SZENT ISTVÁN UNIVERSITY

Doctoral Training Programme in Veterinary Science

Occurrence of the honey bee viruses in Hungary, investigations of the molecular structure of certain viruses

Ph.D. Dissertation

Tamás Bakonyi DVM

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Szent István University
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Programme Coordinator:
Péter Rudas, DVM, DSc
professor

Supervisor and co-supervisors

Miklós Rusvai DVM, PhD
professor
SzIU, Faculty of Veterinary Science, Department of Microbiology and Infectious Diseases

Balázs Harrach DVM, PhD
director
Veterinary Medical Research Institute of the Hungarian Academy of Sciences

György Berencsi MD, PhD
professor
"Johan Béla" National Epidemiological Institute

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Dr. Péter Rudas	Dr. Tamás Bakonyi

1. Introduction

Viral infections of the honey bee (*Apis mellifera* L.) became into the scope of the scientific interest in the last few years due to its pathogenic role and the increase of the importance of the host species. Although the first insect virus, the sacbrood virus, (SBV) was isolated from bees and since that time 17 other bee viruses have been isolated so far, there are very few data available on the molecular structure, classification, genetic diversity, distribution and importance of the bee viruses.
The presence of bee viruses has been reported from several countries worldwide. Most of them (i.e. acute bee paralysis virus, ABPV; black queen cell virus, BQCV; Y bee virus, YBV; deformed wing virus, DWV) are present in high proportions of the bee colonies. The inapparent infection is often activated by predisposing- and weakening factors, which leads to depopulation and sudden collapse of the bee colonies. The most important predisposing factor is the parasitic mite *Varroa destructor* (previously *V. jacobsoni*), which is a vector of several honey bee viruses, and also weakens the pupae and adult bees by its feeding activities and activates the hidden viral infections. Besides Varroa mites, the presence of other pathogens, parasites (i.e. *Nosema apis*), intoxications and the environmental pollution may also act as an activator of viral infections.
In the case of bee diseases, the colonies or apiaries are the subjects of the diagnostic tests and treatment instead of the individual bees. The conventional diagnostic methods have limited value in the case of bee diseases: the findings of physical examination and the symptoms of the disease are often less informative. Furthermore, as bees do not produce antibodies against pathogens, the indirect determination of

infections (widely used in other fields of veterinary practice) is not possible.
It is difficult to isolate bee viruses due to the lack of permanent cell lines of bee origin. The only method to propagate the honey bee viruses is the experimental infection of bee pupae or newly emerged bees. Since honey bee virus infections are widespread, often without causing symptoms, an experimental infection may activate persistent infections with other viruses present in the apparently healthy pupae resulting in a mixture of different viruses in the pupae-extracts. Furthermore the experimental infection is season dependant and rather time consuming.
The conventional methods for the diagnostics of bee viral infections are the electron microscopy (EM) and the agargel-immunodiffusion (AGID) test. The morphological appearance and physico-chemical features of most of the honey bee viruses are quite similar; the use of EM is therefore difficult for the identification of bee viruses. Several methods have been developed to detect viral antigens in clinical samples, such as immunodiffusion, enzyme-linked immunosorbent assay (ELISA), chemiluminescent Western blotting, and radioimmuno-assays. The disadvantage of these techniques is that they require specific antisera. Raising specific antisera is complicated regarding the difficulties of production pure virus suspension in large amounts.
The development of the new molecular diagnostic techniques gave an opportunity to investigate the nucleic acid structure of several honey bee viruses in details. Complete genome sequences have been determined in the case of SBV, BQCV and ABPV, while from KBV partial genome sequences were determined. Sensitive and reliable diagnostic methods were developed for the detection of viral nucleic acid of KBV, SBV, ABPV and BQCV applying polymerase chain reaction following reverse transcription (RT-PCR). The new methods

are suitable for the direct detection of the viral nucleic acid from bee samples, and for the phylogenetic analysis and comparison of the strains' nucleotide sequences.
In spite of the importance of beekeeping in Hungary, very little was known on the occurrence and distribution of bee viruses in the country, although clinical symptoms and EM findings supported the presence of certain bee viruses in Hungarian apiaries (i.e. SBV; Chronic Bee Paralysis Virus, CBPV).
This thesis presents the results of the studies on the occurrence of ABPV, in connection with an outbreak caused by the virus in Hungary.

Aims of the investigations:

- Detection, isolation and identification of honey bee viruses from Hungarian apiaries.
- Development of RT-PCR for the detection of the nucleic acid of certain bee viruses.
- Survey on the occurrence of the aforementioned viruses in Hungarian apiaries.
- Phylogenetic analysis of certain bee viruses.

2. Materials and methods

2.1. Samples

In July, 1997 sporadic dysentery and death was observed in an apiary near by Budapest. Three colonies were tested for the presence of bee viruses. The ABPV strain isolated from these colonies was used for the development of a new diagnostic method based on RT-PCR.
To determine the occurrence of bee viruses, a survey was undertaken in five regions of the country. Apparently healthy adult bees were sent by twelve volunteering beekeepers. Within a two years' period 114 bee samples have been tested.

Additional samples were tested from colonies showing sudden collapse or high winter mortality in eight apiaries. *V. destructor* mites collected from the samples were also checked for the presence of bee viruses.
Phylogenetic analyses were performed on 13 Hungarian, 4 Polish (G. Topolska, Warsaw), 3 German (W. Ritter, Freiburg) and 1 Austrian (N. Nowotny, Vienna) isolates of ABPV., The RT-PCRs developed for the detection of SBV and BQCV were tested on strains isolated in Poland (G. Topolska, Warsaw), due to the lack of positive samples.

2.2. Virus propagation and purification

Following parasitological investigations 50-100 bees were homogenized in phosphate buffered saline and cleared from bacteria by centrifugation. Supernatants were injected into 8-10-days-old pupae from apparently healthy colonies of the same apiary. Pupae were processed for EM investigations or after 4 days of incubation, they were homogenized and the homogenates were purified by cesium-chloride gradient (1.2-1.5 g/ml) ultracentrifugation.

2.3. Electron microscopic investigations and agar-gel immunodiffusion

The ultrathin sections of infected pupae and the gradient ultracentrifuge purified and dialyzed virus suspension were examined in a JEM-JEOL 100S transmission electron microscope. Serial dilutions of the gradient ultracentrifuge purified virus suspension were reacted with specific antisera raised in rabbits against the most frequent bee viruses in AGID tests.

2.4. RNA isolation and reverse transcription

Viral RNA was purified from the supernatants of the bee sample- and pupa-homogenates, and it was transcribed to complementary DNA (cDNA) by the oligo (dT) method. In the case of the phylogenetic analyses the reverse transcription and the polymerase chain reaction were performed in a continuous system using virus-specific primers.

2.5. Polymerase chain reaction and agarose-gel electrophoresis

Specific oligonucleotide primers were designed based on the nucleotide sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov) for the amplification of the nucleic acid of ABPV, KBV, SBV and BQCV. Primers designed on the structural protein gene region of ABPV were employed in the study on the occurrence of the virus in Hungarian apiaries using a RT-PCR assay In the phylogenetic analysis of Central European ABPV strains, the complete structural protein gene region was amplified with six, overlapping products. The non-structural protein gene region of ABPV was amplified with another six primer pairs producing overlapping products. The primers used in the discriminative RT-PCR assay for the identification of a new bee virus isolate related to KBV, were designed on the basis of the non-structural protein gene region of KBV. In the RT-PCR assays for the detection of SBV and BQCV, the primers were designed on the 5' non-coding section of the virus genomes. Amplifications were performed in a MJ Research MiniCycler.
The PCR products were electrophoresed in agarose gel, the bands were visualized by UV translumination and the pictures were documented by a Kodak Digital Science 1D software.

2.6. Nucleotide sequencing and sequence analysis

To confirm the specificity of the primers used in the PCR assays for the detection of ABPV and identification of KBV, after electrophoresis, the products were sliced out from the gel, they were purified by QIAquick Gel Extraction Kit, and were sent for sequencing to the Biological Research Centre of the Hungarian Academy of Sciences (Szeged). In the case of the phylogenetic analyses, direct sequencing PCR of the products was followed by fluorescence-based sequencing in an ABI Prism 310 automated sequencing system. Nucleotide and deduced amino acid sequences were corrected and aligned using FastA, ClustalW, BioEdit and Align Plus software programmes, and the phylogenetic analyses were performed using the PHYLIP package. Bootstrap resampling analysis was generated with the SEQBOOT program to prove the stability of the trees. Distance matrices were generated by the DNADIST/Neighbor-Joining and Fitch programs, using a translation/transversion ratio of 2.0.

3. Results

3.1. Virus propagation and purification

After the gradient ultracentrifugation of the extracts of the experimentally infected pupae, two distinct, opalescent zones were observed with a buoyant density of 1.32-1.33 g/ml and 1.37-1.38 g/ml, respectively.

3.2. Electron microscopic investigations and agar-gel immunodiffusion

Ultrathin sections of various organs of the affected bees and experimentally infected pupae revealed the presence of

icosahedral virus particles with 30 nm in diameter, arranged in crystalline arrays, in the cytoplasm of cells. Masses of virus particles of similar size were observed in the extracts of experimentally infected pupae purified by caesium chloride gradient centrifugation (Figure.1).

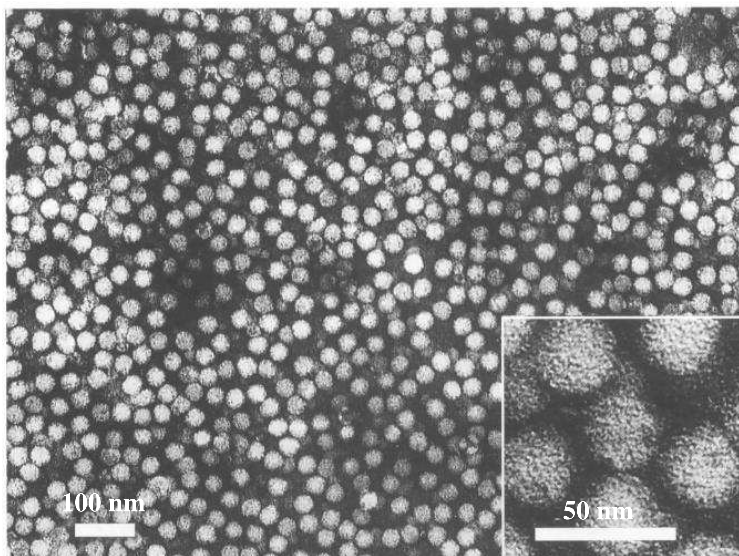

Figure 1.: EM picture of ABPV particles. (© Dept. of Pathology and Forensic Veterinary Medicine, Faculty of Veterinary Science, SzIU.)

The AGID tests of the zone of 1.37-1.38 g/ml buoyant density gave a strong positive reaction with ABPV antiserum. Homogenates of the bees, which were positive for ABPV nucleic acid by RT-PCR, were negative in AGID tests.

3.3 Development of RT-PCR for the detection of ABPV

The RT-PCR reaction on the RNA gained from the gradient ultracentrifuge purified virus suspension amplified a 398 nucleotide (nt) long part of the structural protein gene region of ABPV. The reaction gave positive result even in the 10^4

dilution of the virus suspension. The presence of ABPV nucleic acid was also demonstrated directly from bee homogenates by the aforementioned RT-PCR method.

3.4. Survey on the occurrence of ABPV in Hungarian apiaries.

Investigating 114 apparently healthy bee colonies of 12 apiaries from five regions of the country, the ABPV nucleic acid was detected in 14 colonies (12.2%) of 8 apiaries (66.6%), by RT-PCR, in two years. The simultaneous presence of infected and non-infected colonies in the same apiary was confirmed by repeated investigations in more apiaries. The presence of the viral nucleic acid was also demonstrated in 87.5% of the apiaries, where the colonies showed clinical symptoms (7 out of 8 apiaries). *V. destructor* and *N. apis* infestations have also been observed in most of these colonies. The ABPV nucleic acid was also demonstrated from the mites of two out of three apiaries heavily infested with *V. destructor.* The presence of other bee viruses (KBV, SBV, BQCV) could not be detected in the samples.

3.5. Nucleotide sequencing and sequence analysis

3.5.1. Control of the specificity of the diagnostic RT-PCR

The specificity of the RT-PCR assay used in the survey was confirmed by the sequencing of the product followed by an overall homology search in the GenBank database. The 398 nt long sequence has the highest identity (93%) with the structural protein gene region of the complete ABPV genome sequence (accession number AF150629) between nt positions 8107–8504.

3.5.2. Phylogenetic analysis of Central-European Acute Bee Paralysis Strains

Regarding the wide distribution of ABPV in Central Europe with various clinical manifestations, phylogenetic analysis was performed on isolates to reveal the variability of the ABPV genome, and the molecular relationship between virus strains of different geographic origin. A 3071 nt fragment of the ABPV genome including the entire structural protein gene region, has been amplified and sequenced in ten isolates. The sequenced region comprises 32.4% of the genome and codes for 914 amino acids (aa). The nucleotide sequences were compiled and aligned. They showed identity rates of 94% to 95% compared to the reference strain. The deduced aa sequences showed 97-98% identity. The phylogenetic analysis of the nucleotide sequences revealed three distinct genotypes: the ABPV samples from Austria and Germany were grouped together in one branch, while the Polish and the Hungarian strains formed two other distinct clusters.

Another comparative and phylogenetic analysis was carried out on a shorter (401 nt) fragment of the ABPV structural protein gene, where higher sequence divergence was observed. In this analysis, all ABPV sequences available to date have been included (eleven sequences of probable UK origin deposited in the GenBank database, partial sequences of the samples mentioned above, and additional ten sequences amplified from nine Hungarian and one Polish ABPV specimens). The nucleotide sequences of 32 virus strains between nt positions 8121-8521 showed identity rates between 89% and 96%, respectively. The deduced amino acid sequences had 90-96% identity to the reference strain. In the phylogenetic tree constructed with these sequences, the ABPV strains were separated into at least two major branches. One is composed of the British sequences deposited in GenBank, while the other

branch comprised the isolates from continental Europe; however, every branch could be sub-divided into further distinct clusters. The RT-PCR assays represent the methodical basis for phylogenetic analysis and classification of new ABPV isolates. (Figure 2.)

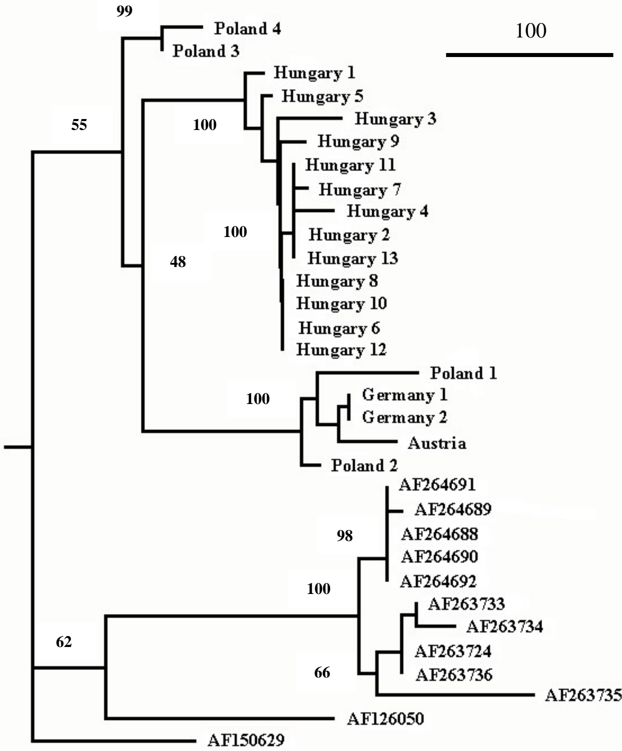

Figure 2.: Phylogenetic tree of ABPV strains
(Numbers represent bootstrap values)

3.5.3. Analysis of the non-structural protein coding regions of the ABPV genome (Numbers represent bootstrap values)

To reveal the genetic variability of the non-structural protein genes, the helicase and protease regions of one Hungarian and one Polish ABPV isolates were analyzed. A 4338 nt long sequence was determined, which covers 45.7% of the genome. The sequences were aligned to the reference complete ABPV genome. Sequence analysis revealed 93.1% identity to the reference strains, while the two Central European strains have shown 97.3% identity to each other.

3.6. Detection of a new variant of KBV in Hungary

Within the identification procedure of a virus isolated from a Hungarian apiary, with ABPV specific immune-serum in AGID test, interestingly, a double precipitation line occurred, indicating the presence of two antigenically related but not identical viruses. Discriminating primer pairs designed in the structural protein-coding region of ABPV were used in RT-PCR investigations. Sequencing of the amplicons proved that the virus suspension contains indeed a mixture of two genetically distinct viruses. Homology search demonstrated a new variant of Kashmir bee virus (KBV) as one of the components (nucleic acid identity 83.6 % to a reference KBV strain) while the other virus was closely related to the prototype ABPV strain (93.6 % nucleic acid identity)

3.7. Development of RT-PCR assay for the detection of SBV and BQCV nucleic acid

Specific primer pairs were designed based on the complete genome sequences of SBV and BQCV for the amplification of

certain parts of the viral nucleic acids. Primers were tested in virus strains and field samples from Poland. Amplifications produced specific products in the case of both viruses. Samples from apparently healthy colonies of Hungarian apiaries were negative for the presence of SBV and BQCV nucleic acid, and samples showing clinical symptoms from Hungary were not available for us so far.

4. Final conclusions, new results

1. We have detected, propagated, identified and described for the first time the acute bee paralysis virus in diseased bees collected from a Hungarian apiary. ABPV is the first honey bee virus isolated in Hungary.
2. We have developed rapid and sensitive diagnostic methods based on RT-PCR for the detection of ABPV, KBV, SBV and BQCV in honey bee samples.
3. We had started a survey on the occurrence of ABPV in Hungary, and demonstrated the presence of the virus in two third of the investigated apiaries. The comparison of the occurrence rate of the virus in healthy colonies and in colonies with symptoms of the disease supported the impact of the virus in the clinical manifestation of bee diseases.
4. We have demonstrated the presence of the viral nucleic acid of the ABPV in the parasitic mite *Varroa destructor*, supporting the theories that the mite is playing a role as a vector in the spread of the virus.
5. We have analyzed the genetic relationships of Central European ABPV strains. Phylogenetic trees constructed on the basis of the alignments of the structural protein gene regions revealed at least two distinct genetic linkages of ABPV, which are sub-divided into several genotypes reflecting the geographic origin of the

isolates. The methods provide a basis of phylogenetic analysis of new ABPV isolates.

6. We have determined the partial nucleotide sequence and the sequence divergence of the non structural protein gene region of a Hungarian and a Polish ABPV isolate.
7. We have detected and isolated a new virus variant in a honey bee sample from Hungary. The phylogenetic analysis of the new variant indicated close relationship with KBV and ABPV strains.

5. Publication list

5.1. *Articles published in refereed scientific journals in English:*

5.1.1. BÉKÉSI L., BALL, B. V., DOBOS-KOVÁCS M., BAKONYI T., RUSVAI M.: Occurrence of acute paralysis virus of the honey bee (*Apis mellifera*) in a Hungarian apiary infested with the parasitic mite *Varroa jacobsoni*. In: *Acta Veterinaria Hungarica*, 1999., vol. XLVII. p. 319-324.

5.1.2. BAKONYI T., FARKAS R., SZENDRŐI A., DOBOS-KOVÁCS M., RUSVAI M.: Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* samples: Rapid screening of representative Hungarian apiaries. In: *Apidologie*, 2002., vol. XXXIII. p. 29-40.

5.1.3. BAKONYI T., GRABENSTEINER, E., KOLODZIEJEK, J., RUSVAI M., TOPOLSKA, G., RITTER, W., NOWOTNY, N.: Phylogenetic analysis of acute bee paralysis virus strains. In: *Applied and Environmental Microbiology*, 2002., Submitted for publication.

5.1.4. RUSVAI M., BAKONYI T., SZENDRŐI A., TOPOLSKA, G., NOWOTNY, N.: Detection of a new variant of Kashmir bee virus in Hungary. In: *Apidologie,* 2002., Submitted for publication.

5.2. *Articles published in refereed scientific journals in Hungarian:*

5.2.1. BÉKÉSI L, BALL, B. V., DOBOS-KOVÁCS M., BAKONYI T., RUSVAI M.: A mézelő méh (*Apis mellifera*) heveny bénulás vírusának hazai előfordulása Varroa-atkával fertőzött méhészetben. In: *Magyar Állatorvosok Lapja*, 1999., vol. CXXI. p. 601-603.

5.2.2. FARKAS R., BAKONYI T., BÖRZSÖNYI L., RUSVAI M.: A mézelő méh (*Apis mellifera* L.) *Varroa jacobsoni* Oudemans fertőzöttségével kapcsolatos kérdőíves vizsgálat hazai méhészetekben. In: *Magyar Állatorvosok Lapja*, 2001., vol. CXXIII. p. 348-353.

5.3. *Conference abstracts:*

5.3.1. RUSVAI M., BAKONYI T.: A mézelő méh (*Apis mellifera*) heveny bénulását okozó vírus hazai izolálása és struktúrális polipeptidjeinek vizsgálata. In: *Akadémiai beszámolók*, 1999., Budapest

5.3.2. BAKONYI T., DOBOS-KOVÁCS M., RUSVAI M.: Nucleic acid investigations of the acute bee paralysis virus and development of a diagnostic PCR assay. In: *Proceedings of the First Joint Meeting of the Slovenian Society for Microbiology and the Hungarian Society for Microbiology*, 2000., Keszthely

5.3.3. RUSVAI M., BAKONYI T., DOBOS-KOVÁCS M., BÉKÉSI L.: Polypeptide composition of an acute paralysis strain isolated in Hungary. In: *Proceedings of the First European Scientific Apicultural Conference,* 2000., Pulawy, Poland

5.3.4. TOPOLSKA, G., BAKONYI T., SZENDRŐI A., RUSVAI M.: Rapid diagnosis of acute bee paralysis virus infection by polymerase chain reaction. In.: *Proceedings of the 38th Scientific Apicultural Congress*, 2001., Pulawy, Poland

5.3.5. BAKONYI T., SZENDRŐI A., RUSVAI M.: A mézelő méh heveny bénulását okozó vírus kimutatása RT-PCR vizsgálatokkal az ázsiai óriás méhatkából. In: *A Magyar Mikrobiológia Társaság Nagygyűlése*, 2001., Balatonfüred

5.3.6. RUSVAI M., BAKONYI T., TOPOLSKA, G., NOWOTNY, N., RITTER, W.: A mézelő méh vírusfertőzései a kelet-közép európai régióban. In: *A Magyar Biológiai Társaság Szimpóziuma,* 2001., Budapest

5.3.7. BAKONYI T., GRABENSTEINER, E., RUSVAI M, NOWOTNY, N.: A heveny méhbénulás vírus közép-európai izolátumainak filogenetikai vizsgálata. In: *Akadémiai beszámolók,* 2002., Budapest