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METAGENOMIC ALGORITHM FOR CREATING THE DIAGNOSTIC KITS FOR THE DETECTION OF RNA VIRUSES OF HONEY BEES

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ABSTRACT

Application of bio-information technology and analysis techniques is necessary for creation of a new generation of diagnostics. Establishment of such tests requires a new approach to selective parts calculations and selection of reagents. Example, presented in this work, allows being more effective on the way in identifying of retroviruses and analysis of their genomes. The use of this diagnosticum in practice found a high degree of infection of tribal and private apiaries in the Ryazan region.

Keywords: retroviruses, metagenomics, virus variability, *Apis mellifera*, mutation kinetics, diagnostics.

INTRODUCTION

Modern computational technologies are actively developing at the moment. This allows to solve a number of important problems of genetics. Analysis of homology of nucleotide, differences of protein sequences, identification of static and high-speed kinetic areas - are highly significant present day problems. The thermodynamic and factor analysis allows choosing automatically the oligonucleotides and fluorescent probes to specific areas of the genome of RNA viruses, which is also interesting.

The model of investigations of genetic variability of honeybee viruses in this paper was presented by the genomic study of KBV (Kasmir bee virus), ABPV (Acute bee paralysis virus), CBPV (Chronic bee paralysis virus), DWV (Deformed bee virus), SBV (Sacbrood bee virus), IAPV (Israel acute paralysis virus), BQCV (Black queen cell virus), SPV (Slow paralysis virus), the disease with these viruses is a highly contagious infection for honeybees. The infection occurs to a latent form, affecting on bee colonies [Berenuy O. *et al*, 2006, DeMiranda J.R., Genersch E. 2010]. For effective infection control it is necessary to develop new modern diagnostic techniques. Diagnosis of classical methods: (ELISA, RID), cannot provide sufficient diagnostic and analytical sensitivity [Зиновьева Н.А. и др., 2010].

MATERIALS AND METHODS

The work was performed at the Laboratory of Molecular Genetics of Animals in Ernst All-Russian Research Institute of Animal Husbandry using samples of 10 bee colonies (N = 30 individuals of adult bees were collected from tick-borne defeat apiaries in the Ryazan region of Russia). The metagenomic search and analysis of nucleotide sequences of viruses (N> 650 of nucleotide sequences which was included in GenBank database) were performed in the program Ugene 1.11.3, by using algorithms ClustalX and Muscle (data not shown, and available at [https://sites.google.com/site/aekalashnikoff/genomics-in-virusology-alexander-kalashnikov/list-of-publications-alexander-kalashnikov, & https://www.researchgate.net/profile/Alexander_Kalashnikov]. The difference between the new and previous methods is that the nucleotide sequences have been filtered according to the maximum differences by geographic origins of isolating, as well as the maximum genetic subdivision.

For each virus were identified several areas with low kinetics of mutations for which the design was done using the Primer algorithm 3.

The formation of dimers and secondary structures was tested using UnaFold (Table 1). BLAST (points) was calculated by the formula:

$$BSc = \frac{freq_{TargetPrimer1} + freq_{TargetPrimer2}}{N_{taxviuses}} + freq_{PRIMERBLAST} + Ufold + Tm$$,

where freq(N) is the number of matches without replacements, tax- the total number of sequences in the taxon, Ufold - a coincidence Ufold (0/1 points), PRIMER-BLAST is the number of matches without replacements in PRIMERBLAST, divided into the total number of matches.

Table 1.

The primer sequences and PCR conditions

Number	Name of Virus	The sequence of the primers5'-3' Forward primer, backward primer, product length, bp	Ta, ^{0}C	Tm, ^{0}C	BSc
1	ABPV	TCAACCAGGCTATAACCAAC	54.7	77.5	1.10
		TCTTGAAAATGCTTCACCAA, 157			
2	BQCV	CCGTTAGTCCTCAACAGACT	54.0	84.5	3.03
		AAACATGGAGCATAGTACGG, 130			
3	CBPV	ATTGTGAAGCCCAAAACCTG	56.8	72-85	1.50
		GAACCGCTTCGGTGGTAATA, 177			
4	KBV	GCAAATTGATGCTCCTAATG	56.8	82.0	1.11
		TCATAGTCTAATGGGGCAAG, 153			
5	DWV	ATATGCTTTTCCAGGTCCAT	55.0	80.4	1.18
		CTCGCTTCTTCTCTTCACTC, 204			
6	SBV	GGTGACCTTCATCCAGTATC	54.7	76.0	2.39
		GGGTTTCATTTTCGATAACC, 186			
7	IAPV	AGATGTAAGCATGCAGATCC	56.4	75.0	2.58
		CTGAGATCTTCAGGACCAAA, 105			
8	SPV	GTGGGCTGATAGGATGTTAC	56.4	75.0	4.00
		ATCAATGTTATGCGTGGAAG, 201			
9	ActB*	AGGAATGGAAGCTTGCGGTA	55-65 opt59	80.5	0.507
		AATTTTCATGGTGGATGGTGC, 182			

* [Chen Y.P. et al, 2005]

DNA isolation from adult bees was carried out individually by DNA sorb B kit (Central Research Institute of Epidemiology, Russia). Reverse transcription reaction was performed using MMLV RT kit (Evrogen, Russia) with the amount of the enzyme 50 U / reaction. Amplification was performed by using the algorithm with the time of primer annealing and product elongation of 30 and 45 seconds respectively (40 cycles) using the SYBR qPCRmix-HS kit (Evrogen, Russia) (New England Biolabs, England, [Mackay I.M., 2002]. The concentration of primers for the amplification of the reference and target genes was 100 nM, and 200 pmol respectively. SYBR fluorescence detection was determined by CFX Touch (Bio-Rad, USA) amplificator at a temperature starting from the melting temperature of a specific product. The average value of the relative amount of viral RNA and integrated proviral RNA were calculated using the Bio-Rad manual [Livak K.J., Schmittgen T.D., 2001]. DNA and RNA concentration was measured using a spectrophotometer Amersham Bioscience (Sweden).

RESULTS AND DISCUSSION

The specificity of all variants of the oligonucleotides in the system of scoring matches has been verified in the BLAST system. For the synthesis and PCR assembly the 35 pairs of primers were selected. Appropriate tests have been set up in the quantitative detection by SYBR system relative to the reference gene ActB. The amplification was performed for proviral DNA and RNA viruses in active form (after the reverse transcription step). Detection of fluorescence was performed starting from the formation temperature of the product in the calculated melting temperature (table 1). The analytical sensitivity of the test was amounted to 1024 times dilution (final concentration of 0.07 mg / l). The measured concentration of DNA samples after the separation was represented 196-1544 and 38-1344 of RNA ng / µl respectively. Later the samples were normalized to a concentration of 50 ug / ml. The

specificity of the primers was verified by melting a curve desired product and by results the electrophoresis in 2% agarose gel stained with Ethidium bromide (data not shown).

The test assays appeared more effective than previously developed for the detection of bee viruses COLOSS [www.coloss.org], detected 20-85% of positive specimens [Bereney M. *et al*, 2006, Калашников А.Е. и др., 2010; 2014]. The average frequency of the proviral DNA and RNA of DWV were not less than 75% and for CBPV more than 80% (Table 2).

Table 2.

The results of determination of an occurrence and the mean amount of RNA viruses

Samples	Name of virus	The frequency of the virus (N = 10 colonies, 30 samples)	The average relative amount of virus, R, p = 0.05
Proviral DNA detection			
1	ABPV	0.2	$3.98 \pm 2.01 \cdot 10^{-3}$
2	BQCV	0.4	$6.41 \pm 2.23 \cdot 10^{-6}$
3	KBV	0.8	$3.95 \pm 1.50 \cdot 10^{-2}$
4	SBV	0.9	$1.79 \pm 0.63 \cdot 10^{-3}$
5	IAPV	0.8	$3.02 \pm 1.23 \cdot 10^{-5}$
6	SPV	0.9	$3.41 \pm 1.68 \cdot 10^{-4}$
Viral RNA detection (as a cDNA, the active form of the virus)			
7	ABPV	0.7	$5.41 \pm 2.08 \cdot 10^{-1}$
8	BQCV	0.3	1.27 ± 0.63
9	KBV	1.0	$2.97 \pm 2.8 \cdot 10^{-1}$
10	SBV	0.9	$3.27 \pm 1.88 \cdot 10^{-2}$
11	IAPV	0.5	$1.66 \pm 1.17 \cdot 10^{-2}$
12	SPV	0.8	$1.81 \pm 1.07 \cdot 10^{-2}$
13	CBPV	0.8	$1.14 \pm 0.689 \cdot 10^{-2}$

Table 3.

Distribution of viruses in samples

Samples	DNA ABPV	RNA ABPV	DNA BQCV	RNA BQCV	DNA KBV	RNA KBV
3	7.18E-07	1.36E+00	5.04E-07	3.30E+00	1.87E-02	2.52E+00
6	*	6.11E-02			9.92E-02	8.33E-03
7			2.36E-06		1.18E-02	1.60E-02
8	7.96E-03				1.12E-01	1.02E-02
9		3.26E-02			2.79E-03	1.90E-02
15					4.73E-02	4.49E-02
17				5.16E-01	2.29E-03	2.00E-01
18		4.54E-01				1.45E-01
19			1.42E-05	7.54E-06		2.23E-03
20		1.73E-01	0.00E+00		2.28E-02	6.00E-03
	DNA SBV	RNA SBV	DNA IAPV	RNA IAPV	DNA SPV	RNA SPV
3	7.58E-06		1.54E-06			7.86E-02
6	1.37E-02	1.40E-04	4.57E-05		2.32E-03	1.12E-05
7		1.34E-02		1.98E-03	1.96E-04	
8	2.22E-03	1.01E-02	8.98E-05	3.48E-03	3.01E-04	6.94E-03
9	5.85E-05	3.04E-03	2.13E-06	2.42E-03	9.85E-05	1.82E-03
15	4.70E-05	1.09E-02	7.03E-05		3.94E-05	1.19E-03
17	1.74E-05	1.63E-01		7.48E-02	8.70E-06	3.77E-02
18	7.34E-05	5.59E-02	2.15E-05		5.17E-05	
19	7.40E-06	2.38E-04	8.27E-06	3.10E-04	2.74E-05	
20	1.69E-05	3.68E-02	2.51E-06		2.13E-05	2.14E-04

* zero values are not shown.

The distribution of various families of bees was excellent with samples of the proviral DNA and RNA and viral concentrations were differed in a sufficiently wide range (Table. 3). The degree of insertion of the viral RNA in the host gDNA is at least parity with the gDNA 10^{-3}-10^{-5}- and minimally, compared to the concentrations of RNA in tissues of the same bee families and individuals. This failure of individual bees as the host (and therefore, considering them further transmitters of infection) was very high. The active form of the virus was detected in the prevailing number of bee colonies and with greater concentrations.

Thus, in this study was verified a new universal way of implementation of metagenomic analysis sequences analysis of existing retroviruses bees in the form of a single database with the identification of areas of low kinetics and according to these specific primers new oligonucleotides were chosen to identify relevant viruses. The high efficiency of test creation was shown, as well as model study of native bee colonies of the apiaries to the quantitative determination of the virus was carried out.

Using of this test will allow to identify effectively previously unknown viruses subtypes, as well as carry out monitoring of apiaries in the shortest time for the quantitative detection of viruses.

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МЕТАГЕНОМНЫЙ АЛГОРИТМ СОЗДАНИЯ ДИАГНОСТИКУМОВ ДЛЯ ВЫЯВЛЕНИЯ РНК-СОДЕРЖАЩИХ ВИРУСОВ ПЧЕЛ

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АННОТАЦИЯ

Применение биоинформационных технологий и технологий анализа необходимо для создания диагностикумов нового поколения. Создание таких тестов требует как нового подхода к расчетам селективной части, так и подбору реагентов. Представленный пример позволяет пойти более эффективным путем при выявлении ретровирусов и анализа их геномов. Применение диагностикума на практике выявило высокую степень инфицированности племенных и частных пасек в Рязанской области.

Ключевые слова: ретровирусы, метагеномика, вариабельность вирусов, *Apis mellifera*, кинетика мутаций, диагностика