УДК 636.09:577.213:575.113:821.83:591.531.2

V. KATSYMON, Senior Research Fellow Department of Molecular Biology and immunology
M. KARPULENKO, Fellow Department of Molecular Biology and immunochemistry
O. GOLOVKO, Post-graduate student
State Scientific Control Institute of Biotechnology and strains of microorganisms, Kyiv

DEVELOPMENT OF DIAGNOSTIC PCR TEST SYSTEM FOR GENOME INDICATING OF DISTEMPER VIRUS

Based on the selected 3 pairs of oligonucleotide primers was constructed PCR test system for indicating the genome of distemper virus, which is characterized by high sensitivity and specificity, and allows to obtain objective results compared with commercial counterparts.

Canine distemper (*Pestis canum*, *Feebris catarrhal infeciosa canum*, plaque) – acute highly contagious disease of carnivorous, which characterized by fever, inflammation of the mucous membranes, pneumonia, dermal rash, conjunctivitis, catarrhal inflammation of the gastrointestinal tract and damage to the nervous system [6]. Animals of all ages are susceptible, especially young. Disease registered at any time of year but in fall and spring often occurs in the form of epidemic diseases. Mortality ranges from 60 to 90 %.

Distemper is known since the time of animals' domestication, and now a wide range of virus lesions encompasses not only dogs but other predators. In vivo 8 from 11 families of Predators squad are susceptible to the distemper infection.

Distemper virus resistant to various physical and chemical factors [7].

The virus has affinity with man measles virus, and one-way antigenic relationship with bovine virus [5]. In immunobiological relation different strains of distemper virus, isolated from sick dogs in different geographical areas, are uniform and differ only in virulence [1].

Pressing task for veterinarians are to find new therapeutic and prevention methods for canine distemper, and a major role in the effective treatment plays timely diagnosis of the disease.

The diagnosis of distemper based on epidemiological data, clinical signs, micro-and macroscopical lesions, and results of laboratory investigation [2].

For the diagnosis of distemper is used laboratory diagnostics, conduct which facilitates the identification of pathogen antigens and antibodies [4].

Current serological methods (HI, CF tests and others) do not give stable results in the diagnosis of distemper [3]. Moreover recent diagnosis a bit complicated, since active immunization of animals has contributed abrasion view of some characteristic features of the disease. Therefore we were tasked to develop a new rapid method of diagnosing distemper.

Objective – to develop a diagnostic PCR test-system for indication of distemper virus genom in biological material of different origin.

MATERIALS AND METHODS

In developing specific primers to detect the virus Canine Distemper Virus (CDV) by PCR have been used the database of GenBank, EMBL (European Molecular Biology Library), DDBJ (Japanese database of nucleotide sequences), PDB sequences.

According to the literature were highlighted several marker sequences: «highly conserved region of the NP gene of the Ond-CDV strain» [8] and «consensus sequence of 55 gene H», which are suitable for the development of specific primers, from which in further work was selected area «highly conserved region of the NP gene» RNA CDV.

Then we searched for nucleotide sequences «conserved region of the nucleocapsid protein N gene» by CDV RNA for further analysis of their variability, and the search for conserved areas for the primers determination. Using the computer program «Vector NTI» and «PerlPrimer» developed several pairs of primers, from which was selected one pair CDV F5 (direct primer) and CDV R6 (reverse primer) and through Internet (program BLAST) tested their specificity. The critical homology with nucleotide sequences of other groups of bacteria, viruses, and eukaryotes have not been identified.

PCR was performed on four channel thermocycler «Tertsyk» production firm «DNA technology» (Russia, Moscow). The reaction mixture was placed 25 ml 67 mM Tris-HCl (pH 8,8), 16,6 mM $(NH_4)_2SO_4$, 2,0 mM $MgCl_2$, 0,01 % Tween-20, 100 mmol dATF, dGTP, dTTF, dTsTF, 50 pmol of each specific primer, 2 units Taq polymerase, 5 mkl samples of the selected cDNA. To prevent evaporation of the sample in each reaction mixture were layered on top 30 ml of mineral oil. Amplification consisted of 35 cycles. Each amplification cycle consisted denaturation of cDNA at 950 °C for 45 seconds , annealing of primers at 580 °C – 30 seconds, the synthesis of complementary chains at 74 °C – 40 seconds (at last cycle this stage was extended to 5 minutes). Detection of reaction products was performed by electrophoresis in 1,5 % agarose gel (ethidium bromide stained) using tris-borate buffer at a voltage gradient of 10 V/cm. The results were evaluated by viewing the gel after electrophoresis on transilluminator under UV light in the presence (or absence) of red- orange nucleic acid fragment size. The specificity of the amplified nucleic acid fragment was determined by its position (size) relative to the standard marker fragments.

RESEARCH RESULTS

A key step in the creation of highly specific PCR diagnostics to detect Canine Distemper Virus (CDV) (as well as in the

УВАГА! ТРИВАЄ ПЕРЕДПЛАТА НА ЖУРНАЛ НА 2014 РІК!

© V. Katsymon, M. Karpulenko, O. Golovko, 2014

Table 1 – **Oligonucleotides used for synthesis**

Name	Sequence (5' –> 3')	Fragment size	Target
CDV F1	ACAGGATTGCTGAGGACCTAT	287	Highly conserved region of the NP gene of the Ond-CDV strain
CDV R2	CAAGATAACCATGTACGGTGC		
CDV F3	TTCTG AGGCA GATGA GTTCT TC	829	Conserved segment of CDV N gene (Onderstepoort)
CDV R4	CTTGG ATGCT ATTTCTGACA CT		
CDV_F5	AGGAGCAAGTTTGGATTCTGAGG	827	Nucleocapsid protein N gene
CDV_R6	GACACTAGCTGAGCCTCTTCC		

creation of other PCR diagnostic) is the sequence selection for oligonucleotide primers. This stage is based on the previous studying of literature, Internet data and further direct development of oligonucleotide primers pair, using special software. Oligonucleotide pair must meet certain requirements:

– have a high specificity for binding with strictly defined areas of the pathogen genome;

– have a similar temperature annealing and constant binding to nucleic acid under certain conditions of PCR;

– not create rigid secondary structures;

– not be complementary to each other.

3 pairs of oligonucleotide primers were synthesized, including (for the control proprietary primers) outlined – CDV F1 and CDV R2 [8]; CDV F3 and CDV R4 [9] and proprietary – CDV F5 and CDV R6 (Table 1). Synthesis of primers on our order executed in the firm «Liteh» (Russia, Moscow).

The check of primers first was carried out using annealing rate – 55 °C and 60 °C (Figure 1). The results of the studies show satisfactory properties of proprietary primers pair – CDV F5 and CDV R6. Operating annealing temperature of primers was selected 58 °C.

Sensitivity was determined in dilution titer of Virus febris contagiosae canis (Figure 2).

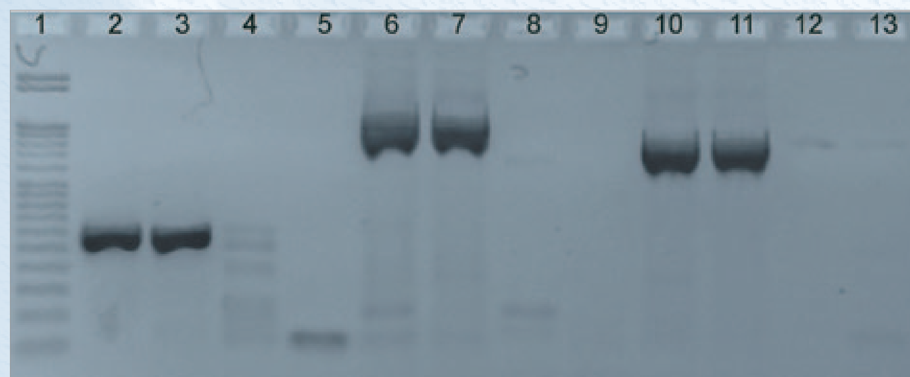

Fig. 1. Testing of annealing temperature
1 – molecular weight marker (GeneRuler 50 bp DNA Ladder); **2–5** – praimers ***CDV F1 i CDV R2;*** **6–9** – praimers ***CDV F3 i CDV R4;*** **10–13** – praimers ***CDV F5 i CDV R6.***
2, 6, 10 – PKZ (T=**55** °C, *Virus febris contagiosae canis* (strain CDVU 39) min $10^{4,2}$ $TCID_{50}$);
3, 7, 11 – PKZ (T=**60** °C, *Virus febris contagiosae canis* (strain CDVU 39) min $10^{4,2}$ $TCID_{50}$);
4, 8, 12 – blood serum; **5, 9, 13** – saline

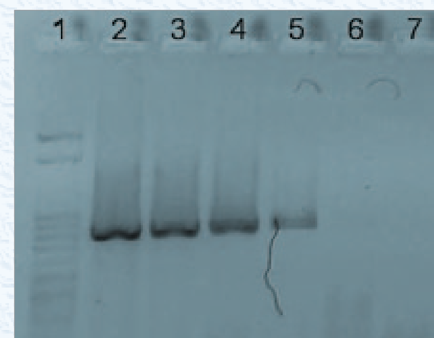

Fig. 2. Sensitivity determination
1 – molecular weight marker (GeneRuler 50 bp DNA Ladder); **2** – *Virus febris contagiosae canis* $10^{4,7}$ $TCID_{50}$; **3** – *Virus febris contagiosae canis* $10^{3,7}$ $TCID_{50}$; **4** – *Virus febris contagiosae canis* $10^{2,7}$ $TCID_{50}$; **5** – *Virus febris contagiosae canis* $10^{1,7}$ $TCID_{50}$; **6** – *Virus febris contagiosae canis* $10^{0,7}$ $TCID_{50}$; **7** – negative control

Table 2 – **Validation of developed primer pairs**

Vaccine name	Strain	CDV F5 CDV R6	Polichum	CITO TEST CDV Ag
Biocan PPUPY – D	CDVU 39	+	+	+
Biocan PPUPY	CDVU 39	+	+	-
Biocan DHPPI	(Unknown)	+	+	-
Мультикан-4	Штам № 37	+	+	+
Мультикан-8	(Unknown)	+	+	+
Nobivac Puppy	Onderstepoort	+	+	+
Nobivac DHPPI	Onderstepoort	+	+	+
Duramune Max 5 CvK\4L	Onderstepoort	+	+	+
Vanguard plus 5\L	Snyder Hill	+	–	+
The solvent for vaccine «Bio-Test Laboratory»	NKZ	–	–	–

It was also tested the annealing temperature on pathological material (data not shown).

Conducted validation of the developed primer pairs compared with a commercial PCR test system «Polichum» (produced by «Amplisens», Russia) and the serological test system «CITO TEST CDV Ag». Results of the study are presented in Table 2.

Currently the developed test systems is under further examination with pathological material, and the development of regulatory documents for the purpose of registration of the test system in Ukraine is in progress.

CONCLUSIONS

1. As a result of the work developed primers for the detection of distemper based on the polymerase chain reaction.

2. Optimal annealing temperature of primers is 58 °C.

3. Elobarated test-system is highly sensitivity – $10^{1,7}$ $TCID_{50}$ *Virus febris contagiosae canis.*

4. A comparison of the developed test system with the PCR test system «Polichum» (produced by «Amplisens», Russia) and the serological test system «CITO TEST CDV Ag» based on which it is at least not inferior to commercial test systems.

LITERATURE

1. **Белов А.Д.** Болезни собак / А.Д. Белов, Е.П. Данилов и др. – М.: Колос, 1995. – С. 259–270.
2. **Гертман М.И.** Чума плотоядных / М.И. Гертман // Диагностика, лечение и профилактика инфекционных болезней собак / Составитель Ф.Г. Гизатуллина. – Челябинск: Челябинский Дом печати, 1998. – С. 17–23.
3. **Игнатов П.Е.** Очерки об инфекционных болезнях у собак / П.Е. Игнатов. – М.: Валта, 1995. – С. 13–41.
4. **Притулин П.И.** Экспресс-иммунологические методы при индикации и диагностике инфекционных болезней животных / П.И. Притулин, Н.В. Привалова // Сб. науч. тр. ВИЭВ. Актуальные проблемы ветеринарии в промышленном животноводстве. – 1984. – Т. 60. – С. 35–41.
5. **Слугин В.С.** Чума плотоядных / В.С. Слугин // Эпизоотология и инфекционные болезни; под ред. А.А. Конопаткина. – 2-е изд., перераб. и доп. – М.: Колос,1993. – С. 620–623.
6. **Сюрин В.Н.** Диагностика вирусных болезней животных / В.Н. Сюрин, Р.В. Белоусов, Н.В. Фомина. – М.: Агропромиздат, 1991. – С. 228–237.
7. **Шуст Д.Ф.** Справочник собаковода-любителя / Д.Ф. Шуст, А.Д. Носкова, Т.В. Третьяк. – К.: ГТГОО «А.С.К.», 1993. – С. 90.
8. **Frisk A.L.** Detection of Canine Distemper Virus Nucleoprotein RNA by Reverse Transcription-PCR Using Serum, Whole Blood, and Cerebrospinal Fluid from Dogs with Distemper / A.L. Frisk, M. König et al. // Journal of Clinical Microbiology. –1999. – Nov. – P. 3634–3643.
9. **Wang F.X.** Differentiation of canine distemper virus isolates in fur animals from various vaccine strains by reverse transcription-polymerase chain reaction-restriction fragment length polymorphism according to phylogenetic relations in china / F.X. Wang et al. // Virology Journal. – 2011. – Vol. 8. – P. 85.

Одержано 6.02.2014

Розроблення діагностичної тест-системи ПЛР для ідентифікації геному вірусу чуми м'ясоїдних. *В.В. Кацимон, М.С. Карпуленко, О.А. Головко*

На основі підібраних трьох пар олігонуклеотидних праймерів сконструйована тест-система ПЛР для індикації геному вірусу чуми м'ясоїдних, яка характеризується високою чутливістю та специфічністю і дозволяє отримувати об'єктивні результати порівняно з комерційними аналогами.

Разработка диагностической тест-системы ПЦР для идентификации генома вируса чумы плотоядных. *В.В. Кацимон, М.С. Карпуленко, О.А. Головко*

На основе подобранных трех пар олигонуклеотидных праймеров сконструирована тест-система ПЦР для индикации генома вируса чумы плотоядных, которая характеризуется высокой чувствительностью и специфичностью и позволяет получать объективные результаты по сравнению с коммерческими аналогами.

З версією статті українською мовою можна ознайомитися на web-сторінці журналу – **http://vmu.org.ua**

УВАГА! ТРИВАЄ ПЕРЕДПЛАТА НА ЖУРНАЛ НА 2014 РІК!