

Molecular Diagnosis and Characterization of Honey Bee Pathogens

Eva Forsgren

*Faculty of Natural Resources and Agricultural Sciences
Department of Ecology
Uppsala*

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Cover: Honey bee with symptoms of deformed wing virus infection
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Abstract

Bees are crucial for maintaining biodiversity by pollination of numerous plant species. The European honey bee, *Apis mellifera*, is of great importance not only for the honey they produce, but also as vital pollinators of agricultural and horticultural crops. The economical value of pollination has been estimated to be several billion dollars, and pollinator declines are a global biodiversity threat. Hence, honey bee health has great impact on the economy, food production and biodiversity worldwide.

A broad spectrum of specific pathogens affect the honey bee colony including bacteria, viruses, microscopic fungi, and internal and external parasites. Some of these microorganisms and parasites are more harmful than others and infections/infestations may lead to colony collapse. Knowledge of the biology and epidemiology of these pathogens are needed for prevention of disease outbreak.

The use of molecular methods has increased during recent years offering a selection of powerful tools for laboratories involved in honey bee disease diagnostics and research. Novel diagnostic techniques also allow for new approaches to honey bee pathology where specific and critical questions can be answered using modern molecular technology.

This thesis focuses on molecular techniques and their application within honey bee pathology. Specific questions concerning five disease-causing agents all capable of inducing population depletion of honey bee colonies were addressed. The studied pathogens included two different bacterial species, (*Melissococcus plutonius* and *Paenibacillus larvae*) that cause serious disease in honey bee larvae, one virus (deformed wing virus, DWV) that causes deformity during pupal development and finally, two honey bee pathogenic microsporidia (*Nosema ceranae* and *Nosema apis*) that cause disease in adult bees. Resolving questions concerning prevalence and characterization of these pathogens using newly developed molecular methods has contributed to a better understanding of host-pathogen interactions and epidemiology in the honey bee system. Furthermore, the attained knowledge contributes to improved disease diagnostics and disease prevention in the beekeeping industry and expands the general field of honey bee epidemiology.

Keywords: *Apis mellifera*, *Melissococcus plutonius*, *Paenibacillus larvae*, deformed wing virus, *Nosema ceranae*, PCR, qPCR, rep-PCR, PFGE.

Author's address: Eva Forsgren, SLU, Department of Ecology,
P.O. Box 7044, 750 07 Uppsala, Sweden
E-mail: eva.forsgren@ekol.slu.se

Dedication

To my parents.
Till mina föräldrar, Walter och Laila.

Life is half spent before we know what it is.
Jacob Cats, Moral Emblems, 1632

Contents

List of Publications	7
Abbreviations	9
1 Introduction	11
2 The honey bee	13
2.1 The honey bee colony	14
2.2 Social immunity and pathogen transmission	14
2.2.1 Step 1 and 2. Pathogen uptake and intake	15
2.2.2 Step 3. Establishment of the pathogen in the nest	15
2.2.3 Step 4. Vertical and horizontal transmission between individuals	15
2.2.4 Step 5. Vertical and horizontal transmission between colonies	15
2.3 Individual immunity	16
3 Honey bee parasites and pathogens	19
3.1 Bacteria	19
3.1.1 <i>Melissococcus plutonius</i>	20
3.1.2 <i>Paenibacillus larvae</i>	22
3.2 Parasitic mites	23
3.2.1 <i>Varroa destructor</i>	23
3.2.2 <i>Tropilaelaps mercedesae</i>	24
3.3 Viruses	24
3.3.1 Deformed wing virus	24
3.4 Microsporidia- <i>Nosema</i>	26
3.4.1 <i>Nosema apis</i>	26
3.4.2 <i>Nosema ceranae</i>	27
4 Methods	29
4.1 Nucleic acid detection	29
4.1.1 DNA and RNA extraction	30
4.1.2 Polymerase chain reaction	30
4.1.3 Real-time PCR	32
4.1.4 Repetitive PCR	36
4.1.5 Pulsed Field Gel Electrophoresis	36
4.1.6 DNA sequencing	36

5	Conclusions and future challenges	39
	References	41
	Svensk sammanfattning	53
	Acknowledgements - Tack	57

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Forsgren E, Cassel-Lundhagen A, Imdorf A, Fries I (2005). Distribution of *Melissococcus plutonius* in honey bee colonies with and without symptoms of European foulbrood. *Microbial Ecology* 50, 369-374.
- II Genersch E, Forsgren E, Pentikäinen J, Ashiralieva A, Rauch S, Kilwinski J, Fries I (2006). Reclassification of *Paenibacillus larvae* subsp. *pulvificiens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *International Journal of Systematic and Evolutionary Microbiology* 56, 501-511.
- III Forsgren E, de Miranda R J, Isaksson M, Wei S, Fries I (2009). Deformed wing virus associated with *Tropilaelaps mercedesae* infesting European honey bees (*Apis mellifera*). *Experimental and Applied Acarology* 47, 87-97.
- IV Forsgren E, Fries I. Comparative virulence of *Nosema ceranae* and *Nosema apis* in European honey bees (Submitted manuscript).

Papers I-III are reproduced with the permission of the publishers: Springer Verlag (I & III) and the Society for General Microbiology (II).

Related publications

Belloy L, Imdorf A, Fries I, Forsgren E, Berthoud H, Kuhn R, Charrière, J-D (2007) Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood *Apidologie* 38, 136-140.

Forsgren E, Stevanovic J, Fries I (2008). Variability in germination and in temperature and storage resistance among *Paenibacillus larvae* genotypes *Veterinary Microbiology* 129, 342-349.

Forsgren E (2009) European foulbrood in honey bees. *Journal of Invertebrate Pathology*, in press.

Abbreviations

AFB	American foulbrood
CCD	Colony collapse disorder
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
C _q	Quantification cycle
dNTP	Deoxyribonucleotide triphosphate
DWV	Deformed wing virus
EFB	European foulbrood
FRET	Fluorescence resonance energy transfer
NFQ	Black-hole non-fluorescent quencher
OIE	Office International des Epizooties (World Organization for Animal Health)
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
Rep-PCR	Repetitive PCR
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-qPCR	Quantitative real-time RT-PCR
QC	Quality control
qPCR	Quantitative real-time PCR

1 Introduction

Honey bees are important not only for the honey they produce; they also are vital pollinators of agricultural and horticultural crops. It has been estimated that one-third of the human diet can be traced directly or indirectly to bee pollination (Delaplane & Mayer, 2000; McGregor, 1976) and the economic value of pollination is estimated to several billion dollars (Anonymous, 2009; Anonymous, 2008b). Furthermore, a decline in pollinators is a global threat to biodiversity and honey bees are key generalist pollinators (Jaffé *et al.*, 2009). Hence, the honey bee health has a great impact on economy and biodiversity worldwide.

Colony depletion and disease outbreak in managed honey bees is not unusual and has been of great concern for man all through history. However, in recent years there has been an increasing worldwide focus on honey bee health since serious colony losses and a decline in bee populations have been reported all over the world. In 2006, the term Colony Collapse Disorder (CCD) was used to describe a phenomenon of large-scale, unexplained loss of managed honey bee colonies (vanEngelsdorp *et al.*, 2007). Numerous causes for CCD have been proposed (including pathogens), but no single factor alone seems to be responsible. The leading hypothesis today is that CCD may be a syndrome caused by a combination of many different factors working together synergistically (Anonymous, 2009), although honey bee pathogens clearly play an important role in colony collapse.

A large diversity of microorganisms are associated with honey bees (Olofsson & Vasquez, 2008; Gilliam, 1997) most of them commensals, but some are pathogens (disease causing organisms) affecting adult bees and brood. Some of these pathogens are more harmful than others and infections may lead to colony collapse. Knowledge of the biology and epidemiology of

these infections is urgently needed for prevention of disease outbreak and colony losses.

Up until recently, reliable analytic techniques for the study of many honey bee pathogens have been lacking. For example, the cultivation-dependent techniques used to study honey bee pathogenic bacteria have proved to be very insensitive (Forsgren *et al.*, 2008; Hornitzky & Smith, 1998). Modern diagnostic technologies offer new approaches to honey bee pathology where specific and critical questions can be answered. These include novel immunological, biochemical and molecular techniques. The use of molecular diagnostics in particular has increased during recent years and has become a powerful tool in laboratories involved in disease diagnostics and research in many fields. However, despite major advances made over the last few years, basic knowledge about some of the honey bee pathogens is still lacking. The increasing worldwide interest in honey bee health together with more focused basic and applied research in this important field will hopefully provide deeper insight. This is much needed if we are to mitigate the negative effects on honey bee populations caused by escalating disease outbreaks.

The work presented in this thesis aimed to investigate epidemiological aspects, such as transmission or virulence, of five disease causing agents, all capable of inducing population depletion and colony collapse. This work has provided important knowledge concerning prevalence as well as characterization of these honey bee pathogens and has further resulted in a better understanding of epidemiology and host-pathogen interactions in the honey bee system. Moreover, the results may help produce more coherent and reliable results from standardized analytic methods. This is a key factor for broadening our general knowledge of infections in honey bees and to further improve diagnostic tools for disease prevention in the beekeeping industry.

2 The honey bee

Two species of the genus *Apis* (the true honey bees), have long attracted the special attention of man. These are the European honey bee, *Apis mellifera* (Figure 1), and the physically smaller but very similar Asian honey bee, *Apis cerana* (Figure 3). These species have been of particular interest to man because they produce large amounts of honey and can be kept as “domesticated animals” in movable nests or hives. During the past centuries, the European honey bee has been transported all over the world and is widely distributed. The Asian honey bee is restricted to South East Asia, China, eastern Russia and Japan but is to some extent being actively replaced by *A. mellifera*.

Honey bees are well studied insects. Many detailed descriptions of the biology of the honey bee can be found in the literature, for example Seeley (1995) and Winston (1987). Some of the basic features of the honey bee, *A. mellifera* in temperate climate are described below.



Figure 1. Honey bee (*Apis mellifera*) on lavender. Photo: Barbara Locke.

2.1 The honey bee colony

Feral honey bee colonies usually build their nests in a hollow space, for example, a log cavity. The nest is a shelter for the bees and the comb, with its hexagonal cells, is used for brood rearing and storage of pollen and nectar. Many characteristics of feral colonies have been incorporated into the design of man-made bee hives for apiculture.

A honey bee colony typically consists of three castes: a single reproductive female - the queen, a few hundred drones and between 40-60,000 worker bees when the colony population peaks in summer. Development of all three castes involves four life stages: egg, larva, pupa and adult. The queen lays either fertilized eggs (i.e., diploid) that normally develop into workers or queens, or unfertilized eggs (i.e., haploid) that develop into drones in the bees wax cells. During larval development, the larvae spin cocoons and change into pupae after workers have capped their cells with wax. The pupae transform into adults within the cells and then chew through the wax cell capping and emerge.

The worker bees are non-reproductive females that exhibit a temporal division of labor throughout their life beginning “in-hive tasks” such as building combs, feeding and tending the brood. After two to three weeks they begin to forage for pollen and nectar. During the summer season, the life-span of the individual bee is no more than 4-5 weeks. The drones are reproductive males with the sole function to mate with virgin queens and they are ejected from the colony at the end of the season.

Beside the individual level reproduction briefly described above, there is a colony level reproduction (i.e., swarming). The old queen leaves the colony with a majority of the workers that locate a suitable nest-site where the new colony settles. The remaining workers and a newly reared virgin queen are left behind; the virgin queen mates with drones during a mating flight, and starts laying eggs, bringing the swarm cycle to completion.

2.2 Social immunity and pathogen transmission

The honey bee colony can be considered a superorganism where each individual bee is dispensable just like a single cell in a human body. Division into two or several colonies is fundamental for colony fitness (Moritz & Southwick, 1992), and the two levels of reproduction are important when discussing pathogen transmission and colony immunity.

Transmission of pathogens can be either horizontal (between individuals within a single generation) or vertical (between an individual from one generation to another in the next generation). In honey bee colonies, these

modes of transmission work on two levels; intra- and inter-colony level (Fries & Camazine, 2001). The colony immune response is based on consequences of pathogenic pressure at both individual and colony level (Cremer *et al.*, 2007). A short résumé of the steps of pathogen invasion into a honey bee colony (*Figure 2*) is as follows;

2.2.1 Step 1 and 2. Pathogen uptake and intake

A new infection occurs when a pathogen enters the colony either vertically from the mother colony or from outside. Viral infections can be introduced into a colony with mechanical and biological vectors, but horizontal transmission and uptake independent of vectors also exists. Foragers may bring pathogens into the colony from the external environment. Drifting (when forager bees enter a colony other than their own) is example of how an infected bee can introduce a pathogenic agent from a neighbouring colony. Specialised guard worker bees control the nest entrance and may prevent the entry of infected foragers (Drum & Rothenbuhler, 1985).

2.2.2 Step 3. Establishment of the pathogen in the nest

Irrespective of whether a pathogenic agent enters the colony actively or via vertical transmission, the pathogen has to establish itself in the internal environment. Some pathogens successfully multiply within the individual bee or larvae, but hygienic behaviour among honey bees such as constructing nests from antimicrobial materials (Gilliam *et al.*, 1988) or the quick removal of dead and infected individuals (Spivak & Reuter, 2001) are obstacles a pathogen needs to overcome in order to establish an infection in the colony.

2.2.3 Step 4. Vertical and horizontal transmission between individuals

The probability of a healthy bee becoming infected is a function of (a) the infectiousness of the infected individual, *i.e.*, the number of infectious particles it can transfer to its nestmates, (b) contact rate and type of interaction, and (c) the susceptibility of the non-infected individuals. Both horizontal and vertical transmission occurs within the colony (de Miranda & Fries, 2008). Collective defense mechanisms to prevent pathogens from spreading between group members are implemented at any of these steps (Cremer *et al.*, 2007).

2.2.4 Step 5. Vertical and horizontal transmission between colonies

Honey bee pathogens are transmitted vertically when colonies reproduce by colony fission (swarming). Horizontal transmission to neighbour colonies is

also likely to occur with infected drifters, when an infected colony is robbed out or with beekeeping practices that can involve interchanging equipment between colonies.

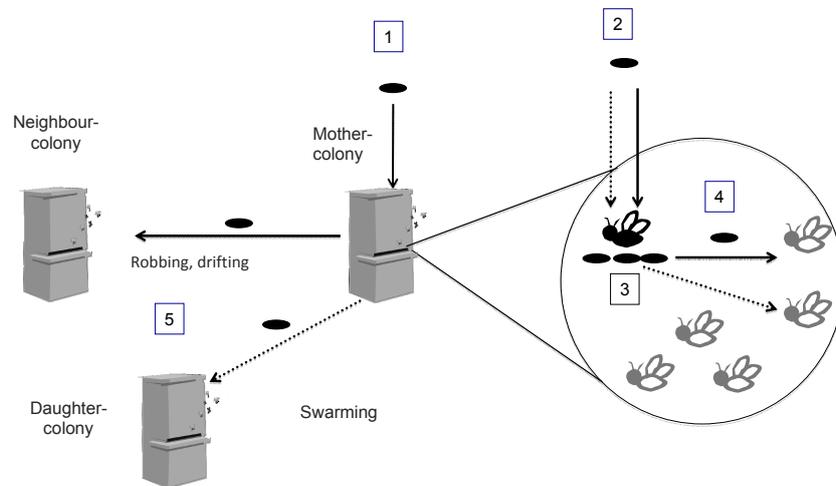


Figure 2. Pathogen transmission in honey bees. 1. Pathogen entry into host colony. 2. Pathogen entry into individuals. 3. Multiplication within individuals. 4. Transmission between individuals. 5. Transmission between colonies. Solid arrows indicate horizontal transmission. Dotted arrows indicate vertical transmission.

2.3 Individual immunity

Insect immunity shows many similarities to the innate immune response of vertebrates. Innate immunity is the first line of internal defense in a host. This defense allows all animals (and plants) to detect infectious agents and to activate a set of responses for controlling the infection (Dostert *et al.*, 2008). Innate immunity involves a diverse set of actions such as the secretion of antimicrobial peptides, phagocytosis, melanization and enzymatic degradation of pathogens (Hoffman, 2003; Hultmark, 2003). The study of innate immunity in both vertebrate and invertebrate models has shown that some of these mechanisms are ancient, and have been conserved throughout evolution (Hoffman, 2003).

Recent sequencing of the honey bee genome (Weinstock *et al.*, 2006) made analysis of the immune components in honey bees possible. A comparison between the *A. mellifera* genome and the sequenced *Drosophila* and *Anopheles* genomes revealed that honey bees possess only one-third as many genes involved in insect immunity. It has been suggested that the diminished immune capacities in individual bees reflect either the strength of their social immunity or a tendency to be attacked by a more limited set of pathogens (Evans *et al.*, 2006).



Figure 3. A colony of Asian honey bees, *Apis cerana*. The queen is seen in the middle of the picture surrounded by workers. Photo: Ingemar Fries.

3 Honey bee parasites and pathogens

Honey bee diseases have been a concern of man for thousands of years, and Aristotle (384–322 B.C.) described such disorders. Considering the high genetic relatedness and high density in the colony, honey bees are afflicted by relatively few diseases. However, some specific pathogens and parasites affecting adult bees, larvae and pupae have adapted to the honey bee colony, and are capable of causing colony collapse. Although many of the pathogens may kill the individual they infect, their effects on the colony are more unpredictable. Some infections may lead to colony collapse, whereas others (although lethal to the individual) are less virulent at the colony level.

This thesis includes work concerning five of the most important disease causing organisms of honey bees which are all capable of causing depletion in honey bee colonies. Results from the studies included in the thesis are presented in chapter 3 and 4, highlighted in bold letters.

3.1 Bacteria

The name “foulbrood” was first introduced as a descriptive name of a honey bee brood disease characterized by a foul smell (Schirach, 1769). The symptoms of foulbrood disease were further described in the late 19th century (Cheshire & Cheyne, 1885), but it was not until 1912 that it became clear that there were actually two honey bee brood diseases assigned the same name (White, 1912); European foulbrood (EFB) caused by the bacterium *Melissococcus plutonius* (Bailey, 1983; Bailey, 1956; **Paper I**) and American foulbrood (AFB) caused by the spore forming bacterium *Paenibacillus larvae* (**Paper II**). Both species are gram-positive bacteria belonging to the phylum *Firmicutes* (Figure 4). *P. larvae* belong to the genus *Paenibacillus* in the family *Paenibacillaceae* and *M. plutonius* belongs to the monospecific genus *Melissococcus* within the *Enterococcaceae* family.

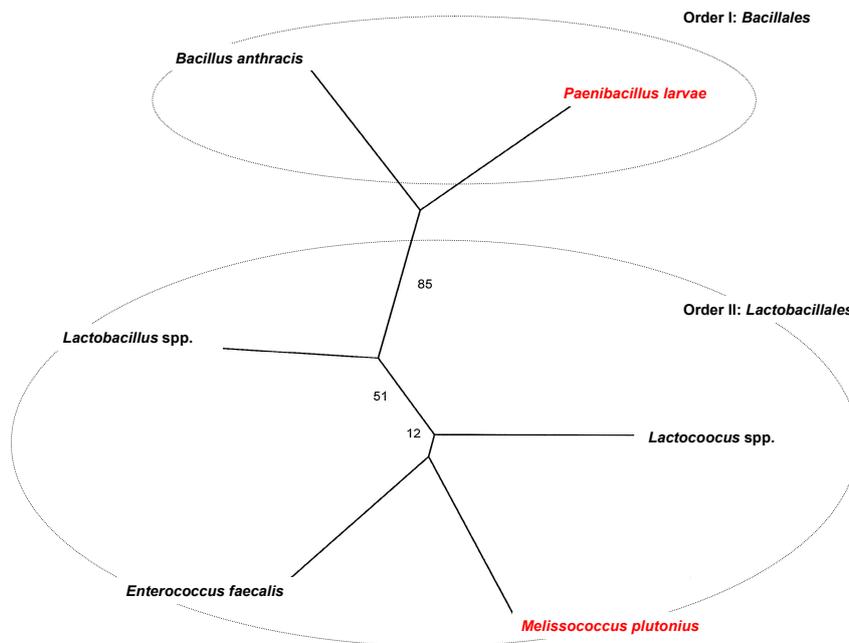


Figure 4. Phylogenetic tree showing a selection of bacterial species in section XXIV (The Bacilli and Lactobacilli) within the phylum Firmicutes.

3.1.1 *Melissococcus plutonius*

The causative agent of EFB, *M. plutonius*, is a Gram-positive, lanceolate coccus, sometimes pleomorphic and rod-like. The bacterial cells occur singly, in pairs or in chains of various lengths. *M. plutonius* is microaerophilic to anaerobic and needs carbon dioxide for growth. European foulbrood was originally thought to be caused by *P. alvei* (Cheshire & Cheyne, 1885) but was later described as caused by the bacterium *Bacillus pluton* (White, 1912). The organism was first cultivated and characterized by Bailey (1956) who concluded that *Streptococcus pluton* would be a more suitable name for the bacterium. Later, a new monospecific genus *Melissococcus* was created and the type species was named *Melissococcus pluton* (Allen & Ball, 1993; Bailey & Collins, 1982a; Bailey & Collins, 1982b). The name was later modified to *Melissococcus plutonius* (Truper & de Clari, 1998). Cai and Collins (1994) made a comparative sequence analysis revealing that *M. plutonius* is a close phylogenetic relative of the genus *Enterococcus*. Furthermore, isolates of *M. plutonius* are remarkably homogenous based on morphological, physiological and immunological (Allen & Ball, 1993; Bailey & Gibbs, 1962) as well as genetic studies (Djordjevic *et al.*, 1999; Dancer & Barnes, 1995).

Several other bacteria may be associated with EFB (*Achromobacter curidice*, *Enterococcus faecalis*, *Paenibacillus alvei* and *Brevibacillus laterosporus*), and at one time been considered the primary pathogen of EFB. However, no experimental data have been presented to justify such claims. In contrast, infectivity tests causing disease in bee colonies with cultured *M. plutonius* have been reported (Bailey & Locher, 1968; Bailey, 1963) although more severe clinical symptoms were observed when using extracts from naturally EFB infected larvae (Bailey, 1963; Bailey, 1960).

EFB affects mainly uncapped brood, killing honey bee larvae usually when they are 4 to 5 days old. The infected larva move in the brood cell, and instead of the normal, coiled position, the larva die displaced in its cell, twisted around the walls or stretched out lengthways. The color of the larvae changes from pearly white to yellow, then brown and finally, when they decompose, grayish black (Bailey, 1960). Some larvae may also die after the cell is sealed, resulting in sunken capping resembling the symptoms of AFB. If a high proportion of the larvae die, the brood pattern appears patchy and sometimes gives off a foul or sour smell.

The conventional view of EFB is that it is caused by the pathogen *M. plutonius*, but it has also been suggested that *M. plutonius* is a ubiquitous commensal in honey bees (Dancer, 2004). Few studies have been made on actual outbreaks of EFB, and new techniques enables studies of the occurrence and spread of *M. plutonius* in honey bee colonies with and without disease symptoms. Considering the increased incidence of EFB in some parts of the world, deeper knowledge of the epidemiology and virulence of the causative agent is of great importance. In **Paper I**, we investigated the occurrence and distribution of *M. plutonius* in diseased and healthy larvae in colonies with and without symptoms of EFB using a hemi-nested PCR (Djordjevic *et al.*, 1998).

The obtained data showed a strong correlation between occurrence of *M. plutonius* and clinical symptoms of EFB, and we could conclude that *M. plutonius* is not ubiquitous, but indeed an infectious pathogen. Furthermore, even in colonies with severe symptoms of disease, not all individuals are colonized by the bacterium. *M. plutonius* can be detected in larvae and pupae without any clinical symptoms although mainly detected in diseased larvae within limited areas of the brood (**Paper I**). In a related article, Belloy *et al.* (2007) found that the bacterium is more frequent among adult bees compared to larvae in diseased colonies and that more than 90% of the adult bees are carriers of *M. plutonius* in symptom free colonies found in apiaries with the disease. Adult worker bees transmit the bacterium not only within the colony, but also between colonies and apiaries (Belloy *et al.*, 2007; McKee *et al.*, 2003).

3.1.2 *Paenibacillus larvae*

American foulbrood (AFB) is a common bacterial disease affecting apiculture worldwide (Ellis & Munn, 2005). AFB is classified on list B of the World Organization for Animal Health (OIE, Organization International des Epizootic). The definition of “List B” diseases is transmissible diseases which are considered to be of socio-economic and/or public health importance within countries and is significant in the international trade of animals and animal products.

The disease is caused by *Paenibacillus larvae* (**Paper II**), a spore-forming, Gram-positive rod-shaped bacterium. The vegetative bacterial cell is about 2.5–5 µm by 0.5–0.8 µm and motile due to its peritrichous flagellae. The inactive forms of the bacterium, the endospores, are extremely resilient and may still be infectious after decades (Matheson & Reid, 1992).

The bacterium was first described as *Bacillus larvae* (White, 1906) and has been reclassified several times. A closely related species, *Bacillus pulvificiens*, was described as the causative agent of a rare disease called “powdery scale disease” (Katznelson, 1950), and the two sister species were later named *Paenibacillus larvae* and *Paenibacillus pulvificiens* (Ash *et al.*, 1993; Ash *et al.*, 1991). In 1996, a revised classification to *Paenibacillus larvae* subspecies *larvae* and *Paenibacillus larvae* subspecies *pulvificiens*, respectively, was suggested (Heyndrickx *et al.*, 1996). However, following analysis of brood samples sent to the honey bee diagnostic laboratory at SLU, Uppsala, it was obvious that clinical symptoms of AFB could also be caused by *P. pulvificiens*. This initiated the collaborative study presented in **Paper II**. After a final revision the bacterium is now classified as one species, *Paenibacillus larvae*, without any subspecies differentiation (**Paper II**).

Strains of *P. larvae* can be subdivided into genotypes based on rep-PCR (see 4.1.4) using ERIC-primers (Genersch & Otten, 2003; Versalovic *et al.*, 1994). Genotypes differ in prevalence (Loncaric *et al.*, 2009; Peters *et al.*, 2006; Genersch & Otten, 2003) as well as in virulence, course of infection (Genersch *et al.*, 2005) and sensitivity to heat treatment and storage (Forsgren *et al.*, 2008). Other characteristics, such as colony morphology and biochemical properties of the genotypes are further described in **Paper II**.

Young larvae become infected through ingestion of contaminated food, and the bacterial spores germinate and proliferate in the midgut lumen before they start to breach the epithelium and invade the haemocoel (Yue *et al.*, 2008). The colonization of the larval midgut has been suggested to be one of the key steps in the pathogenesis of *P. larvae* (Yue *et al.*, 2008). Newly hatched larva may become infected by as few as 10 spores (Woodrow, 1942), but the dosage-mortality relationship is greatly

influenced by larval age, larval genetic constitution and bacterial strain (Genersch *et al.*, 2005). Thus, the number of spores needed to cause disease in individual larvae is very variable (Ashiralieva & Genersch, 2006).

The symptoms of the disease are typical; the remains from dead brood in sealed cells consist of a brownish, sticky substance sometimes with a foul smell. If drawn out with a matchstick, the larval remains will form a glue-like thread. If these remains are left to dry, it will eventually form a black scale in the bottom of the cell. These larval remains have been reported to contain as many as 2.5 billion spores (Sturtevant, 1932).

Data presented in **Paper II** showed that all *Paenibacillus* species/genotypes caused symptoms of AFB in larval bioassays and the polyphasic taxonomic study did not support subspecies differentiation. The extensive experimental study resulted in a final classification of the bacterium as one species, *P. larvae* without any subspecies differentiation and a revision of the OIE-manual (Anonymous, 2008a), where the causative agent of AFB has been changed from *P. larvae* subspecies *larvae* to *P. larvae* without subspecies differentiation.

3.2 Parasitic mites

Mites (Acari or Acarina) are truly ubiquitous and have successfully colonized nearly every known terrestrial, marine, and fresh water habitat. Many kinds of mites parasitize insects and animals where they live and multiply on almost any external or internal surface of their host. Several species have specialized to a life within the tracheal system of insects, including locusts and bumble bees, and at least 40 species have been associated with honey bees (Eickwort, 1988). To date, eight species are known to parasitize *A. mellifera* (Anderson & Morgan, 2007; Anderson & Trueman, 2000; Bailey & Ball, 1991).

3.2.1 *Varroa destructor*

The ectoparasitic mite, *Varroa destructor* (Anderson & Trueman, 2000) is an obligate honey bee parasite that feed on the hemolymph of the brood and adults. The parasitism of the mite involve different mechanisms that may weaken the colony, and the role of *V. destructor* as a vector of bacterial, fungal and viral diseases in particular is well documented (Cox-Foster *et al.*, 2007; Yang & Cox-Foster, 2007; Shen *et al.*, 2005; Liu, 1996; Glinski & Jarosz, 1992). Honey bee viruses that normally occur at low, non-epidemic levels have become epidemic as this new route of transmission has appeared (Sumpter & Martin, 2004). The depletion of honey bee colonies infested by

V. destructor is commonly attributed to viral infections, and the mite also act as a biological (reproductive) vector for some viruses (Gisder *et al.*, 2009; Yue & Genersch, 2005), including deformed wing virus, DWV (see 3.3.1 and **Paper III**)

3.2.2 *Tropilaelaps mercedesae*

Other external parasites of honey bees are mites in the genus *Tropilaelaps*. *Tropilaelaps clareae*, whose natural host is the giant honey bee, *Apis dorsata*, has also been found on *A. mellifera* throughout South East Asia (De Jong *et al.*, 1982). Recent data redefines the *Tropilaelaps* mite that can reproduce on *A. mellifera* brood as two separate species, *T. clareae* and *Tropilaelaps mercedesae* (Anderson & Morgan, 2007). The other two species (*Tropilaelaps koenigerum* and *Tropilaelaps thaii*) appear to be harmless to *A. mellifera* (Anderson & Morgan, 2007). Heavy colony losses caused by *Tropilaelaps* spp. have been reported (Camphor *et al.*, 2005) and *Tropilaelaps* infestations may be considered more damaging to *A. mellifera* than *Varroa* infestations. The *Tropilaelaps* mites cannot feed on adult bees and die within a few days without access to brood (Woyke, 1987), limiting its expansion to temperate climates with extended brood-free periods. Hence, global climate changes may have a direct effect on the occurrence, emergence and spread of *Tropilaelaps* mites.

3.3 Viruses

Virus infection in bees was first described by White (1913) who observed that a filterable agent from diseased larvae could cause sacbrood disease in honey bees. Since then, at least 18 honey bee viruses have been reported worldwide (Ribi re *et al.*, 2008; Chen & Siede, 2007; Ellis & Munn, 2005). Many viruses associated with insects can cause asymptomatic infections, and honey bees may carry virus which generally provoke no visible symptoms. The recent introduction of the *V. destructor* to *A. mellifera* colonies worldwide has changed the situation. Several studies implicate that the combination of this parasitic mite and certain viral infections may pose a serious threat to the health of honey bee colonies (Nordstr m *et al.*, 1999; Ribi re *et al.*, 2008; Berenyi *et al.*, 2006; Chen *et al.*, 2006; Ball, 1993; Ball & Allen, 1988).

3.3.1 Deformed wing virus

Deformed wing virus is one of the few viruses that cause well-defined disease symptoms in infected bees. As the name of the virus indicates, one of

the characteristic symptoms is deformed or poorly developed wings in the newly emerged bees from affected colonies.

Deformed wing virus belongs to the genus Iflavirus, consists of a single, positive strand RNA genome and produces a 30nm icosahedral particle (Lanzi *et al.*, 2006). Previous studies of the genetic variability and population structure have described DWV to be rather homogenous (Berenyi *et al.*, 2007; Fujiyuki *et al.*, 2006), but the results presented in **Paper III** revealed considerable heterogeneity and polymorphism.

Overt DWV infections are invariably associated with *V. destructor* infestations (Martin, 2001) while in the absence of the mite, DWV seem to persist as a covert infection (deMiranda & Genersch, 2009). The mite acts as a mechanical vector but is also a potential biological vector, since DWV is able to replicate within mites (Yue & Genersch, 2005; Ongus *et al.*, 2004). Development of deformed bees with crippled wings is positively correlated to the DWV titers in the parasitizing mites (Gisder *et al.*, 2009; Bowen-Walker *et al.*, 1999).

Colonies of European honey bees infested by *Tropilaelaps* spp. can occasionally contain bees emerging with crippled wings (Sharma *et al.*, 1994; Sihag & Singh, 1991; Burgett & Akwatanakul, 1985). Both the *Tropilaelaps* and *Varroa* mites feed on honey bee brood, and *Varroa* mites transmit viral particles when feeding on the hemolymph of pupae or adult bees. To investigate whether DWV could be linked to *Tropilaelaps* infestation of European honey bee colonies, brood and adult bees from one *Tropilaelaps* spp. infested *A. mellifera* colony was assayed for DWV (**Paper III**). A quantitative real-time RT-PCR assay (RT-qPCR) was developed to quantify DWV in infested brood and *Tropilaelaps* mites.

Recently presented data redefines the *Tropilaelaps* reproducing on *A. mellifera* brood as two separate species (Anderson & Morgan, 2007). We used these new species criteria to identify the *Tropilaelaps* species in our samples as *T. mercedesae* (**Paper III**).

Our results strongly indicated that infestation of *A. mellifera* by *T. mercedesae* is linked to DWV infections similar to those that occur with *Varroa* infestations, and that the viral load in infested pupae is positively correlated to the amount of virus present in the corresponding mites (**Paper III**). The extremely high DWV titers in the infesting mites compared to corresponding infested pupae suggests viral replication in the mites. Direct evidence for DWV replication within the *T. mercedesae* similar to the *Varroa*-DWV association has recently been presented (Dainat *et al.*, 2009). Thus, it is highly probable that symptoms in *A. mellifera* colonies infested by *T.*

mercedesae are due to DWV infections similar to the interaction between DWV, *A. mellifera* and *V. destructor* (Sumpter & Martin, 2004).

3.4 Microsporidia-*Nosema*

Microsporidia are obligate intracellular parasites belonging to the cluster Fungi (Sina *et al.*, 2005). Thus, taxonomically microsporidia are highly specialized parasitic fungi dispersing between hosts as spores. *Nosema* is a genus within microsporidia causing infection in the ventriculus (nosemosis) of adult honey bees. Adult bees contract infectious spores mostly through combs soiled with feces from infected bees (Bailey, 1955), or through contaminated food/water (L'Arrivée, 1965). Digested spores germinate in the midgut extruding the polar filament with great force. To initiate infection, the cell membrane of an epithelial cell must be penetrated by the polar filament and the diplokaryotic sporoplasms become injected into the host cell cytoplasm. Following injection, the sporoplasm matures into a mother cell (meront) that multiply inside host cells. Two types of spores are produced, internal spores germinating inside the host cell and responsible for the between-cell transmission and external spores representing the transmission between hosts. When released into the midgut, the external spores may germinate and reinfect the epithelium, but most of them accumulate in the bee's rectum and are voided with the feces providing new sources of infection (Fries, 1993).

In *A. mellifera*, nosemosis was for decades thought to be exclusively caused by *Nosema apis*, but recent studies have shown that another *Nosema* species, *Nosema ceranae* (earlier considered restricted to *A. cerana*) also infect *A. mellifera* (Huang *et al.*, 2007; Fries *et al.*, 2006; Higes *et al.*, 2006). Infection experiments have demonstrated that both parasites are cross-infective across host species, but that *N. apis* develops less well in *A. cerana* compared to *N. ceranae* in *A. mellifera* (Fries, 1997; Fries & Feng, 1995).

3.4.1 *Nosema apis*

Nosema apis was first described by the German researcher Enoch Zander (1909). *N. apis* is known to be distributed in honey bees worldwide (Ellis & Munn, 2005; Matheson, 1993), and causes serious problems to apiculture in temperate climates. At colony level, infections of *N. apis* are correlated with winter loss of colonies, poor spring build-up and reduced honey yield (Fries, 1988).

There is no specific outward sign of disease in individual bees infected with *N. apis*, although the ventriculus of heavily infected bees may appear

opaque-white and swollen (Fries, 1997). Survival data comparisons from cage experiments suggest that dosing newly-emerged bees with *N. apis* may result in a relatively fast death for some bees and a slower death for most of the bees. Also, spore-loads may vary greatly, with no clear relationship to survival time (Malone & Giacon, 1996).

3.4.2 *Nosema ceranae*

Nosema ceranae is a microsporidian parasite presently known to infect both the Asian honey bee, *A. cerana*, and the European honey bee, *A. mellifera* (Higes *et al.*, 2006; Fries *et al.*, 1996). Recent studies suggest that *N. ceranae* made a host switch to *A. mellifera* over 10 years ago, displacing *N. apis* as the most common *Nosema* infection in many regions of the world (Chen *et al.*, 2008; Klee *et al.*, 2007; Paxton *et al.*, 2007). It has also been suggested that *N. ceranae* may contribute to the recent large numbers of colony losses in Europe and the United States (Cox-Foster *et al.*, 2007; Martin-Hernandez *et al.*, 2007; Oldroyd, 2007).

The little data yet available on colony level virulence of *N. ceranae* infections is contradictory. Several studies from Spain suggest that *N. ceranae* is a highly virulent parasite at the colony level and that infection eventually lead to colony collapse unless controlled with antibiotics (Higes *et al.*, 2008; Martin-Hernandez *et al.*, 2007). However, *N. ceranae* infections are prevalent in many areas where colonies are left untreated without significant colony losses linked to *Nosema* infections (Invernizzi *et al.*, 2009; Chen *et al.*, 2008; Siede *et al.*, 2008).

The effect from infections of *N. ceranae* on the individual bee has been investigated by Higes *et al.*, (2007a). In cage experiments, bees infected with *N. ceranae* all died within eight days post infection. However, this high and rapid mortality has not been confirmed in other reports using cage experiments and individual feeding (Mayack & Naug, 2009; Paxton *et al.*, 2007). Although the ID₅₀ value for *N. ceranae* may be somewhat lower, *N. ceranae* infections do not induce higher mortality than infection caused by *N. apis* in our experiments (**Paper IV**). Furthermore, when investigating the course of infection, the spore load in the individual bee built up more slowly with *N. ceranae* compared to *N. apis*, and 12 days after infection the number of spores was almost the same for both parasites (**Paper IV**). This is consistent with results from Paxton *et al.* (2007) where both parasites reach approximately 30 million spores in the midgut after 10-12 days, and does not imply a faster or more effective spore production in *N. ceranae*.

Mixed infections with *N. ceranae* and *N. apis* in individual bees are found in some areas (Chen *et al.*, 2009; Fries & Forsgren, 2009; Paxton *et al.*,

2007) In order to study within-host competition, bees were individually fed the two *Nosema* species in three relative frequencies (**Paper IV**) but no clear competitive advantage was found for either of the two parasites. Even though earlier studies suggest that infections caused by *N. ceranae* are lethal for adult bees, no competitive advantage within the individual bee could be seen (**Paper IV**). The data actually suggest a tendency for the least common variant to be favored, which could reflect a scenario of negative frequency-dependent selection within the host (Weeks & Hoffmann, 2008). This is to our knowledge the first within-host study of multiple *Nosema* species infections in honey bees.

Collective data on the virulence of *N. ceranae* infections (at colony level as well as individual level) is contradictory. The discrepancies may be due to a number of yet unresolved factors such as climatic differences, different isolates of the parasite, nutritional conditions, larval immune response, hygienic behavior or interaction between *Nosema* and the intestinal microbiota of the honey bee larvae. Synergistic effects of all the factors mentioned above may also be of importance. Furthermore, differences in experimental and detection methods may lead to inconsistent results (see section 4.1.3).

4 Methods

Detection of a disease causing agent is of the utmost importance since accurate diagnoses in a timely manner facilitate preventive actions to reduce or impede the spread of a disease.

According to the Merck Manual of Diagnosis and Therapy (Anonymous, 2006), major options for laboratory diagnosis of a pathogen can be classified into following categories:

- Culture based methods
- Microscopy
- Immunological methods
- Non-nucleic acid based methods
- Nucleic acid based methods

Methods within the above mentioned categories all have advantages and limitations and are frequently used within diagnostic microbiology and various research fields. Recent advances in molecular technology are making it increasingly easy to study microbes. Depending on the target molecule, the molecular detection methods can be separated into two categories; nucleic acid or protein detection techniques. Nucleic acid detection methods and its applications in honey bee pathology will be presented in the following chapter.

4.1 Nucleic acid detection

The success of nucleic acid-based methods relies on detecting a DNA (or RNA) sequence that distinguishes the target organism from all other known microbes and from background material including DNA from the host (animal, plants and other species). Thus, the selection of a species or strain-

specific DNA signature is crucial. This “signature sequence” is a nucleotide sequence unique to a specific taxonomic group, for instance a species or a genotype (Phillippy *et al.*, 2007). Upon choosing the organisms DNA signature, a variety of molecular detection techniques can be used to detect the specific marker. An ideal assay is rapid, easy to perform, inexpensive and have high reproducibility. The choice of method may be based on sample type, technological resources among others.

DNA-based detection can be classified into two major groups: non-amplified (including hybridization-based approaches) and those using DNA amplification by polymerase chain reaction (PCR). Within these groups, the number of alternative methods is continuously increasing.

4.1.1 DNA and RNA extraction

All nucleic acid-based protocols require DNA/RNA extraction; hence the quality of the extracted nucleic acid is a critical factor for all these detection techniques (Albuquerque *et al.*, 2009). Since RNA is easily degraded by the ubiquitous presence of endogenous enzymes, the choice of storage method prior to detection is crucial (de Miranda, 2008; Chen *et al.*, 2007). Errors deriving from storage and sample processing become especially evident with quantitative data (see 4.1.3.). In order to determine the quality of the extracted RNA templates used in **Paper III**, the amplification of a reference gene (RP49) was included in the assay (de Miranda & Fries, 2008) to ensure accuracy of the quantitative results. The expression of the reference gene (RP49) was unchanged through the experiment thus indicating equal RNA quality in all samples.

Although technical advances have simplified the DNA/RNA extraction, allowing for safer, automated and rapid methodologies, PCR inhibition in complex biological samples may still be a problem (Mackay, 2007; Lantz, 1998; Wilson, 1997). Interference from PCR inhibitors in honey has been reported (Piccini *et al.*, 2002; Dobbelaere *et al.*, 2001). The protocol published by McKee and co-workers (2003) and used for larval DNA extractions in **Paper I** proved to be inappropriate for honey samples and adult bees. Using a commercial DNA isolation kit for purifying DNA from honey (**Paper I**) or adult bees (Belloy *et al.*, 2007) resolved the PCR inhibition problems in this qualitative PCR.

4.1.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is widely employed and allows for rapid, specific and sensitive amplification of target DNA from test samples. The PCR method utilizes a pair of “primers” (short, single stranded pieces

of DNA) designed to hybridize a particular strand of a DNA target. The primers span a target region that is exponentially duplicated during subsequent reactions. The continuous doubling of the DNA is accomplished by enzymes (polymerases) that string together nucleotides to new, complementary DNA strands. The end product of this duplicated region is referred to as “amplicon”, and usually identified using electrophoresis.

The PCR is user friendly and theoretically simple, although DNA/RNA extraction and PCR reactions must be carefully evaluated and optimized (Wilson, 1997). When PCR is used only for detecting the presence or absence of a specific DNA signature, the method is referred to as qualitative PCR (resulting in a yes or no answer).

A variant of PCR is the RT-PCR referring to the detection of RNA by converting it into complementary DNA (cDNA) with reverse transcriptase (RT) followed by PCR. This method is widely used for detection of honey bee virus since the vast majority of these viruses possess an RNA genome (Blanchard *et al.*, 2008; de Miranda *et al.*, 2008; Blanchard *et al.*, 2007; Genersch *et al.*, 2006; Chen *et al.*, 2005; Grabensteiner *et al.*, 2001). The reliability of the RT-PCR is to a high degree defined by the reverse transcriptase part of the reaction, since the performance of this enzyme is far more variable than that of the thermostable DNA polymerase used for the PCR. The development of a more heat tolerant reverse transcriptase and a single reaction buffer made it possible to carry out the cDNA synthesis and PCR amplification in the same tube. The one-step RT-PCR technique was implemented and used in **Paper III**.

After designing primers for a specific sequence of the target organism, PCR can be used for microbe detection either from cultured isolates or clinical samples. In honey bee pathology, a number of PCR and RT-PCR applications have been described for detection of pathogens in adult bees, brood, honey, pollen and wax (e.g. Chen *et al.*, 2009; Eyer *et al.*, 2009; de Miranda *et al.*, 2008; Blanchard *et al.*, 2007; Gauthier *et al.*, 2007; Higes *et al.*, 2007b; Genersch *et al.*, 2006; Chen *et al.*, 2005; Tentcheva *et al.*, 2004; Lauro *et al.*, 2003; McKee *et al.*, 2003).

When testing the specificity and sensitivity of a PCR protocol, obtaining promising results with pure cultures of your target organism does not necessarily mean similar efficiencies when analyzing different types of samples. The presence of a large amount of genomic background DNA in complex biological samples is likely to reduce both specificity and the detection level of the assay (Iqbal *et al.*, 1997). With the PCR method used for detection of *M. plutonius* in **Paper I**, (Djordjevic *et al.*, 1998), we were able to detect 10fg (10^{-14}) of bacterial DNA extracted from pure culture of

the bacteria. However, when the reaction was carried out on an artificially mixed template, *i.e.* purified *M. plutonius* DNA + purified DNA from healthy larvae, the detection level decreased 10-fold to 100fg (10^{-13}). Furthermore, the PCR assay published by Djordjevic *et al.* was used not only for increased detection resolution, but also for increased specificity. In contrast to a PCR assay published by Govan *et al.* (1998), the specificity of the primers used in **Paper I** was verified. The authors used DNA purified from other honey bee pathogens and a range of saprophytic organisms commonly recovered from honey bee colonies as templates in the PCR reaction. No nonspecific amplicons were produced and the assays specificity for *M. plutonius* was verified.

Multiplex PCR uses several primer pairs targeting different loci in a single PCR reaction. This makes it possible to target more than one pathogen in a single assay. However, the applicability of the method is limited and detection levels may be lower partly due to a higher risk for accumulation of nonspecific products (Gyarmati, 2008). Multiplex PCR systems are published for detection and quantification of *N. apis* and *N. ceranae* (Chen *et al.*, 2009; Martin-Hernandez *et al.*, 2007) and multiple virus infections (Teixeira *et al.*, 2008; Topley *et al.*, 2005; Grabensteiner *et al.*, 2001). Although the primers presented and used in uniplex qPCR in **Paper IV**, the intention when designing a common reverse primer was to equalize the conditions for both amplifications in a qualitative duplex PCR.

4.1.3 Real-time PCR

In contrast to conventional PCR methods, real-time PCR detects the amplicon as it accumulates (in “real” time) and determines the number of new DNA molecules formed in each reaction. It is the detection process that discriminates real-time PCR from conventional “end-point” PCR assays. Real-time PCR needs a fluorescent reporter that binds to the amplicon and reports its presence by fluorescence. As the amount of product accumulates, a fluorescent signal develops that initially increases exponentially. The number of amplification cycles required for the samples response curve to reach a particular fluorescent threshold is called the quantification cycle (Cq) value. Differences in Cq values reflect the differences in initial amount of template.

There are broadly two major variants for amplicon detection: specific or non-specific. Most of the specific real-time PCR chemistries involve hybridization of a fluorophore-labeled oligoprobe using fluorescence resonance energy transfer (FRET) and a black-hole non-fluorescent quencher (NFQ). The non-specific assays uses dyes that will interact with

any and all double stranded DNA, e.g. SYBRgreen I. In general, the specific and non-specific fluorogenic chemistries detect the amplicon with the same sensitivity (Mackay, 2007), and assays based on SYBRgreen I chemistry can be combined with melting (dissociation) curve analysis in order to discriminate between specific and nonspecific amplicons.

Real-time PCR does not always mean quantitative PCR. The majority of real-time PCR applications in microbiology are for qualitative detection of microbes. However, this technique also allows for quantification of target nucleic acids for many purposes. It is assumed that a direct relationship exist between the amount of target nucleic acid and the actual number of microorganisms in a sample, and to strengthen this association the amplification variability should be minimized. The impact of the variables in each assay is more visual than with ordinary PCR, thus permitting improved assay optimization, standardization and normalization (Ferre, 1992).

The amount of the target DNA molecule or template in a sample can be quantified either relatively or as absolute values/numbers. Relative quantification describes changes in amount of the target sequence compared to a reference sequence. Absolute quantification determines the exact number of target molecules in relation to a specific calibrator unit. The quantification calibrator is most commonly created using a cloned amplicon (**Paper III**), a portion of the target organism's genome or the purified amplicon itself (**Paper IV**). Dilutions should be carried out into defined concentrations of unrelated carrier nucleic acid, e.g. yeast tRNA (Bustin *et al.*, 2009) essential for quantitative recovery of small amounts of nucleic acids in dilute solutions. To date, the method developed and presented in **Paper III** is the only published method for absolute quantification of DWV particles in a sample.

The quality of quantitative data relies upon a number of PCR variables such as

- i) variation in template amount between samples
- ii) poor or variable quality template (variations in purification and/or storage
- iii) inhibitors (of both fluorescence and amplification)
- iv) variation in performance of DNA polymerase,
- v) variation in amplification between sample and calibrators (Mackay, 2007).

Although using standardized and effective nucleic acid extraction methods, some inhibiting compounds may still remain and affect the kinetics of the PCR. The choice of enzymes and buffers may therefore be critical.

As a part in the optimization process of the qPCR described in **Paper III**, two commercially available one-step RT-PCR kits were compared (ABsolute™ MAX QRT-PCR SYBR® Green Mix from ABgene™ and Bio-Rad iScript™ One-Step RT-qPCR Kit). RNA extractions of virus-infected and uninfected adult bees were made using the column based RNEasy™ protocol (Qiagen) as described in **Paper III**. RNA from the infected bee was diluted in the uninfected bee extraction to look for possible inhibition. The results are indeed very different. No inhibitory effect from the bee RNA extraction could be seen using the kit from Bio-Rad (*Figure 5*), whereas the RT-qPCR reaction using the ABgene-kit was clearly inhibited by the bee homogenate (*Figure 6*).

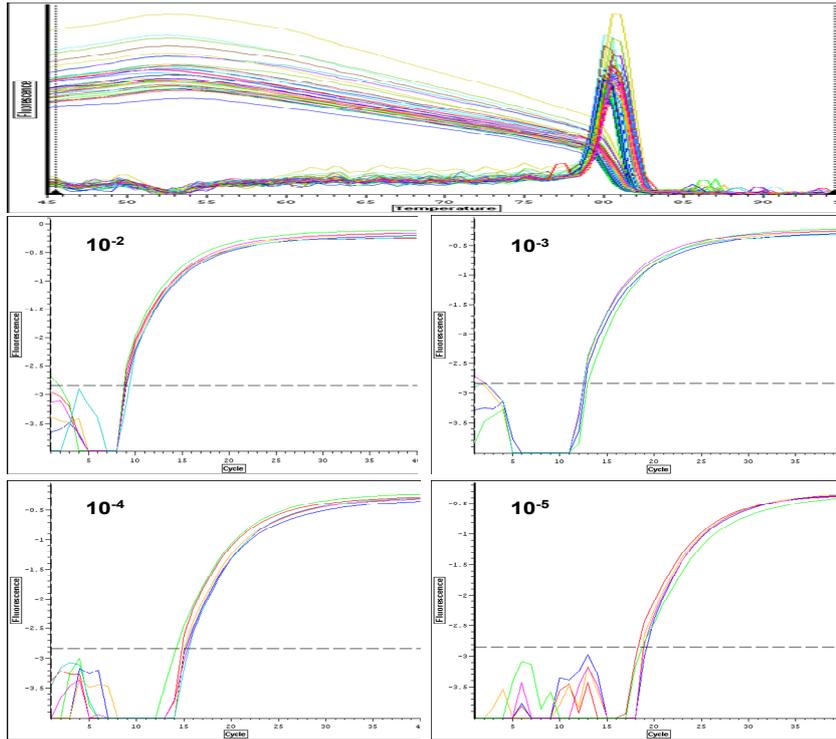


Figure 5. Real-time PCR using Bio-Rad iScript™ One-Step RT-PCR Kit and DWV specific primers detecting RNA from DWV infected bee diluted in RNA preparation of an uninfected bee. Dilutions 10^2 , 10^3 , 10^4 and 10^5 are shown. No inhibitory effect from the bee RNA extract is observed. The C_q value is the same for infected bee diluted in H₂O as for infected bee diluted in uninfected bee RNA extraction. No nonspecific products or primer-dimers can be detected by the melt-curve analysis.

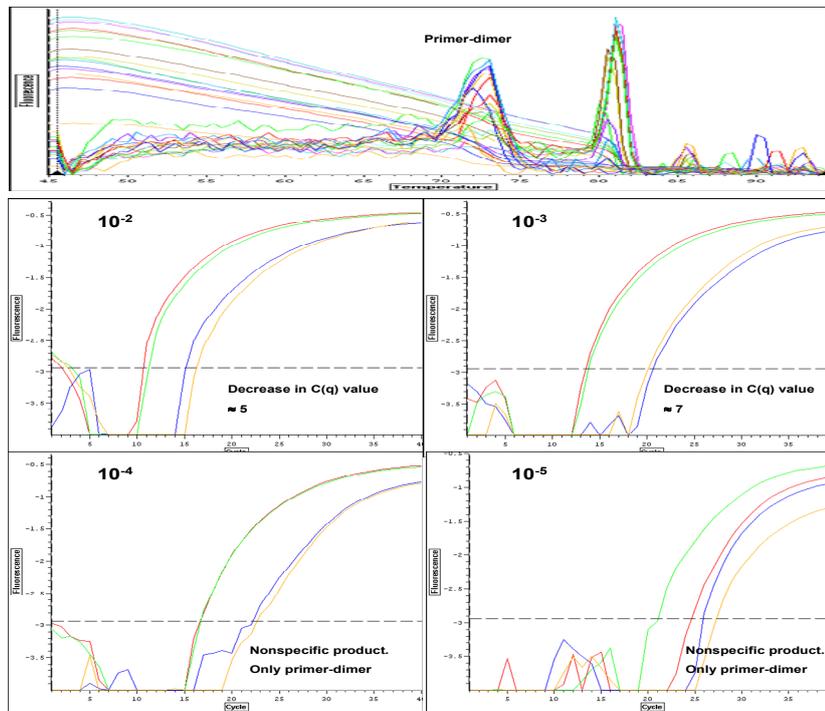


Figure 6. Results from a real-time PCR using a One-Step RT-PCR Kit from ABgene and DWV specific primers detecting RNA from DWV infected bee diluted in RNA preparation of an uninfected bee. Dilutions 10^2 , 10^3 , 10^4 and 10^5 are shown. The difference in Cq value for the DWV infected bee diluted 10^2 in uninfected bee RNA extraction compared to dilution in H_2O is 5 cycles. In the 10^3 -dilution, the inhibition lower the Cq value by 7 cycles. In the 10^4 - and 10^5 -dilution, the inhibition is total, and the only produced amplicon is nonspecific primer-dimer.

Quantification is based on the assumption that a direct relationship exists between the amount of the target nucleic acid and the actual number of microorganisms in a sample. However, in microsporidia the presence of multiple non-homologous copies of rRNA is common and genetic analyze of a single spore of *N. bombi* demonstrated non-homologous multiple copies of rRNA (O'Mahony *et al.*, 2007; Gatehouse & Malone, 1998). This concern was addressed before performing the qPCR for *N. ceranae* and *N. apis* presented in **Paper IV**. A pilot study using generic primers for *Nosema* (Klee *et al.*, 2006) was performed as follows:

Spores of *N. apis* and *N. ceranae* were purified and counted under a microscope. Spore solutions of each parasite were adjusted to 1.5×10^6 spores per ml and DNA was extracted from four aliquots of each solution to be

used as templates in a real-time PCR using SYBRgreen I and the generic primers. The average C_q value for *N. apis* templates was 28.03±1.24 and the average value for *N. ceranae* templates was 28.32±1.03. The result indicates that the method used in **Paper IV** was not biased in favor of any of the two *Nosema* species.

4.1.4 Repetitive PCR

Repetitive PCR (rep-PCR) is a molecular typing method based on PCR where strain specific patterns are obtained from PCR amplification of repetitive DNA elements (Versalovic *et al.*, 1994). Two main sets of repetitive elements are commonly used for bacterial typing; the repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC) sequences (Wu & Della-Latta, 2002; Olive & Bean, 1999). A third repetitive element initially thought to be unique to *Streptococcus pneumoniae* is the BOX sequence, now also found in a number of other bacterial species (Olive & Bean, 1999).

Rep-PCR has become the most widely used method of DNA typing, and has also successfully been used to differentiate strains of *P. larvae* (**Paper II**; Loncaric *et al.*, 2009; Alippi *et al.*, 2004; Genersch & Otten, 2003; Alippi & Aguilar, 1998).

4.1.5 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) of chromosomal DNA is considered the “gold standard” of molecular typing methods (Olive & Bean, 1999). It is a frequently used nonamplified molecular typing method applicable to a wide range of microorganisms, including *P. larvae* (**Paper II**; Pentikäinen *et al.*, 2009; Wu *et al.*, 2005).

Preparation of bacterial chromosomal DNA are embedded into small agarose plugs, digested with a restriction enzyme producing different numbers of fragments depending on species or isolate. The plugs are then inserted into an agarose gel and subjected to an electrophoresis with the capacity to separate very large DNA fragments ranging from 10 to 800 kb (Schwartz & Cantor, 1984). The patterns are visualized and data analysis of the gel results accomplished by any of a number of the commercially available software packages e.g. GelCompar II (Applied Maths, Belgium).

4.1.6 DNA sequencing

All molecular genetic methods are based on differences in DNA sequences, thus sequencing would appear to be the best approach to differentiate between microbes. However, for several reasons this is not always the case.

DNA sequencing is often directed at a small region of the chromosome, thus examining only a small portion of the sites which can potentially vary between the organisms. This DNA region must further meet several criteria before it can be used for differentiation, and for bacteria and fungi very few sequences exist that meet these criteria (e.g. the variability of the sequence must be sufficient to differentiate between strains and should not be horizontally transmissible). By contrast, DNA sequencing is considered the gold standard for viral typing (**Paper III**).

Next-generation DNA sequencing technology offer novel and rapid ways for genome-wide characterization with applications within biology, medicine and other fields (Ansorge, 2009; MacLean *et al.*, 2009). These new sequencing methods generate data allowing the assembly of whole microbial genome sequences in days, which may offer new possibilities for honey bee pathogen diagnosis and characterization in the future.

5 Conclusions and future challenges

Using molecular techniques, we could conclude that *M. plutonius* (EFB) is not ubiquitous in honey bees, but is indeed an infectious pathogen. Consequently, there are apiaries and areas free of EFB justifying preventive actions to reduce the spread of the disease. Furthermore, not all individuals within a diseased colony are infected by the bacterium; it is mainly found in diseased larvae within limited areas of the brood. Hence, correct sampling of brood is crucial for correct laboratory diagnosis. The majority of the adult bees are carriers of *M. plutonius* in symptom free colonies found in infected apiaries, and adult worker bees transmit the bacterium between colonies and apiaries. Thus, high densities of honey bee colonies and apiaries promote transmission of EFB.

The collaborative study on *Paenibacillus* subspecies/genotypes clearly demonstrated that all tested strains of the bacterium caused characteristic disease symptoms of AFB in infected larvae. The taxonomic study did not support subspecies differentiation, and the bacterium was reclassified as one species, *P. larvae* without any subspecies differentiation. Furthermore, a revision of the OIE-manual was made where the causative agent of American foulbrood is now defined as *P. larvae*. This will allow for correct diagnosis of diseased brood in the future.

Deformed wing virus is present in *T. mercedesae* and brood infested by the mite. Similar to *Varroa* infestations in *A. mellifera*, *T. mercedesae* is also linked to DWV infections. Although *Tropilaelaps* spp. has a limited distribution range today, environmental changes may have a direct effect on the occurrence, emergence and spread of the mite. This could also imply major consequences for the spread of viral diseases.

Even though it has been suggested that infections caused by *N. ceranae* are lethal to adult bees, there seems to be no substantial differences in individual level virulence between *N. apis* and *N. ceranae* in cage

experiments. *N. ceranae* infections do not induce higher mortality than infection caused by *N. apis*, and no competitive advantage within host was found for either of the two parasites.

The increased need for detection and characterization of newly emergent or previously unknown endemic pathogens is a challenge to expand our understanding of infectious diseases in honey bees. The driving factors behind choosing a microbial detection method should be sensitivity, specificity and speed. Hence, nucleic acid detection technologies are quickly displacing the traditional phenotypic assays in diagnostic microbiology. However, there is still a lack of standardization among existing in-house assays which may result in the production of incomparable data. For example, the current lack of consensus in how to best perform and interpret qPCR data together with insufficient reporting of the experimental details in many publications have recently been highlighted (Bustin *et al.*, 2009). Following the MIQE-guidelines (Minimum Information of Quantitative real-time PCR Experiments) should indeed be useful in order to obtain better experimental practices for more reliable qPCR results.

The importance of validated quality control (QC) systems for inter-laboratory tests on nucleic acid analysis and real-time PCR has been proven in various fields. Improved collaboration and expanded/refined quality control systems is important when continuing the challenging study of honey bee pathogens, thus permitting more meaningful comparisons. There is a need for reference standards such as plasmid calibrators, primers, probes and exogenous RNA calibrators for comparison of different reversed transcriptase protocols. Expansion in this field would be greatly facilitated if such standards would be available i.e., from an independent source. Overcoming this challenge would facilitate cooperation and lead to more focused efforts to solve the puzzle of honey bee colony losses world-wide.

The efforts made in the field of honey bee pathology summarized in this thesis show the great importance of a deeper understanding and further development of robust and harmonized methods i.e., nucleic acid detection tools. Furthermore, with the tools demonstrated here, new and refined questions of epidemiological importance can be resolved. Improved knowledge of honey bee pathogens is a key factor in the challenge to ensure honey bee health worldwide. Healthy honey bees will in turn sustain biodiversity and food/feed production, thus affecting the long term health and well-being of humans and animals worldwide.

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Svensk sammanfattning

Det Europeiska honungsbiet, *Apis mellifera*, har länge använts av människan för att producera vax och honung. Den största nyttan av biodling är dock inte i första hand de produkter som bisamhället producerar. Bins ekologiska betydelse som pollinatörer av växter är svår att övervärdera, och honungsbiet intar en särställning bland dessa pollinerare. En av de stora fördelarna med honungsbin är att det är möjligt att flytta bisamhällen till områden där det råder brist på pollinerare; en företeelse som blir allt vanligare världen över. En tredjedel av den föda vi människor konsumerar beräknas vara direkt eller indirekt beroende av insektspollinering vilket medför att biodling också har stor ekonomisk betydelse. Väl fungerande biodling och friska bisamhällen har därför både stort ekonomiskt och ekologiskt värde.

Bisamhällen kan drabbas av många olika sjukdomar och farsoter vilket har varit ett bekymmer för människor i alla tider. De senaste åren har ökande globalt fokus riktats mot honungsbin och deras hälsa. Anledning är att allvarliga och ökande förluster av bisamhällen har rapporterats över hela världen. År 2006 användes termen Colony Collapse Disorder (CCD) för att beskriva ett fenomen av storskaliga och oförklarliga förluster av bisamhällen i USA. Man har föreslagit flera olika faktorer som orsak till CCD, men ingen enskild faktor har hittills kunnat pekats ut. Den ledande hypotesen idag är att CCD är ett syndrom orsakat av en kombination av många olika samverkande faktorer. Det är dock troligt att sjukdomsalstrande organismer utgör en mycket viktig faktor.

Mikroorganismer kan vara både skadliga och nyttiga. Nyttogörande mikroorganismer finns inte bara fritt i naturen; de återfinns också i stora mängder på och i alla levande varelser. Varje djurart, inklusive honungsbiet, har en för arten karakteristisk normalflora av mikroorganismer. Det finns många olika nyttogörande mikroorganismer i ett bisamhälle, och förhållandevis få som orsakar sjukdom. De sjukdomsalstrande

mikroorganismerna (patogenerna) i bisamhället inkluderar virus, bakterier och svampar. Dessutom finns en rad parasiter (exempelvis kvalster) som kan orsaka skador hos både biyngel och vuxna bin. Några sjukdomar är mer allvarliga än andra och dessa kan leda till att hela bisamhällen dukar under. Att studera hur bisamhället och dess sjukdomsalstrare fungerar tillsammans är viktigt för att kunna förebygga och förhindra skador och förluster av bisamhällen.

Det har länge saknats pålitliga analytiska metoder för att studera patogener hos honungsbin. Till exempel har odlingsbaserade tekniker som används för att studera bakterier visat sig vara mycket okänsliga. Modern diagnostik erbjuder nya metoder för att besvara specifika och viktiga frågor om sjukdomsalstrare i bisamhället. Användningen av i synnerhet molekylära metoder har ökat på senare år och blivit ett användbart verktyg inom både laboratediagnostik och forskning. Men trots att stora framsteg gjorts saknas fortfarande grundläggande kunskaper om flera av honungsbiets sjukdomsalstrare. Det ökande intresset i världen för honungsbiets hälsa tillsammans med en mer fokuserad forskning inom det här området kommer förhoppningsvis att ge ökad kunskap; kunskap som är viktigt för att kunna förebygga och förhindra de negativa effekterna av sjukdomsutbrott.

Syftet med den här avhandlingen har varit att utveckla molekylära detektionsmetoder och med hjälp av dessa undersöka bl.a. överföring och sjukdomsalstrande förmåga (virulens) hos fem mikroorganismer som orsakar allvarliga sjukdomar i bisamhället. I studien ingår två olika bakteriearter, (*Melissococcus plutonius* och *Paenibacillus larvae*) som orsakar sjukdom hos biyngel, ett virus (deformed wing virus, DWV) som gör att bin kläcks med missbildningar, samt två mikrosporidier (*Nosema ceranae* och *Nosema apis*) som orsakar tarminfektioner hos vuxna bin. Här följer en kort sammanfattning av respektive studie.

Europeisk yngelröta - *Melissococcus plutonius*

Europeisk yngelröta (EFB) är en sjukdom hos biets yngel. Den vedertagna åsikten är att sjukdomen orsakas av bakterien *Melissococcus plutonius*. Det har dock förekommit påståenden om att bakterien skulle vara allmänt förekommande i flertalet bisamhällen. Sjukdomen är vanlig i vissa delar av världen, och är ett ökande problem bl. a. i Schweiz och England. Målet med vår studie var att studera bakteriens förekomst och utbredning hos sjuka och friska larver i samhällen med och utan sjukdomssymptom. Dessutom ville vi testa hypotesen att *M. plutonius* skulle vara en bakterie som alltid förekommer i bisamhället.

Slutsatserna vi kunde dra var att *M. plutonius* inte är allmänt förekommande hos honungsbin utan faktiskt en smittsam sjukdomsalstrare. I praktiken betyder det att det finns bigårdar och områden som är fria från Europeisk yngelröta och att förebyggande åtgärder kan minska spridning av sjukdomen. Man hittar bakterien i huvudsak hos sjuka larver inom begränsade områden i yngelramarna. Därför är en noggrann provtagning av sjukt yngel avgörande för att man ska kunna ställa en korrekt diagnos. I symptomfria kolonier i smittade bigårdar är en majoritet av de vuxna bina bärare av *M. plutonius*. Bin kan alltså överföra bakterien mellan kolonier och bigårdar, och hög bitäthet (som t.ex. i Schweiz) främjar spridning av Europeisk yngelröta.

Amerikansk yngelröta - *Paenibacillus larvae*

Amerikansk yngelröta (AFB) är en allvarlig yngelsjukdom som normalt leder till döden för infekterade larver. Orsaken till sjukdomen är en bakterie som namngivits som *Paenibacillus larvae* underart *larvae*. En annan, närbesläktad bakterie, *Paenibacillus larvae*, underart *pulvificiens*, har inte ansetts orsaka AFB. På laboratoriet vid SLU hittades dock yngelprov med kliniska symptom på AFB som visade sig innehålla *P. larvae* underart *pulvificiens*. Det gav anledning till att undersöka om namngivningen och klassificeringen av de två underarterna var motiverad.

Studien av *Paenibacillus* visade att samtliga testade stammar av bakterien orsakar Amerikansk yngelröta hos infekterade larver. Resultaten gav inget stöd åt en differentiering i underarter, vilket ledde till att vi kunde omklassificera *Paenibacillus larvae* till en art. Bakterien som orsakar AFB definieras nu enbart som *Paenibacillus larvae*.

Deformed wing virus (DWV)

Det finns ett flertal virus som angriper honungsbiet, och många av dem har samband med varroakvalstret (*Varroa destructor*). Genom att parasitera på vuxna bin och yngel överför kvalstret viruspartiklar från biets hemolymfa. Det virus som utan jämförelse har störst betydelse i samband med varroakvalstret är DWV. Infektioner av DWV kan i samband med kraftiga angrepp av varroa leda till bisamhällets kollaps. Ett annat kvalster, *Tropilaelaps mercedesae* kan orsaka stora skador på samhällen av *A. mellifera* i Asien. Kvalster av denna art förmår inte parasitera vuxna bin och kan därför inte överleva utan tillgång till yngel. Syftet med vår studie var att utveckla en kvantitativ bestämningsmetod för DWV för att undersöka om tropilaelapskvalstret sprider infektioner av DWV på samma sätt som varroakvalstret.

Vi utvecklade en metod för att bestämma antalet viruskopior i ett prov, och kunde, med hjälp av denna, visa att DWV finns i tropilaelapskvalstret såväl som i det angripna ynglet. Liksom vid varrooangrepp i Europeiska bin, sprider tropilaelaps också DWV. Även om tropilaelapskvalstret har ett begränsat utbredningsområde i dag, kan den globala uppvärmningen ha en direkt inverkan på förekomst och spridning av detta kvalster.

Nosemasjuka - *Nosema apis* och *Nosema ceranae*

Mikrosporidierna *Nosema apis* och *Nosema ceranae* är intracellulära parasiter som orsakar tarminfektion (nosemasjuka) hos vuxna bin. Det finns idag relativt lite information om skillnader i skadeverkningar av de båda mikrosporidierna, men vissa uppgifter tyder på att *N. ceranae* har en högre sjukdomsalstrande förmåga. Studier gjorda i Spanien har t.o.m. indikerat att infektioner av *N. ceranae* dödar vuxna bin på någon vecka.

Vi ville studera skillnaderna i sjukdomsalstrande förmåga mellan de två *Nosema*-arterna, och infekterade därför individuella bin med blandningar av *N. apis* och *N. ceranae* i olika proportioner. För att ha möjlighet att senare mäta andelen *N. apis* respektive *N. ceranae* i dessa bin utvecklade vi en artspecifik kvantitativ analysmetod.

Resultaten från studien visade inga större skillnader i sjukdomsalstrande förmåga mellan *N. apis* och *N. ceranae* i individuella bin. Infektioner av *N. ceranae* orsakade inte högre dödlighet än infektion av *N. apis*, och ingen av de två mikrosporidierna hade några konkurrensfördelar inom bin infekterade av båda *Nosema*-arterna.

Slutsats

Det är viktigt att vidareutveckla och standardisera diagnosmetoder för infektionssjukdomar hos honungsbin. Med hjälp av, i synnerhet molekylära metoder, kan vi besvara specifika frågor och öka den generella kunskapen om sjukdomsalstrande mikroorganismer i bisamhället. Det är nödvändigt med ökande kunskaper om hur bisamhället och dess sjukdomsalstrare fungerar för att kunna förebygga och förhindra skador och förluster. Vi behöver friska honungsbin för att upprätthålla den biologiska mångfalden, och vi behöver dem också för livsmedels och foderproduktion världen över. Betydelsen av friska bisamhällen kan inte överskattas!

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