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Molecular epidemiology of selected viral, bacterial and fungal disease of honeybees in the Czech Republic.

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Declaration
Except of the individual papers and patent in which the authorship and the copyright are mentioned

this PhD dissertation is exclusively my own work.

Štěpán Ryba
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I dedicate this dissertation …

… to my parents for instilling the importance of hard work and higher education.

… to my wife who supported my research and writing efforts over the years.

… to all my colleagues and friends without whom this work would be hardly feasible.

PREFACE

Current modern scientific research is known for the huge flood of new information, rapid development of new techniques, and the establishment of new scientific disciplines. Information, which was acquired twenty years ago, is often outdated or incomplete, if not wrong. Therefore, in current scientific projects, we cannot avoid team work of people where each of them is skilled in certain methods or know certain scientific issues in depth. Therefore, since information in natural sciences is continuously changing and developing often with increasing speed, it is also important to find the right colleagues to collaborate with on new projects. This even applies to my dissertation, which I would not have been able to complete without the hard work and assistance of many people.

First of all, I need to express my gratitude and deep appreciation to my supervisor Pavel Stopka, whose friendship, knowledge, and wisdom have supported me over the many years of my master and doctorate studies. As in any aspect of life, in the academic community you must be fortunate about who you work with. Often one small coincidence can influence the rest of a person's life. When I met Pavel Stopka for the first time, it was he who recognized my enthusiasm for viruses and everything related to them. I am grateful to Pavel Stopka for his great help in guiding my thesis and for his help in finding new scientific questions and answers.

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about anything. My thanks also go to the other employees and co-workers of the Bee Research Institute, especially to Martin Kamler and Marcela Haklová. I would like to express my thanks to the company R-Biopharm Germany, for providing many pieces of advice, support and testing material for our research.

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If I look back at several of my last years of research at Biodiversity Research Group, I have to acknowledge that due to an undisputed co-operation with colleagues, several research projects

were successfully finished, published, and presented. At the same time, I can also see many research plans and proposals of scientific projects which finished only as collected samples in the freezer. These were not completed due to the lack of time, money, or it was an inability to build a motivated research team. Nevertheless, even at the time when I am writing this dissertation, other samples are leaving the freezer and several interesting research projects are going on.

Štěpán Ryba
Prague, 20th Februar 2012

CONTENTS

SUMMARY

For my dissertation thesis, I chose a relatively small part of all bee diseases, because it would be out of the scope of my possibilities to cover the entire observed area in its entire complexity and depth.

Altogether, the six most common bee viruses which infect the honey bee (*Apis mellifera)* were monitored in the territory of the Czech Republic between 2006 and 2009. Parallel infections of viruses (DWV, ABPV and BQCV) in bee adults and parallel co-infection of viruses with fungal diseases caused by *Nosema apis* and *Nosema ceranae* were confirmed by PCR tests. A new sensitive method of detection of the causative agent of the American foulbrood (*Paenibacillus larvae*) from bee debris was developed for the practical use of detection of AFB disease in bee populations. Various approaches for the extraction of spores from bee debris and lyses of spores were compared. The sensitivity of PCR tests for the presence of *Paenibacillus larvae* in debris was compared with the classic cultivation method. The PCR method for the detection American foulbrood was further studied and developed to be more efficient. A new method, based on a matrix-like sample re-arrangement and a use of pooled samples, has been developed for testing 1000 samples in 35 PCR reactions. Another goal was to develop a robust and fast screening method for American foulbrood based on the cultivation test using paper sheets RIDA®COUNT with a specific cultivation medium, specific selection conditions for *Paenibacillus larvae* and chromogen visualization of the grown bacterial colonies. This method has been evaluated in the laboratory conditions and its sensitivity has been verified. Finally, the differential diagnostics of both microsporidia on a molecular level was implemented in the area of detection of bee fungal diseases and a possibility of microscopic differential diagnostics of *Nosema apis* and *Nosema ceranae* was confirmed. This microscopic method can be used in field conditions. The results of a microscopic examination were validated parallel with the PCR test.

SOUHRN

Pro svou disertační práci jsem vybral relativně malou část ze všech včelích onemocnění, protože by bylo nad rámec mých možností pokrýt celou sledovanou oblast v celé její komplexnosti a hloubce.

Na území ČR bylo monitorováno během let 2006 – 2009 celkem 6 nejobvyklejších včelích virů, které se objevují u včely medonosné *Apis mellifera.* Pomocí PCR testů byly potvrzeny souběžné infekce virů (DWV, ABPV a BQCV) v dospělcích včel a souběžně ko-infekce virů s houbovým onemocněním vyvolaným *Nosema apis* a *Nosema ceranae.*

Pro praktické využití detekce onemocnění AFB v populaci včelstev byla vyvinuta nová citlivá metoda detekce původce moru včelího plodu *Paenibacillus larvae* ze včelí měli. Byly porovnány různé přístupy extrakce spor z včelí měli a lyzování spor. Citlivost PCR testu na přítomnost *Paenibacillus larvae* v měli byla porovnána s klasickou kultivační metodou. Metoda PCR detekce moru včelího plodu byla dále studována a zefektivňována. Pomocí přeuspořádání vzorků do vytvořených matic a vytvoření směsných vzorků, byla vyvinuta metoda, u které bylo možné testovat 1000 vzorků pomocí 35 PCR reakcí. Dalším přístupem k vytvoření robustní a jednoduché metody pro screening moru včelího plodu bylo vytvoření, laboratorní ověření a srovnání citlivé detekční metody, založené na kultivačním testu na papírových kartách RIDA®COUNT se specifickým kultivačním médiem,specifickými selekčními podmínkami pro *Paenibacillus larvae* a chromogenem zvýrazňující narostlé kolonie bakterií.

Konečně v oblasti detekce včelích houbových onemocnění byla zavedena diferenciální diagnostika obou mikrosporidií na molekulární úrovni a dále potvrzena možnost mikroskopické diferenciální diagnostiky *Nosema apis* a *Nosema ceranae*. Tato mikroskopická metoda je použitelná v polních podmínkách. Výsledky mikroskopického vyšetření byly validovány paralelně s PCR testem.

LIST OF PAPERS

This thesis consists of the following nine papers (two published articles, three submitted manuscript being under the review at the time of printing this thesis, one patent and three published papers unrelated to the topic of dissertation) that are referred in the text of the thesis given below:

Published articles

1. **Ryba S.,** Titera D., Haklova M., Stopka P. (2009) A PCR method of detecting American Foulbrood (*Paenibacillus larvae*) in winter beehive wax debris. *Vet. Microbiol*. **139:** 193-196. (IF = 3,256; 2011)
2. **Ryba S.,** Titera D., Schodelbauerova-Traxmandlova I., Kindlmann P. (2012) Prevalence of Honeybee Viruses in the Czech Republic and Coinfections with other Honeybee disease. *Biologia.* **67:** 1-6 (IF = 0,609; 2010)

Submitted articles

3. **Ryba S.,** Kindlmann P., Titera D., Haklova M., Stopka P. (2012) A new low-cost procedure for detecting disease in low-incidence samples: a case study of American foulbrood. *J. Eco. Entomology.*
4. Kamler M., Tyl J., **Ryba S.,** Titera D. (2012) A scientific note on the microscopical discrimination of Nosema apis and Nosema ceranae spores: a possible rapid screening method for nosemosis type C. *Res.in Vet. Sci.*
5. **Ryba S.,** Kristufek V., Titera D. (2012) The use of RIDA®COUNT for monitoring the American Foulbrood pathogen. *Apidologia.*

Patent related to the topic of dissertation

6. Kristufek V., **Ryba S,** Krenek A., Mulec J. (2012) Method of monitoring the infection pressure of American foulbrood, the equipment necessary for this method, and the use of test cards for monitoring the infection pressure of American foulbrood.

Published articles unrelated to the topic of dissertation

7. Stopkova R., Zdráhal Z., **Ryba S.,** Sedo O., Sandera M., Stopka P. (2010) Novel OBP genes similar to hamster Aphrodisin in the bank vole, *Myodes glareolus*. *BMC Genomics.* **11:** 45 (IF = 4,21; 2011)
8. **Ryba, S.,** Tacner J., Havlickova M., Stopka P. (2012) Stability of influenza virus as evaluated by integrity of its RNA, *Acta Virologica*. **56:** 131-134 (IF = 0,547; 2010)
9. **Ryba, S.,** Stopka P. (2012) Monitoring and prevalence of influenza A virus in the population of mallard duck. *Folia Zoologica.* **61:** 118-120 (IF = 0,548; 2010)

GENERAL INTRODUCTION

The most important role of insects on the planet is probably pollination of many cultivated and wild growing plants (Aizen et al., 2009a). Thus, they significantly participate in the process of reproduction. If we focus only on insect pollinators, they are mainly social and solitary bees. Socially living bees, especially the honeybee (*Apis mellifera*, L.), represent the most valuable pollinators of plants in our ecosystems (Morse and Calderon, 2000; Klein et al., 2007). Throughout history we have used their products such as honey, wax, royal jelly, pollen, poison and propolis, for treatment purposes and consumption. Honey was the only available sweetener for civilized nations in Africa, the Middle East and in Europe long before the sugar beet started to be grown (Voorhies et al., 1933). Due to increasing demand in bee products, it is known that bees were domesticated in ancient Egypt sometime before 2,600BC. Bee-keeping spread from Egypt to ancient Greece around 650BC and from there spread further to ancient Rome. From there on the practice of bee-keeping was spreading all over Europe. Finally, ancestors of medieval European bee-keepers spread bee-keeping to the rest of the world. This highly adaptable species originally settled the area from Northern Europe to Central Asia and covered all Africa (Ruttner, 1988; Sheppard and Meixner, 2003; vanEngelsdorp and Meixner, 2010). Nevertheless, since the 17th century, the original habitat of the honeybee has spread to almost all inhabitable areas of the globe. Bees accompanied people on most of their discovery travels and some of the first settlers always took hives with them (Crane, 1975). At that time, transportation by ship was responsible for the rapid expansion of honeybees. Today, the honeybee (*Apis mellifera*) is the most commonly farmed bee in the world. Unfortunately, along with the bees, their pests and many pathogens were also transferred to new territories (vanEngelsdorp and Meixner, 2010). With respect to the crucial importance of bee-keeping as a tool influencing sustainable and profitable agriculture, the health of bee colonies plays an important role for our agricultural and non-agricultural ecosystems. Due to this fact, more and more bee-keepers and vets started to pay more attention to the pathogens of bees and to possible ways of prevention and treatment of bee diseases (Aizen et al., 2009b; Gallai et al., 2009). Among the most serious bee diseases which can negatively influence productivity and survival of farmed bees are: bacterial, fungal, viral, microsporidial diseases as well as parasitic mites, predators, and pests (Morse and Flottum, 1997). This thesis deals with some of the most significant diseases and parasites. Great stress and attention is paid to the selected bacterial diseases such as American foulbrood, viral diseases, diseases caused by microsporidia and interactions with a serious bee parasite – a mite *Varroa destructor*.

Bees and mite Varroa destructor

The parasitic mite *Varroa destructor* (Anderson and Trueman, 2000) is currently among the biggest pests of honeybees. This mite moved from its original host, the Asian bee *Apis cerana* to the European bee *Apis mellifera*. Due to its new host, this mite spread to almost all continents (it has not been reported from Australia or the Southern Island of New Zealand), where it caused dramatic losses in farmed bee colonies (Finley et al., 1996;. Martin et al., 1998; vanEngelsdorp et al., 2007; Rosenkranz et al., 2010). In the infected bee colonies, the mite´s female and its descendants feed on the hemolymph of pupa which leads to serious nutrition deficits for the developing bee. Furthermore, this parasitism leads to a secondary viral infection (Duay et al., 2003; Amdam et al., 2004; Garedew et al., 2004; Rosenkranz et al., 2010).

In bee colonies which are infected by the mite *Varroa destructor*, symptoms of parasitism develop quickly and if they are not cured, the colony becomes weaker and dies. A strong attack of the mite is clinically shown in reduced vitality and development of the colony, its malnutrition, malnutrition of individual bees, a shortened life of bees, the occurrence of deformed or undeveloped wings and creeping bees under the beehive entrance which do not fly. Many of these symptoms are physiologically caused by viruses, which this mite transfers in its body and efficiently transfers them by impaling of the pupa cuticle (Shimanuki et al., 1994; Hung et al., 1996). Varroa mites are vectors of several viruses, from which a majority occurred in bees before the invasion, nevertheless without penetration of viruses to hemolymph during suction by the mite, their manifestation in bees, would be without symptoms (Bowen-Walker et al., 1999; Yue and Genersch, 2005; Rosenkranz et al., 2010; de Miranda and Genersch, 2010).

We know that several of about 18 known bee viruses from the family *Picornaviridae* and *Dicistroviridae* interact with the *Varroa destructor* mite. Pupae infected by such a virus, which is transferred by the *Varroa destructor* mite are supposed to suffer from a disorder of the immune system and they are prone to the viral infection (Ellis and Munn, 2005; Yang and Cox-Foster, 2005; Chen and Siede, 2007). Despite the fact that for example DWV virus can be transferred directly from bee to bee, such a transferred virus is manifested mostly without symptoms. The clinical symptoms, such as crippled wings or shortened abdomen are manifested after the viral infection is transferred through the mite (Bowen-Walker et al., 1999; Yue and Genersch, 2005; Yue et al., 2006, 2007; Tentcheva et al., 2006). It was proven by the DWV virus that for the efficient infection of the host, it is required that the virus is replicated in the mite´s tissues (Bowen-Walker et al., 1999; Shen et al., 2005; Tentcheva et al., 2006; Yue and Genersch, 2005). This means that the number of viral

copies transferred to the host (pupa) will dramatically increase and it is also possible that the developing bee is partially immunosuppresed or the virulence is increased (Shen et al., 2005; Gisder et al., 2009; Rosenkranz et al., 2010; de Miranda and Genersch, 2010). As well as DWV, the ABPV virus was known long before the parasitical mite *Varroa destructor* was introduced. Even clinical symptoms or high death rates of bee colonies were not usually recorded. Despite this situation, after the *Varroa destructor* mite was brought to Europe, ABPV prevalence in Europe sharply increased and *Varroa destructor* was identified as an efficient carrier of the virus (Bailey and Gibbs, 1964; Ball, 1983; Allen and Ball, 1996). A recent study confirmed the dependence between the high number of mites, presence of ABPV virus and increased death rate in bee colonies in winter (Siede et al., 2008). On the other hand, bee colonies which were infected by the ABPV virus, but without presence of mites in autumn, stayed through the winter in good shape (Siede et al., 2008). Members of the "viral complex" from the families of *Picornaviridae* and *Dicistroviridae* are supposed to play a certain role or they are potential causative agents of the CCD (Cox-Foster et al., 2007; Chen and Evans, 2007; Rosenkranz et al., 2010). Screening of bee viruses in pathologically suspicious bee colonies was also a part of this thesis.

Nosemosa

Nosemosa is a disease caused by parasitic microsporidia *Nosema ceranae* and *Nosema apis* and nowadays it has spread worldwide. Both mentioned microsporidia are intracellular parasites and they are spread among bee colonies as spores. They have a unique organ used for invasion into host cells. The principal of the infection is based on mechanical injection of polar fibre coming out during spore germination. By physical penetration, fibre penetrates through a membrane of the host cell. Though the leading fibre, the infectious sporoplasma is injected into cytoplasm of the host cell, where the parasite is replicated, and later on, starts the production of new spores (Fries, 2010). On the molecular level, microsporidia are nowadays classified as fungi (Wang et al., 2006; Singh, 1975). This means that taxonomically microsporidia represent highly specialized parasitic fungi (Larsson, 1986; Sina et al., 2005; Higes et al., 2006; O'Mahony et al., 2007; Chen et al., 2009; Cornman et al., 2009). Until the 20th century, the nosemosa disease in European honeybee *Apis mellifera* was connected only with the microsporidia *Nosema apis*, and it resulted in a weakening and size reduction of the bee colony and visible faeces on the comb in the springtime. In 1996 a morphologically similar microsporidia *Nosema ceranae*, originally parasiting on Asian bee (*Apis ceranae*), was described (Klee et al., 2007). The experiments with cross-infection, using spores of both *Nosema ceranae* and *Nosema apis*, proved that the parasites are able to infect both species

(Fries and Feng, 1995; Fries et al., 1996). However, *Nosema ceranae* was developing better in *Apis mellifera* bees than in *Apis ceranae* bees. *Nosema ceranae* likely infected the *Apis mellifera* honeybee at least two decades ago (Klee et al., 2007; Paxton et al., 2007; Invernizzia et al., 2009) and since then it has been gradually displacing the original parasite of *Nosema apis*. The finding that many microsporidia are able to use more hosts is not much surprising. For instance, this phenomenon is well illustrated in another microsporidia, *Vairimorpha necatrix*, (Pilley, 1976) which is able to develop successfully in various hosts from the family *Lepidoptera* (Darwish et al., 1989; Kramer, 1965; Nordin and Maddox, 1974; Pilley, 1976; Fries, 2010). The first finding of *Nosema ceranae* in Europe and first losses of bee colonies were confirmed (Higes et al., 2006). Ten *Nosema ceranae* positive samples from Spanish apiaries were found in 2005 (Huang et al. 2007). Also in other tested samples from 2005, besides *Nosema apis* also *Nosema ceranae* was confirmed in *Apis mellifera* bee colonies. Detection of microsporidia of *Nosema ceranae* and *Nosema apis* is mainly based on PCR test, but this can be mutually distinguished microscopically based on morphometric signs (PAPER 4).

American foulbrood (AFB)

American foulbrood is the most serious and the most devastating disease to the honeybee (Genersch, 2010). Before the discovery of the parasitic mite *Varroa destructor*, this belonged to the most serious problem of commercial bee-keeping (White, 1906). Earlier, beekeepers were not able to distinguish this disease from a very similar disease – European foul brood (Forsgren, 2010). Due to this fact, it is also regarded as less virulent than AFB. In the 18th century this disease was characteristic with a strange foul smell, and from here comes the name. Later, the causative agent was assigned to the family *Bacillus* and subsequently reclassified into the genus *Paenibacillus* (Ash et al., 1991).

As the name of the disease indicates, American foulbrood attacks mainly the larval stadia of the honey bee. The causative agent is a gram-positive, spore forming rod-shaped bacteria of *Paenibacillus larvae*. The only infectious form which is able to cause the disease is an extremely tenacious endospore. Spores are infectious only for young larvae. The most sensitive to infection are larvae in first stages of development 12-36 hours after eggs hatching, when the sufficient lethal dose for a larva is 10 spores (Wilson, 1971; Hitchcock et al., 1979). About 12 hours after eating the spore by a larva, spores start germinating in the intestine (Yue et al., 2008). Subsequently, the intestinal epithelium penetrates and the germinated spores go into the hemocell. And as in-situ hybridization showed, these places become the primary location of bacterial growth (Woodrow,

1942; Woodrow and Holst, 1942; Bamrick and Rothenbuhler, 1961; Brodsgaard et al., 1998; Genersch et al., 2005).

Paenibacillus larvae bacteria also colonize the intestines and massively multiply there. It is known that these bacteria use the content of the intestines as a source of nutrients (Neuendorf et al., 2004). They are able to metabolize components of carbohydrate metabolism and they metabolize various sugars (including glucose and fructose). After a certain length of time, depending on the preliminary dose of spores, the intestinal epithelium is broken and the bacteria migrate into the hemocell (Yue et al., 2008). One of the characteristic features of *Paenibacillus larvae* is that they secrete a highly active extracellular protease during the vegetative growth and this is supposed to be responsible for breaking the integrity of the epithelial barrier and degradation of intercellular junctions. Thus, they provide the way to the invasion of other bacteria into the hemocell. The protease plays a further role in the decay of the rest of the dead larvae (Holst and Sturtevant, 1940; Holst, 1946; Dancer and Chantawannakul, 1997; Hrabak and Martinek, 2007). The surface structures of larva are decomposed and the entire rest of the dead larva is changed into brown, semi-fluid and glue-like matter. Spores are created and there can be up to 4 billion spores in one larva. The semi-fluid stage of larva can change after drying into a hard foulbrood scale which adheres to the cellular wall. Spores are equipped with a 7-layer shell and they can be infectious for more than 35 years and they can be resistant to extreme conditions such as changes in temperature, drought and humidity (Hasemann, 1961). Spores are available for distribution to the further host which can be another larva on a honeycomb, neighbouring hive, or apiary within the flying distance of the bee colony (Gregorc and Bowen, 1998; Genersch, 2010). This disease is not lethal only for the particular larva, but due to its virulence, it can weaken or destroy the entire bee colony. This disease can be spread in two main modes: vertically and horizontally (Ewald, 1983; Ebert and Here, 1996; Ebert, 1998; Fries and Camazine, 2001). This determines the size of virulence of the bacteria. The disease itself can be spread through infected hives, bees, combs, sending infected queens, nucleus colonies, honey, etc (Lindstrom et al., 2008a;). With respect to the high infection rate, rate of spreading inside the bee colony, between the colonies on the apiary, and between apiaries, AFB belongs to the disease which has to be immediately reported to the relevant veterinary authority. Usually the bee colony with clinical symptoms of AFB is destroyed (burning of the colony, hive, wooden parts and disinfection of all metal and plastic parts with hot lye) to prevent the disease from spreading (**Fig.1**). While some countries still allow the use of prophylactic antibiotics for suppression of AFB such as the USA, many other countries prefer a completely different approach

and this means radical liquidation (Lodesani and Costa, 2005; Martel et al., 2006; Genersch, 2010).

Fig.1: Veterinary liquidation of bee colonies affected by AFB (April 2012, České Vrbné). Poor conditions of old bee hives and other breeding facility show the overall hygienic level of the apiary.

Antibiotics are not efficient for *Paenibacillus larvae* spores. Proven methods include reviving bee colonies where the clinical stage has not started yet, or shook swarm method (von der Ohe, 2003; Pernal, 2008), further improving methods of breeding colonies of social immune response (Wedenig et al., 2003; Evans, 2004), as well as bio control with antagonistic bacteria (Alippi and Reynaldi, 2006; Evans and Armstrong, 2006), by using medication like essential oils of various plants (Fuselli at al., 2006, 2007, 2008, 2009), effects of bee natural disinfectants – propolis (Bastos et al., 2008) and finally a molecular manipulation of *Paenibacillus larvae* (Murray and Aronstein, 2008). A common problem connected to the use of antibiotics is the increasing occurrence of resistant *Paenibacillus larvae* strains and a presence of the residues of antibiotics in honey and wax (Miyagi et al., 2000; Mussen, 2000; Kochansky et al., 2001; Lodesani and Costa, 2005). Nowadays, most European countries focus on the prevention against AFB before the clinical symptoms of this disease are seen (Govan et al., 1999; Dobbelaere et al., 2001; Lauro et al., 2003; Alippi et al., 2004; deGraaf et al., 2006; Reynaldi et al., 2006). One of the ways can be detection of *Paenibacillus larvae* spores in honey during the commercial purchasing of honey. By specifying the infected apiaries from which the honey comes, preventive zootechnical measures may be applied. In the part of the thesis focused on epidemiology of *Paenibacillus larvae*, my colleagues and I dealt with the issue of quick detection of *Paenibacillus larvae* from debris (PAPER 1), detection of *Paenibacillus larvae* in a large number of samples by rearrangement into matrix (PAPER 3) and creating the methodology of sheet tests which allow for a fast and undemanding global detection of *Paenibacillus larvae* (PAPER 5, PAPER 6).

AIMS OF THE THESIS

The objective of this dissertation was to develop several methods available for users which could be used for an easy (some of them also outdoor) detection of pathogens. Subsequently these methods were used for evaluation of an epidemiological study of selected pathogens in the Czech Republic. All papers, methods and patents which are a part of this dissertation, I developed with the idea to use their results in practice either by vets or bee keepers. In other words, I was interested both in the real result of the possibility of fast and exact focus on infected bee colonies and also the overall effect of the monitoring of pathogens in bee-keeping practice. The main goals were as follows:

1. Develop sensitive and reliable PCR method for detection of AFB from bee debris, verify its accuracy and compare it to the cultivation test.

2. Develop sensitive and reliable method for detection of AFB based on PCR, which could be used for the wide screening of *Paenibacillus larvae* pathogen, and which would enable an evaluation of a greater number of samples.

3. Develop sensitive and reliable method for detection of AFB based on the cultivation test which would be easy, cheap and available. A special selection medium would be used for this method as well as resistance of *Paenibacillus larvae* spores, reaction of bacteria with chromogen would be used.

4. Develop a reliable and sensitive method of detection of six bee viruses based on RT-PCR. Due to the knowledge of sequenced viruses, create a set of specific primers for these 6 bee viruses.

5. Develop a sensitive and reliable method for detection of *Nosema apis* and *Nosema ceranae* to distinguish both pathogens on molecular level.

6. Compare reliability of the method of microscopic and molecular distinguishing of *Nosema*

apis and *Nosema ceranae*.

7. Screen bacterial (AFB), viral and fungal (*Nosema*) diseases in the territory of the Czech Republic and find frequency of particular diseases in larvae and adults in bee colonies which were pathologically suspicious for the first time.

GENERAL METHODS

Origin and type of samples

In order to detect honeybee (*Apis mellifera* L.) pathogens, the material was collected from the apiaries of beekeepers (volunteers) who are interested in issues concerning the death of bee colonies. Furthermore, some material comes from my own 6 apiaries and also from the Bee Research Institute at Dol.

The sampling material included dead or killed adult bees – workers, queens and drones, as well as bee pupae, bee larvae, suspicious unhatched queen cells with pupa, *Varroa destructor* mites, parts of all honeycombs, bee wax, honeybee eggs, bee hive debris, bees cultured in-vitro and grown cultures of bacteria *Paenibacillus larvae* on Petri dishes.

Cultivation methods

Several described procedures for cultivation of be bacterial pathogens on Petri dishes (Titera and Haklova, 1999) with MYPGPn medium were used. Also *Paenibacillus larvae* bacteria were cultivated on RIDA®COUNT cards with supplemented nutrient MYPGPn medium.

Molecular approaches

Several molecular techniques were used for isolation, cleaning, transcription and detection of nucleic acids. First, bacterial DNA from the causative agent of the American foulbrood for the purpose of *Paenibacillus larvae* detection was isolated (Bakonyi et al., 2003). Subsequently, in order to detect *Nosema apis* and *Nosema ceranae*, total genomic DNA from bee abdomen was extracted. The isolation of RNA from samples determined for detection of bee viruses belongs to further molecular approaches. These approaches included standard RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), genomic DNA and complementary DNA PCR, cloning, sequencing. The analysis of the data obtained was performed using several bioinformatic tools including the GenBank BLAST (http://www. ncbi.nlm.nih.gov/), BioEdit Sequence Alignment Editor, EXPASY Translate tool (http://www.expasy.ch/).

Microscopic methods

We used light microscopy to evaluate presence of *Nosema ceranae* and *Nosema apis* spores. The samples were prepared according to the standard protocol (OIE, 2008). Pictures were taken from the light microscope Olympus BX41, equipped with microEye UI for taking a picture which is

enlarged 1,000 times. Pictures were further processed in the program QuickPHOTO CAMERA 2.2. and the width and length of 350 selected spores was documented.

Methods of lyses of spores

Paenibacillus larvae spores are covered by seven protective layers which have to be degraded prior to the DNA isolation. To break the shell, a technique of enzymatic lyses with lysozyme in treatment with proteinase-K was used (dAlesandro et al., 2007). Then, the method of lyses of spores based on degrading of the wall of a spore by using NaOH/SDS was used (Vary, 1973).

Methods of extraction of spores

Paenibacillus larvae spores strongly adhere on wax particles. For this reason, it was necessary to use a method of extraction of *Paenibacillus larvae* spores from bee debris as the initial material for detection of AFB disease. Toluene extraction of spores from bee debris was used (Titera and Haklova, 2003). Then the method with Tween80 (Bzdil, 2007) and new methods of isolation of spores into warm and cold water and into ethanol were used.

Modelling statistical methods

A method of rearrangement of tested samples into matrixes was used for the purpose of detection for a large number of samples. By evaluation of all mixed samples for the presence of detected agents, positive mixed samples were determined and particular positive samples in the matrix were found based on their position in the matrix (see PAPER 3).

Other methods

Methods of cryopreservation of tissues of bee adults, pupae, larvae and eggs were used for the purpose of preservation of genetic material and for subsequent research. Also methods for cloning of DNA or cDNA fragments into bacteria for purpose of cryopreservation of samples were used. Further methods are various ways of processing samples prior to diagnostic methods (crushing, etc.), and methods for the preparation of mixed samples.

RESULTS AND SUMMARY OF PAPERS

This dissertation thesis consists of the following nine papers (two published articles, three submitted manuscript being under the review at the time of printing this thesis, one patent and three published papers unrelated to the topic of dissertation) that are referred in the text of the thesis given below:

Paper I:

Ryba S., Titera D., Haklova M., Stopka P. (2009) A PCR method of detecting American Foulbrood (*Paenibacillus larvae*) in winter beehive wax debris. *Vet. Microbiol.* **139:** 193-196. (IF = 3,256; 2011)

The objective of this work was to create a fast and sensitive method of detecting *Paenibacillus larvae* from beehive debris based on PCR that does not require long-lasting cultivation steps. Various methods of extracting spores from beehive debris were compared: the original method of extraction of spores into toluene, and alternative spore extraction methods into Tween 80, into water, into isopropanol and into 95% ethanol. Isolation of DNA from various spore extractions was evaluated too. Best results were provided by isolation of DNA using the QIAamp DNA Mini Kit, without heat treatment. DNA of spores was detected by PCR from 0.25 g of beeswax debris, with the detected titer of 10^5 in 1 g according to the cultivation tests.

Paper II:

Ryba S., Titera D., Schodelbauerova-Traxmandlova I., Kindlmann P. (2012) Prevalence of Honeybee Viruses in the Czech Republic and Coinfections with other Honeybee disease. *Biologia.* **67:** 1-6 (IF = 0,609; 2010)

Six bee viruses, which occur in *Apis mellifera*, were monitored in the Czech Republic between 2006 and 2009. Samples of larvae and pupae collected from hives where American foulbrood was detected were screened for bee viruses and in the 125 samples of larvae, there was no confirmed case of a larva infected with both American foulbrood and a bee virus. Of 145

samples infected with the protozoan *Nosema apis*, there were 23 cases of coinfections with the BQCV virus, 18 with the DWV virus and 11 with the ABPV virus. All coinfections with tree or four viruses were also statistically significant apart from the one between ABPV with CBPV and DWV. The PCA ordination diagram indicates that BQCV occurs mainly with *Nosema apis* and DWV mainly with ABPV.

Paper III:

Ryba S., Kindlmann P., Titera D., Haklova M., Stopka P. (2012) A new low-cost procedure for detecting disease in low-incidence samples: a case study of American foulbrood. *J. Eco. Entomology.*

American foulbrood (AFB) because of its virulence and worldwide spread is currently one of the most dangerous diseases of honeybees. Quick diagnosis of this disease is therefore vitally important. For its successful eradication, however, all the hives in the region must be tested, which is extremely time consuming and costly. Therefore, a fast and sensitive method of detecting AFB is badly needed. Here we present a method, which significantly reduces the number of tests needed by combining batches of samples from different hives. The results of this method were verified by testing each sample. A simulation study was used to compare the efficiency of the new method with testing all the samples and develop a decision tool for determining when best to use the new method. The method is suitable for testing large numbers of samples (over 100) when the incidence of the disease is low (10% or less).

Paper IV:

Kamler M., Tyl J., **Ryba S.,** Titera D. (2012) A scientific note on the microscopical discrimination of Nosema apis and Nosema ceranae spores: a possible rapid screening method for nosemosis type C. *Res. in Vet. Sci.*

Nosemosis of honey bees has a worldwide occurrence. There are two species of microsporidian intracellular parasites, which caused this disease: *Nosema apis* and *Nosema ceranae.* The fact of divers clinical patterns led to consideration as nosemosis type A caused by *N. apis* and nosemosis type C caused by *N. ceranae*. In some countries, nosemosis type C was proved as very pathogenic for honey bee colonies. We tested if the size difference between spores of *N. apis* and *N. ceranae* is appreciable using light microscopy, thus useful in screening of both nosemosis. Each microscopically tested sample was confirmed by PCR. A microscopical evaluation can discrimate *N.apis* and *N. ceranae* also and this method is fast, cheap and usable in fields.

Paper V:

Ryba S., Kristufek V., Titera D. (2012) The use of RIDA®COUNT tests for monitoring the American Foulbrood pathogen. *Apidologia*

American Foulbrood (AFB) is currently one of the most dangerous diseases in honeybees due to its high virulence and worldwide spread. Quick evaluation of the diagnosis of this disease is crucial. Successful eradication in the area indicates a need to test all bee colonies, but this is expensive and time consuming. A fast and sensitive method of detecting *Paenibacillus larvae* using the RIDA®COUNT test (R-Biopharm AG, Germany) was verified in the present study. The test is based on the principle of the cultivation test with MYPGPn medium, coloration of the bacteria with TTC (2,3,5-triphenyltetrazoliumchloride) chromophore, and heat treatment of the sample. Using this new method, color-highlighted colonies of *P. larvae* can be established on the seventh day after inoculating the spores. An identical number of colonies grown with the classic cultivation test on Petri dishes containing MYPGPn medium or RIDA®COUNT-P. larvae (RC-PL) sheets were verified.

Paper VI: PATENT

Kristufek V., Ryba S, Krenek A., Mulec J. (2012) Method of monitoring the infection pressure of American foulbrood, the equipment necessary for this method, and the use of test cards for monitoring the infection pressure of American foulbrood.

The European honeybee (*Apis mellifera* L.) is subject to affliction by a disease known as American foulbrood, the causative agent of which is the Gram-positive bacteria *Paenibacillus larvae* which attacks bee larvae and pupae. This disease is detected and monitored using the PCR test and an ordinary cultivation test; these tests necessitate the complicated preparation of samples, and they must be transported to the laboratory. The submitted invention eliminates these disadvantages by using a mobile test card (1) which is equipped with a detection layer (2) that contains a nutrient medium consisting of MYPGP bullion with nalidixic or pipemidic acid and chromogen 2,3,5-triphenyl tetrazolium chloride (TTC). The sample (7) of bee brood or bee debris comes into contact with the test card (1) by imprint directly in the bee hive (9), by debris fallout on the test card (1) placed on the floor of the hive (9), or by inoculation of a liquid sample (7) on the detection layer (2) of the test card (1). The test card (1) is also equipped with a removable cover layer (3) of transparent oxygen-permeable material, possibly also with a square mesh (11) for deducting the number of cultivated bacteria colonies (10). Following the completion of cultivation, the test card (1) with the sample (7) can be stored in a frozen state.

PAPER 1:

A PCR METHOD OF DETECTING AMERICAN FOULBROOD (PAENIBACILLUS LARVAE) IN WINTER BEEHIVE WAX DEBRIS

Ryba S., Titera D., Haklova M., Stopka P.

Veterinary Microbiology (2009) **139**: 193-196.
(IF = 3,256; 2011)

I have performed all laboratory experiments and analyses presented in this manuscript, written with the help of co-authors.

PAPER 2:

PREVALENCE OF HONEYBEE VIRUSES IN THE CZECH REPUBLIC AND COINFECTIONS WITH OTHER HONEYBEE DISEASE.

Ryba S., Titera D., Schodelbauerova-Traxmandlova I., Kindlmann P.

Biologia. (2012) **67**: 1-6.
(IF = 0,609; 2010)

I have performed all laboratory experiments and molecular analyses presented in this manuscript, written with the help of co-authors. I. Schodelbauerova-Traxmandlova and P. Kinldmann performed all the statistical analyses.

PAPER 3:

A NEW LOW-COST PROCEDURE FOR DETECTING DISEASE IN LOW-INCIDENCE SAMPLES: A CASE STUDY OF AMERICAN FOULBROOD

Ryba S., Kindlmann P., Titera D., Haklova M., Stopka P.

Journal of Economical Entomology
SUBMITTED
(IF = 1,201; 2007)

I have performed all laboratory experiments and analyses presented in this manuscript, written with the help of co-authors. D. Titera and M. Haklova prepared all of the samples, P. Kindlmann performed mathematical models and P. Stopka directed the project and manuscript preparation.

PAPER 4:

A scientific note on the microscopical discrimination of *Nosema apis* and *Nosema ceranae* spores: a possible rapid screening method of emerging nosemosis type C.

Kamler M., Tyl J., **Ryba S.**, Titera D.

Research in Veterinary Sciences
SUBMITTED
(IF = 1.33; 2011)

I have performed all PCR detection and molecular analyses presented in this manuscript, written with the help of co-authors.

PAPER 5:

The use of RIDA®COUNT for monitoring the American Foulbrood pathogen

Ryba S., Kristufek V., Titera D.

Apidologia
(IF = 1,493; 2009)

I have performed half of the laboratory experiments and analyses presented in this manuscript, written with the help of co-authors. Contribution of other people is given in Acknowledgements.

PAPER 6:

PATENT

METHOD OF MONITORING THE INFECTION PRESSURE OF AMERICAN FOULBROOD, THE EQUIPMENT NECESSARY FOR THIS METHOD, AND THE USE OF TEST CARDS FOR MONITORING THE INFECTION PRESSURE OF AMERICAN FOULBROOD

Kristufek V., **Ryba S**, Krenek A., Mulec J.

Patent No. E154441 (17 April, 2012)

I have performed half of the laboratory experiments and analyses presented in this patent, written with the help of co-authors.

GENERAL DISCUSSION

The total number of managed honey bee colonies worldwide is currently estimated for 72.6 million (**Fig. 2**). Although it seems that the number of bee colonies between 1961 and 2009 increased, there is a decrease (**Fig. 3**) in the number of managed bee colonies in developed countries (USA, Canada and some European countries), while the number of managed colonies increase in developing countries (FAO, 2009). The main reasons for increase in the managed bee colonies in developing countries are increasing knowledge of bee keepers, economic aspects of beekeeping and finally, better organization in the registration of highly managed colonies (Aizen and Harder, 2009; Daberkov et al, 2009). Beekeeping has had a long tradition in the Czech Republic, represented by a high level of organization and a high number of bee colonies per 1 km^2.

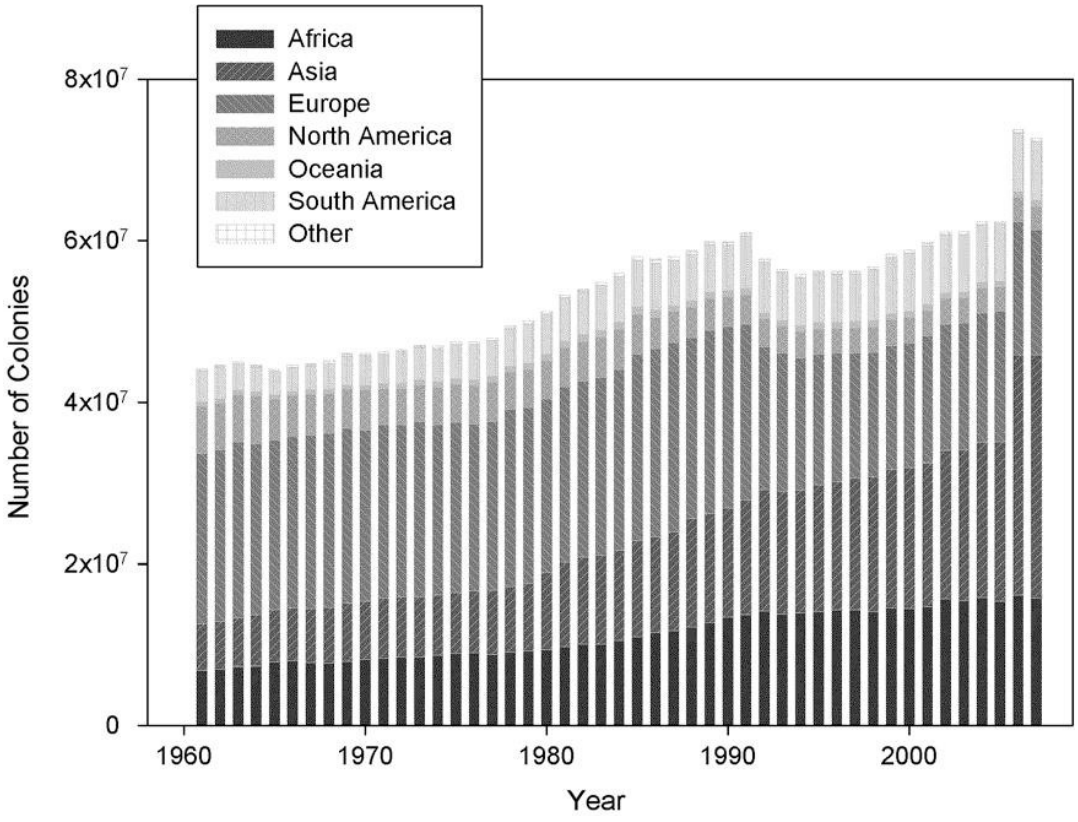

Fig. 2. Total global number of managed honey bee colonies between 1961 and 2007 (FAO, 2009). The large increase in Asian bee populations between 2005–2006 primarily results from countries reporting managed colonies to the FAO for the first time in 2006.

In both historical and current literature devoted to honey bee pathology, we can find a large

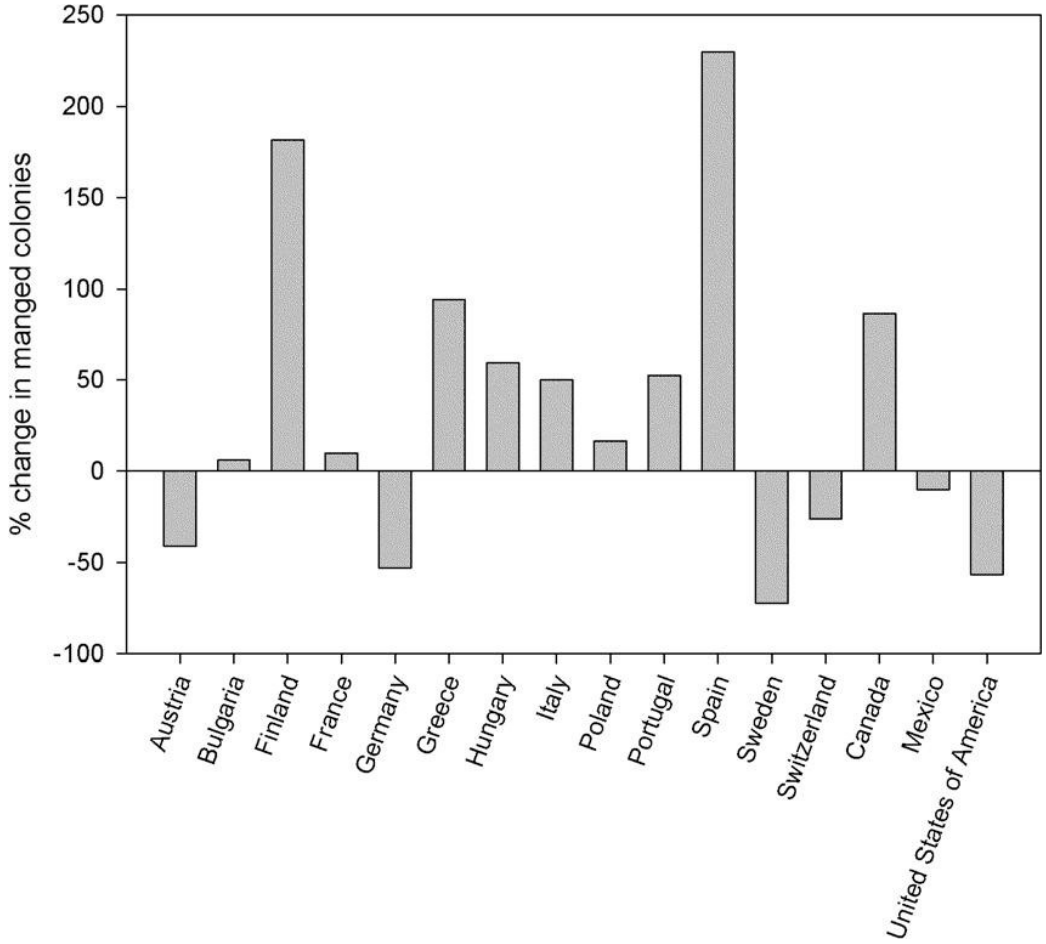

Fig. 3. Percent change in number of managed bee colonies between 1961 and 2006 in selected countries in Europe and North America (FAO, 2009).

number of incidents related to outbreaks of honey bee diseases with subsequent and often total losses which affect large areas, or regional cases of a decline in the number of bee colonies limited to small or island areas (**Fig.4**). In many such cases, the cause of death in bee colonies is not sufficiently explained and there is much speculation about the death or significant loss of strength of bee colonies (Bailey, 1964; Underwood a vanEngelsdorp, 2007; Frazier et al., 2008). The cause of many of these deaths is often caused by the introduction of a parasite or a disease transferred from other species (Higes et al., 2006). Colony Collapse Disorder (CCD) is a condition in the bee colony which was first observed in the USA in 2006. It can be characterized by specific symptoms:

loss of adult bees in the bee colony without visible presence of dead bees both in the hive and in the apiary´s land and absence of predatory behaviour of bees, although there are enough glycide supplies in hives (Cox-Foster and vanEngelsdorp, 2009; Underwood and vanEngelsdorp, 2007). Although the cause of disease remains unknown, the interaction of stressful factors mentioned below has been assumed (Cox-Foster a vanEngelsdorp, 2009; vanEngelsdorp et al., 2008).

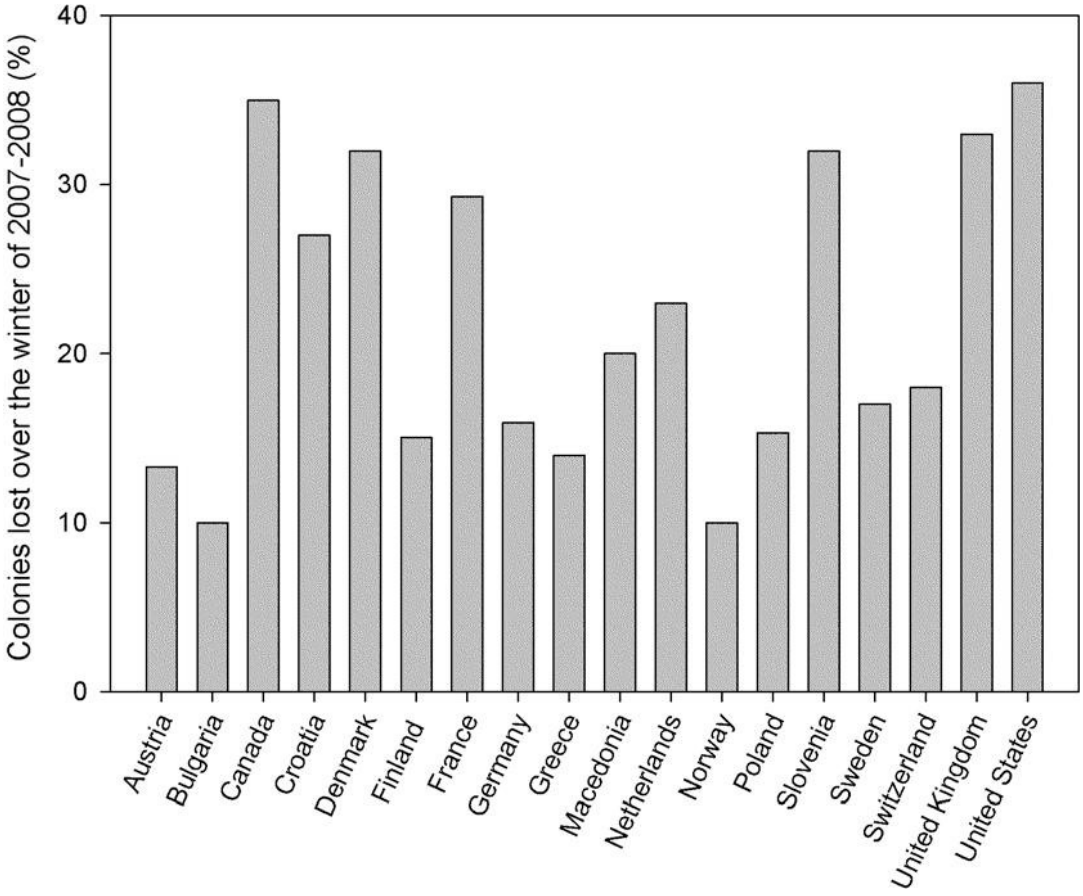

Fig. 4. Percentage of colony winter losses in 2007/2008 in several countries (Pernal, 2008; Coloss, 2009).

Due to modern agriculture involving the use of chemical substances, honey bees can be more often exposed to pesticides that can be brought into the bee colony by foragers. This so-called acute toxicity can be easily found by using cage experiments. On the other hand, sub-lethal effects of pesticides used, such as partial paralysis or disorientation, can significantly influence behaviour in the colony and affect its viability (Croft, 1990, Thompson, 2003; Desneux et al., 2007). Unlike the acute toxicity of honey bees which is clear and easily observed, sub-lethal effects of pesticides on bees are hard to prove (Desneux et al., 2007) and they become evident later or after a long-term exposure. They can affect various life functions both on the level of an individual bee and the entire colony in the long term (Evans et al., 2010).

The consequences of the pesticide use also involve a synergic effect with pathogens as it has been shown for *Nosema* ssp. (Alaux et al., 2010; Vidau et al., 2011). Recently, a high attention has been paid to the impact of acaricides, substances used for the suppression of the mite *Varroa destructor*. These substances do not have a direct effect on bees during the exposure. Nevertheless, with an increasing resistance of the mite *Varroa destructor* and thus increasing doses of varroacid, bee colonies are often more and more exposed (Milani, 1999; Pettis, 2004; Lodesani and Costa, 2005; Martel et al., 2007; Chauzat et al., 2009). The situation is further worsened as the acaricide residues are transferred into the wax and thus get involved in the wax cycle of the bee operation (Martel et al., 2007; Frazier et al., 2008).

A high reproduction potential of honey bees, along with the known methods of production of fertilized queens in bee practice, enable the colonies to produce a large number of descendants (newly fertilized queens) with required properties from a genetically small population of exclusive queens (Lodesani and Costa, 2003). On the other hand, due to the selection and massive breeding of one (even if very convenient) genetic variant, the loss of genetic diversity increases the bee susceptibility to viruses, bacteria and parasites. It was proven in the USA that only 500 main breeders of honey bee queens represent a genome base for all commercially managed bee colonies in the USA. This is a good example of the “bottleneck effect”, when the genetic variability inside the population of bee colonies bred in a large area is reduced (Sheppard, 1988; Delaney et al., 2009). The genetic uniformity of bee colonies in all areas, furthermore intensified by the location of a large number of bee colonies in one place, increases the chance for the transfer of pathogens, their success rate, and related unpredictable (usually winter) losses (Graham et al., 2006; Mattila and Otis, 2007). On the other hand, sufficient genetic variability of bee colonies and their even distribution in the area increases the resistance of bee colonies against pathogens and prevents from spreading easily (Loskotova et al., 1980; Camazine et al., 1998; Tarpy, 2003; Jones et al., 2004, Pettis et al., 2004).

Some breeds and regional variations of the honey bee often did not have the required properties for commercial breeding, placidity, productivity, and did not comply with selection criteria. With an increasing introduction of a new genetic material, underlying the required bee characteristics, into the environment many interesting genetic variations in the territory of the Czech Republic and also in the rest of Europe died out.

Globalization of trade provides the means by which both parasites and pathogens are transported across geographical borders and infect new hosts. The *Varroa destructor* mite, which

originally only infested *Apis ceranae,* but now infests *Apis mellifera*, is a good example. Similarly, the transmission of *Nosema ceranae*, which naturally infects *Apis ceranae*, was discovered a decade ago infecting *Apis mellifera* and now is a threat to apiaries. Screening of AFB disease based on the detection of *Paenibacillus larvae* from bee debris by using PCR represents an alternative and very sensitive method for a proven test on Petri dishes. Test sensitivity on Petri dishes is about 100 spores per 1g of bee debris. Due to a higher sensitivity of PCR (PFU=0.9), PL can be set before the outbreak of clinical symptoms. The alternative of the method of *Paenibacillus larvae* detection based on the matrix re-arrangement of samples allows for a vast screening of *Paenibacillus larvae* by using the PCR method. This also saves a significant amount of time and laboratory materials. Another alternative view on *Paenibacillus larvae* detection represents the use of detection sheets based on the cultivation test, selective conditions, and a coloured reaction of pathogen (PAPER 5, PAPER 6). The spreading of disease caused by *Nosema ceranae* contributes to higher losses in colonies. An alternative method to the classic PCR test is based on the discrimination of *Nosema apis* and *Nosema ceranae* in the light microscope (microscopic discrimination). Beekeeping as well as other areas of human activity goes through a gradual evolution process as more and more modern technologies and knowledge are applied. This process can be documented from the early days of beekeeping until today**.** Each positive step which facilitates any kind of activity and increases its revenues and profitability, has negatively affected bee breeding. This was mostly manifested by increased mortality rates and winter death in bee colonies. Modern zootechnical methods, possibilities of transport of bee colonies to long distances, pesticides in agriculture (as well as in beekeeping operation) and selective techniques in breeding consequently affect vitality of the honey bee. Also a small quantity of original infectious agens introduced to the bee colony can cause the outbreak of the clinical stage of the disease with subsequent fatal effects for the bee colony or the entire breed, and spread to other areas. Routines of detection of bee diseases based on fast, exact and cheap diagnostic techniques are one of the main pillars for an efficient epidemiological screening of managed colonies.

GENERAL CONCLUSION

1. Various approaches to the detection of the American foulbrood from bee debris were compared and several options for spore extraction methods were tested. Based on these findings, a new method of detection of the American foulbrood from bee debris was developed.
2. We developed, evaluated and practice-adjusted general methodology of matrix rearrangement of samples suitable for PCR detection of causative agents of the diseases which rarely and with low incidence occur in the examined population. The case study was verified based on a file of samples of American foulbrood.
3. Detection sheet RIDA®COUNT-Paenibacillus larvae based on the cultivation test on the paper sheet was developed, laboratory-tested and patented. A detection sheet was adjusted for detection of American foulbrood.
4. A sensitive and reliable diagnostic method based on the polymerase chain reaction for detection of the six most common bee viruses (ABPV, BQCV, DWV, SBV, KBV, and CBPV) was developed.
5. Due to the implementation of the methodology for screening of bee viruses, we performed screening of these bee viral diseases for the first time in the territory of the Czech Republic. We evaluated a number of particular detections of disease and shares of reciprocal co-infections.
6. We created a sensitive and reliable methodology for differential detection of *Nosema apis* and *Nosema ceranae* by using PCR.
7. We developed a technique of microscopic determination of *Nosema apis* and *Nosema ceranae* spores and verified it by using a PCR test.
8. By repeating screening during 3 years, we confirmed increasing ratio of bee viruses found in pathologically suspicious bee colonies.
9. We confirmed statistical dependence of common occurrence of *Nosema apis* along with DWV and BQCV viruses and evaluated other pathogens which occur in suspicious samples separately.
10. We verified increasing frequency of *Nosema ceranae* spores over *Nosema apis* spores in the file of taken samples in the territory of the Czech Republic both microscopically and by using PCR techniques.

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

ABPV	–	Acute Bee Paralysis Virus
AFB	–	American foulbrood
BLAST	–	The Basic Local Alignment Search Tool
BQCV	–	Black Queen Cell Virus
CBPV	–	Chronic Bee Paralysis Virus
CCD	–	Colony Collapse Disorder
cDNA	–	copy DNA
DNA	–	Deoxyribonucleic acid
DWV	–	Deformed Wing Virus
EXPASY	–	Expert Protein Analysis System
KBV	–	Kashmir Bee Virus
MYPGP	–	Mueller-Hinton broth, Yeast extract, Potassium phosphate, Glucose, Pyuvate
MYPGPn	–	Mueller-Hinton broth, Yeast extract, Potassium phosphate, Glucose, Pyuvate, nalidixic acid
NA	–	Nosema apis
NC	–	Nosema ceranae
OIE	–	The World Organisation for Animal Health
PCR	–	Polymerase chain reaction
RNA	–	Ribonucleic acid
RT-PCR	–	Reverse transciption - Polymerase chain reaction
SBV	–	Sacbrood Virus

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