Design of primers for identification of honey bee viruses in multiplex-PCR

[1]Alfiya Vasilevna Khammadova, [1,2,3]*Eduard Arkadevich Shuralev,
[2]Nail Ildarovich Khammadov, [1]Bamanga Mamadou Oumarou, [2]Tagir Khadievich Faizov,
[1,3]Malik Nilovich Mukminov

[1]Kazan Federal University, 18 Kremlyovskaya St., Kazan, Tatarstan, 420008, Russian Federation
[2]Federal Center for Toxicological, Radiation and Biological Safety, Nauchniy Gorodok-2, Kazan, Tatarstan, 420075, Russian Federation
[3]Kazan State Medical Academy – Branch Campus of the Federal State Budgetary Educational Institution of Further Professional Education «Russian Medical Academy of Continuous Professional Education» of the Ministry of Healthcare of the Russian Federation, 36 Butlerova St., Kazan, Tatarstan, 420012, Russian Federation

Abstract. This paper is devoted to the design of primer oligonucleotide sequences for their use in the genetic identification of Sacbrood virus, Chronic bee paralysis virus, Black queen cell virus and Deformed wing virus using multiplex-PCR. As a result of the bioinformatic analysis, the design of the oligonucleotide primers was performed; the designed primers had similar annealing temperatures (55 °C), which makes it possible to indicate each of the viruses under the same PCR conditions. Most of the known strains and isolates of these viruses are amplified with this complex of oligonucleotide primers. Nucleotide sequences of designed primers and a universal positive control allow for the genetic identification of each of the biopathogens under the same PCR conditions at a multiplex format.

Keywords: *Apis mellifera* L., Sacbrood virus, Chronic bee paralysis virus, Black queen cell virus, Deformed wing virus.

Introduction

One of the most acute problems in the effective breeding of honey bees (*Apis mellifera* L.) is their viral diseases [1]. About 20 of more than 7,000 currently known viruses are isolated from bees. The size of virions in bee viruses varies from 17 to 450 nm, the shape is diverse: filamentary, polyhedral, ellipsoidal, and spherical.

Bees, like other representatives of the animal world, are susceptible to viral diseases, leading to disruption of the life of the bee family, and sometimes to its death. The danger of viruses is due to their long existence in the host without clinical symptoms, rapid spread both in the bee family and outside it, the presence of ectoparasites and the ability to cause under certain conditions a significant economic damage to beekeeping. The intensity of the spread of viral diseases can be influenced by environmental and geographic conditions [2-4], pollution of the environment by pollutants [5,6], the presence of ticks and mites as vectors of causative agents of infectious diseases of bees [7,8]. Viruses spread very quickly across the territory and cover vast areas, like the spread of European viruses in Asian countries [9], which may also be related to the damage of wild bees [10-12]. Viruses are a cause of the "collapse" of bee colonies, which is characterized by the rapid disappearance of adult individuals while maintaining the brood, sufficient feed and against a

* Corresponding author: Eduard A. Shuralev, PhD, Department of Applied Ecology, Institute of Environmental Sciences, Kazan Federal University, 18 Kremlyovskaya St., Kazan, Tatarstan, 420008, Russian Federation; eduard.shuralev@mail.ru

background of low tick parasitism. The increased multiplication of viruses increases their virulence, which leads to an increase in mortality among insects, and in some cases to the collapse of the whole bee family. Different mechanisms of virus-host interaction affect virus dynamics, virus-virus interactions, superinfections [13].

The most relevant viruses affecting the bees to this day are Sacbrood virus (SBV), Chronic bee paralysis virus (CBPV), Black queen cell virus (BQCV), Deformed wing virus (DWV) and some others [14,15].

To effectively combat the virus diseases of bees, timely and accurate diagnosis is necessary. Previously, the diagnosis of "viral disease" was raised mainly in the presence of typical symptoms. Later, it was established that different strains of the same virus under the influence of various environmental factors can manifest themselves with different symptoms. The method of diagnosis by symptoms proved to be unreliable, especially since many apiaries can simultaneously have several different viruses. Thus, laboratory studies have become the main way to diagnose viral infections [16].

The maximum specificity among various diagnostic methods is the genodiagnosis of viral diseases [17]. When detecting specific markers of particular pathogens, the polymerase chain reaction (PCR) shows maximum efficiency, which allows detecting the virus already at the initial stage of the infectious process development. Good results were obtained in the development of SBV rapid diagnostics by in-situ hybridization [18]. The diagnosis of each of the viral diseases of interest to us requires analysis of the genomes of different types and isolates of viruses, as well as of other biopathogens [19], as well as determination of maximally homogeneous sites and verification of the probability of possible cross reactions.

Objective of this study: the design of oligonucleotide sequences of primers for their further use for the indication and identification of the causative agents of viral diseases of bees by the multiplex-PCR method.

Material and methods.

During the design of primers and probes, the resources of the National Center for Biotechnology Information (NCBI), the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the VectorNTI 9.1.0. program (Invitrogen Corporation) were used. The following problems were posed: the minimum number of dimers and secondary structures; the same annealing temperature of the primers; the minimum number of guanine and cytosine at the 3’ end of each of the primers; only absolute complementarity to primers and probes with the desired sequence is required for positive control, with the ability to synthesize PCR-product of about 100 nucleotide pairs in length; and the probe for PCR should not contain guanine at the 5’ end.

For the purpose of application in multiplex-PCR, the task was to design a unique positive control containing complementary nucleotide sequence to all oligonucleotide primers of the desired viruses.

When working with a sub-set of bees, sample preparation was carried out, including such steps as grinding, homogenization and extraction. For effective grinding and homogenization, bee samples were placed in a disposable polyethylene bag 40×40 mm, 2 ml of a 0.9% NaCl solution was added and, after incubation for 10 minutes at room temperature, frozen at -70 °C. The frozen

sample was ground with a pestle in a sterile ceramic mortar, then 200 µl of homogeneous biomass was taken out by a dispenser. Total 60 ready samples were obtained for further work.

Isolation of nucleic acids was carried out by the method of magnetic sorption with a set of reagents "MAGNO-sorb" version 100-200 (“AmpliSens” Federal Budget Institution of Science “Central Research Institute of Epidemiology”, Rospotrebnadzor) according to the manufacturer's instructions.

In order to exclude the influence of the DNA of the bee and other organisms that got into the sample, the PCR was tested with reagents for the results to detect the RNA-containing viruses we studied, eliminating the reverse transcription stage. Real-time PCR for DNA was performed using RT-PCR kit (Syntol, Moscow, Russia). The final volume of 15 µl PCR mixture contained: 1.5 µl of 25 mM $MgCl_2$ solution; 0.5 µl of 10 pM of each primer solution; 1.5 µl of 2.5 mMdNTP solution; 1.5 µl of 10x buffer EvaGreen+ for PCR; 0.5 µl of Taq polymerase; 5 µl of DNA extract and 3.5 µl of deionized water. PCR was carried out in real time on amplification platform C1000 with an optical module CFX96 (BioRad). The PCR program was as follows: (I) denaturation at 95°C for 2 min; (II) 40 cycles: 10 sec at 95°C, 30 sec at 55°C. The fluorescence was captured on the 55°C segment in each PCR cycle in the FAM channel.

In order to exclude the presence of the desired viruses in the bee samples, PCR was tested with reverse transcription. Real-time PCR for RNA was performed using RT-PCR kit (Syntol, Moscow, Russia) and MMLV reverse transcriptase (Evrogen, Moscow, Russia). The final volume of 15 µl PCR mixture contained: 1.5 µl of 25 mM MgCl2 solution; 0.5 µl of 10 pM of each primer solution; 1.5 µl of 2.5 mMdNTP solution; 1.5 µl of 10x buffer EvaGreen+ for PCR; 0.5 µl of Taq polymerase; 0,2 µl of MMLV; 5 µl of DNA extract and 3.5 µl of deionized water. PCR was carried out in real time on amplification platform C1000 with an optical reaction module CFX96 (BioRad). The PCR program was as follows: (I) reverse transcription at 37°C for 30 min; (II) denaturation at 95°C for 2 min; (III) 40 cycles: 10 sec at 95°C, 30 sec at 55°C. The fluorescence was captured on the 55°C segment in each PCR cycle in the FAM channel.

Results.

Oligonucleotide primer design. During the design of primers and probes, the intercalating probe was selected only for SBV, but for CBPV, BQCV and DWV, the EvaGreen dye was used (Table 1). For the broader functionality of the application of selected specific loci, a forward (F) and reverse (R) primers and probe (P) design was developed that allows PCR to be carried out at a single annealing temperature of the primers for the viral pathogens studied.

Table 1. Primers for PCR indication of bee viral disease pathogens

Name	5` -› 3`sequence	Annealing temperature	Amplicon length
SBVF	agaacgtccactacaccgaaatg	54.8°C	96 bp
SBVR	atagttccttctgtggtcttaattgaca	55.1°C	
CBPVF	atctacgggaatttatggtggaaa	54.9°C	85 bp
CBPVR	gataggtcttatgcacaaaaatccagt	55.3°C	
BQCVF	catgagaagaaccgagaagaaactc	54.6°C	84 bp
BQCVR	ggagggctcactttatctattgctt	55.5°C	

DWVF	gcatgggtgaaggaatgtctgt	55.3°C	90 bp
DWVR	cgcaaacactctctcgatgtactc	54.8°C	

Thus, for the indication and identification of the SBV, CBPV, BQCV and DWV genomes, the combinations of oligonucleotide primers with an optimum annealing temperature of 55.0 ± 0.5 °C were created.

Designed primer specificity analysis. The design of oligonucleotide primers for PCR is always associated with the risk of nonspecific amplification with a DNA heterogeneous to the studied pathogen. To exclude such probability, the nucleotide sequences of both each of the constructed primers, and a complex of forward and reverse primers were compared for the presence of similar sequences of the genetic code. The sequences analyzed were compared with all available sequences of all macro- and microorganisms. To exclude from the list the identified organisms of a certain virus, we used specialized search parameters, namely excluding from search the nucleotide sequences of certain viruses.

The SBV virus, as well as the other viruses studied in this work, is RNA-containing, and errors in the specificity of the produced indication of the given virus can be associated with the homogeneous nucleotide sequence of RNA of heterogeneous organisms. The nucleotide sequence of the reverse primer (SBVR) does not significantly resemble the genetic code of heterogeneous organisms. All direct primer homologies (SBVF) in the RNA sequence of heterogeneous organisms (7 species) having a homology of 68% do not affect the PCR results due to the lack of similar homology in the reverse primer and probe.

During the analysis of the direct primer (CBPVF) for the indication of CBPV, a homogeneous sequence was found in a number of DNA containing non-viral organisms with a specific genome coverage less than 90%. The reverse primer analysis (CBPVR) showed similar results for sequence homology, all organisms detected are DNA-containing and do not synthesize RNA with the sequence we used.

During the analysis of forward and reverse primers (BQCVF and BQCVR) for a BQCV indication, a homogeneous sequence was found for a number of organisms (homology less than 90%). The most significant homologies in the nucleotide sequence that can be noticed were identified in *Sus scrofa* DNA (forward and reverse primer homology), *Propithecus coquereli* mRNA (reverse primer homology), *Otolemur garnettii* mRNA (reverse primer homology), *HIV-1* RNA (reverse primer homology), *Apis cerana* mRNA (forward primer homology), and *Kryptolebia smarmoratus* mRNA (forward primer homology). Homology with both primers is found only in the genome *Sus scrofa* in the form of DNA.

The last analyzed complex of oligonucleotide primers are primers for the indication and identification of DWV. During the analysis of forward and reverse primers (DWVF and DWVR), a homogeneous sequence was found (with a degree of homogeneity of less than 86%) in a number of organisms. The most interesting were nucleotide sequences of RNA. A homologous to the direct primer composition of nucleic acids is found in the following organisms (the percentage of homology is shown in parentheses): *Acropora digitifera* (86%), *Dictyostelium fasciculatum* (81%), *Aegilops tauschii* (81%), *Poecilia reticulata* (81%), *Bombus terrestris* (77%), *Felis catus* (77%), *Ailuropoda melanoleuca* (77%), *Papio anubis* (77%), *Rhinopithecus bieti* (77%), *Gossypium arboreum* (77%), *Oryza sativa* (77%), *Kwoniella pini* (77%), *Cyprinus carpio* (77%), *Sugiyamaella lignohabitans* (77%).

Organisms characterized by a high degree of homology to DWVF do not have a similar homology to the composition of nucleic acids with DWVR, except for: *Felis catus* (66%), *Ailuropoda melanoleuca* (66%) and *Cyprinus carpio* (70%). However, for the successful amplification of nucleic acids of these biological species with the primers developed by us, there are a number of obstacles. As for *Cyprinus carpio*, in addition to the fact that this species does not have contact with the bee, there are other obstacles to the amplification of its RNA with the primers developed: low homology of the nucleotide sequences and homology of the forward and reverse primers are presented to different genes. *Ailuropoda melanoleuca* also has no contact with the bees of our zone, moreover, RNA indication is possible only when analyzing directly biological animal samples, in addition, the homogeneity of the forward and reverse primers is presented to different genes of *Ailuropoda melanoleuca*, which makes amplification, in this case, impossible. *Felis catus* are widely distributed and theoretically may contact with hives, however, as in the case of *Cyprinus carpio* and *Ailuropoda melanoleuca*, the homology of the forward and reverse primers belongs to different genes, and the isolation of *Felis catus* RNA is possible only from biological samples (RNA is a very unstable molecule and is rapidly destroyed outside the host cell).

Analyzing the nucleotide sequence of all primers (combination of forward and reverse ones), a significant degree of homology is not found, which cannot lead to an effective accumulation of amplification products. This fact indicates the success of the primer design stage.

Creating a universal positive control sample. As a result of PCR, the following amplicons are synthesized for the viruses we identify (according to the first DNA chain):

- for SBV:

agaacgtccactacaccgaaatgtccagtgatgagagtggacgaagaatctggaatgttagacgcgcagtgtcaattaagaccacag aaggaactat

- for CBPV:

atctacgggaatttatggtggaaattctgaaaataattgggatttctctgaatcatttactggatttttgtgtataagacctgtc

- for BQCV:

ggagggctcactttatctattgcttaaatcggtaagccacaaatttttctaagtgtcatgagtttcttctcggttcttctcatg

- for DWV:

gcatgggtgaaggaatgtctgtatttggatgatcccaaattcagaatgcgtcgagcgcatgatcaagagtatatcgagagagtgtttgcg

As a positive control of the reaction, it is not necessary to use the entire amplicon sequence, but sufficient to start and end the sequence that are complementary to the nucleotide sequence of the oligonucleotide primers. Linear application of sequences complementary to primers will create a too short portion of the target DNA. To increase the length of the amplified fragment, we applied a combination of positive sequences of different viruses. Thus, one positive control initiates the synthesis of a DNA chain of sufficient size, immediately for all viruses; the sequence of such a control sample is presented below.

As a positive control of amplification of the analyzed viruses, insertion of the developed nucleotide sequence into the vector "pAL2-T" was made; the plasmid map is shown in Figure 1.

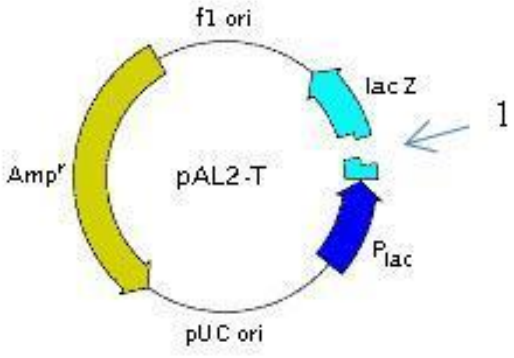

Fig. 1. pAL2-T plasmide map

1 – the universal nucleotide sequence inserted into the plasmid genome in the region of the β-galactosidase enzyme-encoding gene.

An insert to the selected vector was made in the form of a universal sequence that encodes all the amplifiable parts of the viruses of interest to us at once. The nucleotide sequence had the following nucleotide composition 5` -› 3`:

atctacgggaatttatggtggaaagaacgtccactacaccgaaatgcatgggtgaaggaatgtctgtcatgagaagaaccgaga agaaactcactggatttttgtgcataagacctatctgtcaattaagaccacagaaggaactatgagtacatcgagagagtgtttgcgaagcaat agataaagtgagccctcc

Therefore, this approach allows managing the correctness of the PCR setting with any of the analyzed bee viruses, and in the presence of the EvaGreen dye, the amplification is detected in real time. The length of the amplified fragment in the detection of each of the viruses is 120±5 b.p.

Polymerase chain reaction optimization. The practical implementation of the calculated PCR reaction may be often accompanied by some difficulties, associated with small differences in the calculated and real annealing temperature of the primers. To determine the optimum temperature range for annealing the primers, a reaction was carried out with a temperature 53.5-56.0°C (Fig. 2). The optimum annealing temperature for primers for the identification of the genomes of all detectable viruses of bees was 55.0°C. Amplification occurs also at other annealing temperatures of primers (± 1°C), but the most efficient accumulation of amplification products occurs at a temperature equal to the preliminary calculations.

To check the possible cross-reactions with the DNA of the bee, or the bacteria and fungi present on the body of the bee, PCR was set with the isolated nucleic acid preparation without reverse transcription (negative reaction). An artificial circular DNA molecule served as a positive control, with modification in the form of an insert of our amplified sequence. The results of amplification of bees DNA and artificial ring-shaped DNA molecule of SBV, CBPV, BQCV and DWV viruses by real-time PCR method showed that amplification occurs only with positive control DNA (Fig. 3).

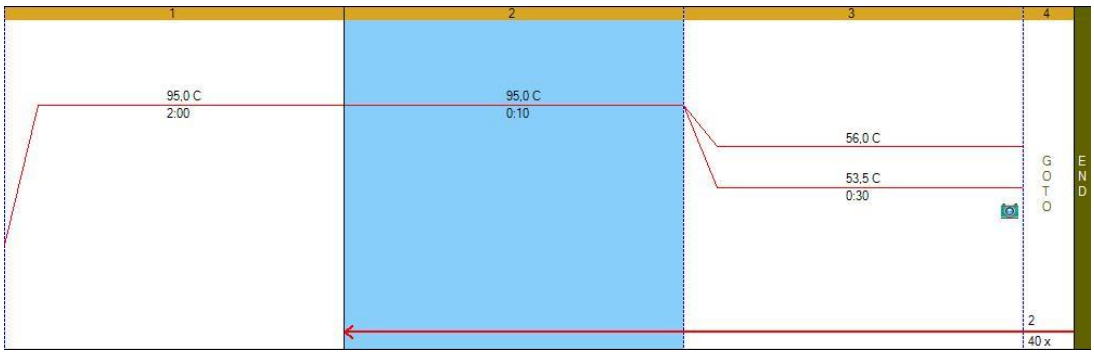

Fig. 2. PCR temperature range

Each series of cells of the amplifiers has its own temperature during the annealing of primers; the lower row of cells (H) has temperature of 53.5°C, the other cells are heated more as they move away from the "H" series toward the "A" series, where the annealing temperature of the primers has a maximum value (56.0°C).

The reverse transcription PCR showed similar results, which indicates the absence of RNA in the samples with a nucleotide sequence complementary to the primers used.

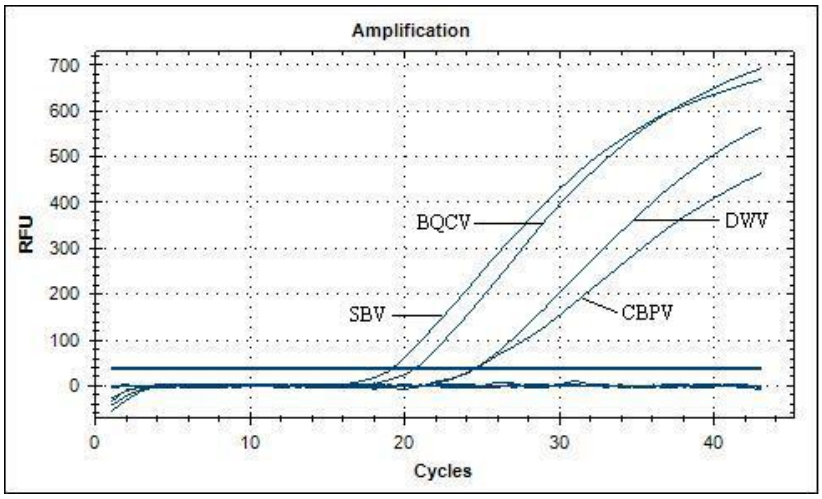

Fig. 3. Graph of the kinetic curve of fluorescence using the real-time PCR method for the detection of nucleic acids of Sacbrood virus (SBV), Chronic bee paralysis virus (CBPV), Black queen cell virus (BQCV), and Deformed wing virus (DWV).

Values above the set horizontal line correspond to positive results, below the line – to negative.

Conclusion. In the course of the research work the following conclusions were drawn:

1. Design and synthesis of primers were performed for Sacbrood virus, Chronic bee paralysis virus, Back queen cell virus and Deformed wing virus, which nucleotide sequence permits the simultaneous analysis of 4 virus targets in a single sample using a multiplex-PCR.

2. Most known strains and isolates of the said viruses are amplified with the designed complex of oligonucleotide primers, without any possible amplification of cross-reactions identified, which indicates their high specificity.

3. The designed universal positive control allows us to evaluate the reaction conditions during amplification of any of the viruses under investigation, which makes it suitable for multiplex-PCR.

ACKNOWLEDGEMENTS

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

References

1. Brutscher L.M., McMenamin A.J., Flenniken M.L. The buzz about honey bee viruses // PLoS Pathog. 2016. V.12(8). e1005757. doi: 10.1371/journal.ppat.1005757.
2. Khanbekova E.M., Rubtsova L.E., Babin Yu.Yu., et al. Viruses and parasites of *Apis mellifera caucasica* Gorb. as related to losses of honeybee colonies in Big Kaukas Mountings in Azerbaijan under different environmental conditions and location // Sel'skokhozyaistvennaya biologiya. 2013. V.6. P.43-54.
3. Mukminov M.N., Nikitin O.V., Ndayishimiye E.W., Shuralev E.A. Assessing the prospects for beekeeping development in Southern Province of Zambia // Veterinariya. 2015. V.10. P.49-52.
4. Ndayishimiye E.W., Nikitin O.V., Mukminov M.N., Shuralev E.A. Assess the suitability of the Copperbelt province of Zambia for the beekeeping management according to the level of polymetallic pollution // Russian Journal «Problems on Veterinary Sanitation, Hygiene and Ecology». 2015. V.3(15). P.80-84.
5. Bilalov F., Skrebneva L., Nikitin O., et al. Seasonal variation in heavy-metal accumulation in honey bees as an indicator of environmental pollution // Res J Pharm Biol Chem Sci. 2015. V.6(4). P.215-221.
6. Cavigli I., Daughenbaugh K.F., Martin M., et al. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination // Apidologie. 2016. V.47. P.251-266. doi: 10.1007/s13592-015-0395-5.
7. Giacobino A., Molineri A.I., Pacini A., et al. *Varroa destructor* and viruses association in honey bee colonies under different climatic conditions // Environ Microbiol Rep. 2016. V.8(3). P.407-412. doi: 10.1111/1758-2229.12410.
8. Bernardi S., Venturino E. Viral epidemiology of the adult *Apis mellifera* infested by the *Varroa destructor* mite // Heliyon. 2016. V.2(5). e00101. doi: 10.1016/j.heliyon.2016.e00101.
9. Tsevegmid K., Neumann P., Yañez O. The honey bee pathosphere of Mongolia: European viruses in Central Asia // PLoS One. 2016. V.11(3). e0151164. doi: 10.1371/journal.pone.0151164.
10. Tehel A., Brown M.J., Paxton R.J. Impact of managed honey bee viruses on wild bees // Curr Opin Virol. 2016. V.19. P.16-22. doi: 10.1016/j.coviro.2016.06.006.
11. Dolezal A.G., Hendrix S.D., Scavo N.A., et al. Honey bee viruses in wild bees: viral prevalence, loads, and experimental inoculation // PLoS One. 2016. V.11(11). e0166190. doi: 10.1371/journal.pone.0166190.

12. Forfert N., Natsopoulou M.E., Paxton R.J., Moritz R.F. Viral prevalence increases with regional colony abundance in honey bee drones (*Apis mellifera* L) // Infect Genet Evol. 2016. V.44. P.549-554. doi: 10.1016/j.meegid.2016.07.017.
13. Carrillo-Tripp J., Dolezal A.G., Goblirsch M.J., et al. In vivo and in vitro infection dynamics of honey bee viruses // Sci Rep. 2016. V.6. 22265. doi: 10.1038/srep22265.
14. Volykhina V.E. Deformed wing virus in *Apis mellifera* L.: prevalence, morphology, and pathogenicity // Sel'skokhozyaistvennaya biologiya. 2015. V.50(4). P.409-419.
15. Sprygin A.V., Babin Yu.Yu., Khanbekova E.M., Rubtsova L.E. Varroa destructor and a threat of viral infections of the honeybee (*Apis mellifera* L.) // Sel'skokhozyaistvennaya biologiya. 2016. V.51(2). P.156-171.
16. Gulukin M.I., Sotnikov A.N., Luchko M.A., et al. Clinical and laboratory diagnosis contagious diseases of the brood of bees // Veterinariya. 2016. V.4. P.25-27.
17. Kalashnikov A.E., Maslennikov I.V., Kolbina L.M., Udina I.G. Genetic differentiation of populations of honey bee (*Apis mellifera* L.) and distribution of RNA-containing viruses at the background of epizootia of *Varroa destructor* on the territory of Udmurtia // Sel'skokhozyaistvennaya biologiya. 2013. V.4. P.88-92.
18. Park C., Kang H.S., Jeong J., et al. In-situ hybridization for the detection of Sacbrood virus in infected larvae of the honey bee (Apis cerana) // J Comp Pathol. 2016. V.154(2-3). P.258-262. doi: 10.1016/j.jcpa.2015.12.003.
19. Ndayishimiye E.W., Khammadov N.I., Osyanin K.A., et al. Bioinformatic analysis of oligonucleotides for molecular genetic indication of honey-bee aspergillosis and ascospherosis pathogens // Veterinarny Vrach. 2015. V.2. P.3-9.

Alfiya Vasilevna KHAMMADOVA, Eduard Arkadevich SHURALEV, Nail Ildarovich KHAMMADOV, Bamanga Mamadou OUMAROU, Tagir Khadievich FAIZOV, Malik Nilovich MUKMINOV
"Design Of Primers For Identification Of Honey Bee Viruses In Multiplex-PCR", Astra Salvensis, V (2017), Supplement no. 1, p. 481-489
https://astrasalva.files.wordpress.com/2018/01/astra-salvensis-supplement-no-1-2017.pdf