, p = 0.0381). In 2005 MLVA type 3-5-6-2 was first detected, which coincided with the introduction of variant 2 strains, and became more common than MLVA type 3-6-6-2. MLVA types 3-5-6-2 and 4-5-7-2 predominated during the epidemic periods of 2005–2009 (38.0%, p = 0.0092 and 43.0%, p = 0.0007, respectively) and 2010–2013 (49.9%, p < 0.0001 and 34.4%, p = 0.0002, respectively) (Figure 7c). MLVA type 4-5-7-2 predominated during the epidemic period of 2016–2017 (48.3%, p = 0.0009). (Figure 7c)

Relationship between P1 and MLVA typing
The MLVA types correlated, with a few exceptions, to either P1 types 1 or 2. MLVA type 4-5-7-2 comprised P1 type 1 strains, except for one variant 2a strain. MLVA type 3-5-6-2 comprised P1 type 2/variant 2 strains, except for two P1 type 1 strains. MLVA type 3-6-6-2 comprised only type 2/variant 2 strains.
Figure 7. Distributions of P1 and MLVA types during different epidemic periods. Figure a) shows the distribution between type 1 and type 2/variant 2 strains, b) shows the distribution between type 2 and different variant 2 strains and c) shows the distribution of MLVA types.
Figure 8. A schematic figure of the major differences between the RepMP2/3 elements situated within the P1 adhesion gene. Variants 2a, 2b and 2e are compared to type 2 according to the position of strain Mac. The grey bars indicate the inserted parts of the RepMP2/3-a element that characterise each of the variant strains. The insertions are identical to the corresponding positions: 128382–128906 (variant 2b), 128382–128954 (variant 2e) and 128688–128954 (variant 2a) – except for 12 bp, which shows a deletion at position 128751–128762 (indicated by a black marking) in variants 2a and 2e.

Epidemiological characterisation of *B. pertussis* (study V)

General characteristics

The average read coverage for each genome ranged between 21.4x and 123.4x, and the assembly resulted in a range of 725–1943 contigs for each of the isolates. The sizes of the genome were estimated to be between 3.61 and 3.99 Mbp. Due to the many repetitive elements present in the *B. pertussis* genome, a closed genome with a single contig is not possible to achieve with short-read sequencing methods but can be overcome by the use of long-read sequencing methods (116, 117, 171).

The mean numbers of the estimated IS elements of the isolates were: 238 IS481 (range 187–274), 6 IS1002 (range 5–7) and 18 IS1663 (range 16–20).

No mutation in the 23S rRNA gene, which is associated with macrolide resistance, was detected in any of the isolates.

Phylogenetic analysis

Phylogenetic analysis divided the 93 isolates into three major clades. Clade 1 (n = 10) comprised all the isolates harbouring the *ptxP1* allele, except two outlier isolates from 1987 and 2010 that also harboured the *ptxP1* allele. The allelic profile of all but one isolate in clade 1 was *ptxP1-pxtA1-fim2-1-fim3-1*, where one isolate harbourued the *ptxA10* instead of *ptxA1*. Clade 2 (n = 27) was characterised by the allelic profile *ptxP3-pxtA1-fim2-1-fim3-2* and clade
3 (n = 54) by ptxP3-pxta1-fim2-1-fim3-1, except for two isolates which harboured a new ptxP allele instead of ptxP3. The new ptxP allele closely resembled ptxP3, except for a point mutation.

A shift from ptxP1 to ptxP3 was detected among the isolates over the years, consistent as described in studies from other parts of the world (100, 109). The proportion of isolates with the ptxP1 allele decreased over time and was replaced by isolates harbouring the ptxP3 allele, comprising all isolates from 2014–2016 (Figure 9a). All isolates from 1986 to 1987 harboured the fim3-1 allele whereas the proportion of strains harbouring the fim3-1 and fim3-2 alleles altered over the other time periods (Figure 9b). The majority (79%) of the isolates from 2014 to 2016 harboured the fim3-1 allele.

Disruption of the pertactin gene

There were three different alleles of prn: prn1, prn2 and prn3, although only five (5.4%) of the 93 isolates harboured an intact prn gene. The prn gene of the other 88 (94.6%) isolates was disrupted, and three different main types of disruptions were detected. For isolates with disruption type 1 (n = 11), the first half of the prn was deleted, similar to the deletion seen in strain J625 (CP022362) described by Weigand et al. (172). In isolates with disruption type 2 (n = 16), there seemed to be an insert of a transposase gene, as seen in strain B3629 (CP011400) described by Bart et al. (115). Disruption type 3 (n = 61) was the most common type, where a likely IS481 sequence has been inserted at about 1700 bp in the gene. The incidence of pertactin deficient strains has been described in many countries, although the proportion of deficient strains described in this study is exceptional (98, 173). Different types of disruptions have been described where IS481-mediated disruptions seem to be most common (98, 99, 115, 116, 172). Assembly difficulties, due to the short-read sequences and the repetitive elements, warrant further investigation with long-read sequencing to confirm and describe the disruption types in more detail.

Geographic distribution

No geographic correlation between the origin of the isolates and a specific clade could be detected. Isolates from each county were present in all clades, and isolates from 2014 to 2016 were not restricted to a specific clade or cluster.

A large cluster in subclade 3A, where the isolates had no SNP difference, contained isolates from all three counties and from two years, 2014 and 2016. This also supports the previous findings that B. pertussis is a highly homogenous species based on nucleotide polymorphism (116, 117).
Figure 9. Changes of the \textit{ptxP} (a) and \textit{fim3} (b) alleles over time.
Conclusions

- The IS481 real-time PCR has a high sensitivity and specificity. However, awareness of other *Bordetella spp*, specifically *B. holmesii*, should be considered and monitored. Low positive results achieved with the IS481 real-time PCR are difficult to verify with another PCR method due to the many copies present within the *B. pertussis* genome.
- When optimising or constructing a real-time PCR method, all parts of the process, including the extraction method, need to be taken into consideration since they may affect the sensitivity of the method.
- The developed duplex real-time PCR method can detect and differentiate *C. pneumoniae* and *M. pneumoniae* with high sensitivity and specificity. The duplex detection of both pathogens did not affect the sensitivity of the method.
- One (0.16%) of the 608 *M. pneumoniae* strains harboured a mutation associated with macrolide resistance. The result does not suggest any reason to change the treatment guidelines in Sweden at the moment, but surveillance needs to be continued, and there should be awareness that macrolide resistance could be the cause of treatment failure.
- A polyclonal distribution of *M. pneumoniae* P1 types 1 and type 2/variant 2 strains was detected over all epidemic periods, but there was a predominance of type 2/variant 2 strains during the epidemic period of 2010–2013. A shift in predominance from type 2 to different variant 2 strains could be seen between the epidemic periods, and a new variant, 2e, was detected in 2016–2017.
- Three *M. pneumoniae* MLVA types dominated over the years. MLVA types 3-5-6-2 and 4-5-7-2 predominated during the epidemic years 2010–2013, and MLVA type 4-5-7-2 predominated during the period 2016–2017.
- The increased incidence of *B. pertussis* in the counties of Dalarna, Gävleborg and Uppsala 2014–2016 was not due to a clonal outbreak.
- Changes in virulence-related genes of the *B. pertussis* isolates were detected: a shift from isolates harbouring the *ptxP3* allele in favour of *ptxP1* was seen, and almost all isolates had a disrupted *prn* gene. The genetic changes may influence the virulence of the bacteria, but whether it is associated with the increased incidence needs to be further studied.
- No mutations associated with macrolide resistance were detected among the *B. pertussis* isolates.
Future perspectives

Multiplex molecular diagnostics for atypical pathogens that cause respiratory infections is advantageous since the symptoms can be similar. The validation and construction of the real-time PCR methods for the detection of *B. pertussis*, *C. pneumoniae* and *M. pneumoniae* described in this thesis has led to improved diagnostics in clinical use. A future project would be to combine the diagnostics of all three pathogens and perhaps include other targets. Multiplex methods receive a higher yield in diagnostics which may lead to better care for the patient and minimised antibiotic consumption. Although molecular detection cannot differentiate between acute infections and long-term and asymptomatic carriage of pathogens, and thus the diagnostic results must always be valued in the context of clinical information.

Serology and culture methods are good complements to molecular methods and have their values especially in epidemiological studies, defining past infections and contributing to defining true positives and enabling the phenotypic detection of antibiotic resistance. Cultured isolates facilitate performing WGS. We hope to resume culturing of *M. pneumoniae*, which at the moment is not performed at any laboratory in Sweden.

WGS is the typing method of the future and will probably be used more as it becomes more available at a lower cost. Concerning the analysis of WGS data, there is a lot more to learn. Different techniques have to be valued against each other and each pathogen has unique genomic features. We learned from our experience of *B. pertussis* that the isolates have few SNP differences, but short-read sequencing needs to be complemented with long-read sequencing to detect rearrangements and confirm and describe the disruption types in more detail. Further analysis with long-read sequencing methods will be performed on selected *B. pertussis* isolates from study V. WGS studies of *M. pneumoniae* will also give higher discriminatory power and may give a better understanding of the genetic differences between strains.

In future typing and macrolide resistance surveillance studies, it would be interesting to include isolates from a wider geographic area and in the context of more detailed clinic information. The progress of antibiotic resistance and the spread of macrolide-resistant *M. pneumoniae* strains are great concerns and they require continued surveillance.

En realtids PCR metod med IS481 som målsekvens jämfördes med odling och serologi diagnostik för detektion av *B. pertussis*. En duplex realtids PCR metod för detektion av *C. pneumoniae* och *M. pneumoniae* konstruerades och jämfördes med två konventionella PCR-er. Båda realtids PCR metoderna upptäckte hög sensitivitet och specificitet.

En typningsstudie utfördes där 624 *M. pneumoniae* stammar, som var insamlade från fyra regioner mellan 1996−2017, analyserades med två olika typningsmetoder: P1 typning och multiple-locus variable number tandem repeat analys (MLVA). En polyklonal förekomst av stammar kunde ses från samtliga epidemiiska perioder, dock dominerade P1 typ 2/variant 2 samt MLVA typ 3-5-6-2 och 4-5-7-2 under 2010–2013. Ett skifte från typ 2 till olika variant 2 stammar kunde ses över tid, och en ny variant, variant 2e, detekterades under 2016-2017. Med hjälp av en PCR-baserad metod kunde mutationen A2063G, vilken visats ge upphov till makrolidresistens, detekteras i en (0.16%) av 608 *M. pneumoniae* stammar.


Utövande av real-time PCR metoderna för detektion av *B. pertussis, C. pneumoniae* och *M. pneumoniae* har lett till förbättrade diagnostiska metoder för kliniskt bruk. Molekylär karakterisering av *M. pneumoniae* och *B. pertussis* har bidragit till kunskaper om stammarnas genetiska förändringar under de epidemiiska perioderna men ytterligare studier krävs för att förstå samband mellan genetiska variationerna samt uppkomst av epidemi eller ökad incidens.
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References


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