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Development of highly pathogenic avian influenza and Newcastle disease surveillance means on the basis of molecular and genetic techniques

B. T. Stegniy, A. P. Gerilovych, A. B. Stegniy

National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine"
83, Pushkinska Str, Kharkiv, Ukraine, 61023

e-mail: antger@vet.kharkov.ua

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Highly pathogenic avian influenza and Newcastle disease pose considerable danger to the countries with developed poultry farming, especially in the circumstances of current complicated worldwide epizootic situation regarding these diseases. **Aim**. This study is aimed at creating a system of methods to identify RNA of influenza and Newcastle disease viruses on the basis of PCR. **Methods.** The methods of molecular diagnostics and bioinformatics were used. **Results**. The system of primers and the methodology for the identification of RNA of influenza A virus (for the M-gene), highly pathogenic avian influenza virus subtype H5N1 in *HA* and *NA* genes, Newcastle disease virus with the amplification of highly variable region gene of *F* have been elaborated. **Conclusions**. The elaborated techniques proved to be effective in the study of both reference and field samples.

Keywords: virus, highly pathogenic avian influenza, Newcastle disease, polymerase chain reaction.

INTRODUCTION

The highly pathogenic avian influenza (HPAI) and Newcastle disease are some of the most contagious diseases and the most impressive causes of economic losses among the viral infections of numerous species of poultry and fowl of all the age groups.

In three recent years the panzootia of avian influenza covered almost the entire Euro-Asian continent. The current epizootic danger is conditioned by the circulation of highly pathogenic variants of influenza A virus subtype H5N1. Considering high instability of the genetic organization of the virus, the genome of which is presented by eight RNA regions, there is a potential risk of rapid variability and recombination of the causative agent, which results in the changes in its antigen structure and diminishes the efficiency of using the vaccine preparations from the previously isolated strains.

The scientists of different countries have noted the presence of several groups of isolates, different in the nucleotide content of the genes of hemagglutinin, neuraminidase, and the main viral tegument [1, 2]. The division of isolates into the corresponding subtaxons by the qualitative content of their genetic material allows, firstly, getting an idea on the ecologic relations in the virus-host system to study the migration ways for the virus transmitters and to predict the epizootic situation, secondly, elaborating efficient molecular and genetic test-systems for the indication and identification of RNA of the causative agent, and, thirdly, creating the strategy of elaborating the means of controlling the disease and eliminating the epizootic sources.

The highly pathogenic avian influenza and Newcastle disease are closely related in the epizootic respect, including the fact that current escalation of the situation is often conditioned by the associated clinical course, the cases of which are described for fowl. Pursuant to the abovementioned, the World Organization for Animal Health recommends having parallel studies to reveal such mixed infections [3].

NSC "Institute of Experimental and Clinical Veterinary Medicine" has been conducting surveillance studies of avian influenza and Newcastle disease in fowl for almost 10 years. A number of epizootic isolates of avian influenza agents were identified during the outbreaks of this disease in the AR of Crimea, which were identified as the representatives of highly pathogenic subtype H5N1 and of Newcastle disease virus.

Our studies have demonstrated that the application of traditional virologic and serological tests is not always sufficient.

This study was aimed at creating a system of methods to identify RNA of influenza and Newcastle disease viruses on the basis of PCR.

MATERIALS AND METHODS

The samples of strains, obtained by the embryonic cultivation of influenza A virus/chicken/Sivash/02/2005/H5N1 and influenza A virus/chicken/Primorsky/01/2006/H5N1 of the highly pathogenic avian influenza virus (HPAIV), isolated during the disease outbreak of chickens in the AR of Crimea, which were inactivated with formalin and β-propiolactone, as well as the samples of embryo-adapted virus – antigen-vaccine AviFluBac-IECVM with the titer of at least 9 $\log_2$ in the hemagglutination reaction, Newcastle disease viruses, strains LaSota (replicating viral vaccine, Lohmann AH GmbH) and LG-85 were used in the work. To test the specificity of the method we used the samples of cDNA of the reference strains of influenza A viruses (H1, H2, H4, H5 (N1 and N3), H7, H10) and B, as well as Gumboro disease, obtained from the collection of the Department of Poultry Diseases, NSC "IECVM".

To create the databases of the sequences of the main genes and RNA fragments of influenza virus and the genomic RNA of Newcastle disease virus, were retrieved from the international databases of EMBL, DDBJ and GenBank, as well as the sequences of the isolates of influenza A virus and Newcastle disease virus, and later divided into subdatabases. Each of them contained only the information on the nucleotide content of the genes of hemagglutinin *(HA)*, neuraminidase (*NA)* and M virus of influenza as well as gene *F* of Newcastle disease virus.

The multiple alignment of sequences, the indices of region entropy, the character of substitutions and the search for conservative regions were performed using BioEdit program v.5.2.9. and its modules – ClustalW and Neighbor.

The conservative regions, identified while analyzing the structure of genes M, hemagglutinin, neuraminidase of influenza A virus and gene *F* of Newcastle disease virus, were analyzed for the presence of probable primer pairs using AmplyX program v.1.1.2 for Win98. The parameters of sequence quality and the optimization regime of the amplification thermocycles were analyzed using OLYGOSoft software package.

The viral RNA was isolated using the commercial set RIBO-sorb-50, manufactured by the Central Scientific Research Institute of Epidemiology of the Russian Directorate for Consumer Rights and Health Protection (RF). The isolated RNA samples were used as a matrix for the ground work on cDNA. The polymerase chain reaction with reverse transcription (RT-PCR) was conducted using the revertase (Fermentas, Lithuania) and Reverta-L set, manufactured by the Central Scientific Research Institute of Epidemiology of the Russian Directorate for Consumer Rights and Health Protection (RF).

The amplification parameters for genes of hemagglutinin and neuraminidase were worked out and improved using the basic AmpliSense K-200 sets of the Central Scientific Research Institute of Epidemiology of the Russian Directorate for Consumer Rights and Health Protection (RF) and Master-Mix (Fermentas, Lithuania). The ratio of introducing the cDNA sample, the concentration of magnesium ions, polymerase, and dNTP, the total volume of the mixture, the temperature of annealing and the number of amplification cycles were empirically selected.

The testing of sensitivity and specificity of the methods was performed using the strains of influenza A virus, which are heterological regarding hemagglutinin and neuraminidase, as well as the samples of biomass of RNA-containing viruses of poultry.

RESULTS AND DISCUSSION

The Bioedit program was used to study the degrees of homogeneity and variability of the sequences of *HA* gene of 98 isolates and the gene HA 87 of the isolates of avian influenza virus, isolated during the main epizootic outbreaks of 1967−2005, registered in the Euro-Asian continent.

The conservative regions of *HA* and *NA* genes were analyzed for the availability of the primer pairs. AmplyX v.1.1.2, the program for primer design, was used to select 18 and 12 possible primer pairs, respectively. The next stage involved the selection of 4 pairs of primers with the PCR-quality of 80–100 % (not having dimers, hairpins, with the GC-pairs ratio of 50 %).

The study of their specificity was conducted in silico applying of all the pairs and *HA* and *NA* genes of 55 strains of subtype H5N1. It was established that only three pairs (two AivH5 and one AivN1) are capable of

amplifying with all the sequences and flanking regions 487 and 425 bp of *HA* and *NA* genes, respectively.

The study of the specificity of the system of developed primers AivH5 demonstrated that the samples of total cDNA of heterogeneous controls (total cDNA of chicken embryos, infected with the strain LaSota of Newcastle disease virus, UM-93 (Gumboro disease virus and influenza viruses of other subtypes which are a part of Flu Vaccine) did not form any stripes after the reaction.

On completing the laboratory testing of the method, the latter was used to create "Test-system for detection of RNA of highly pathogenic avian influenza virus" (TR U 24.4-00497087-060:2007). The commission testing of the test-system for specificity, sensitivity and absence of cross-reaction while studying the material, infected with foreign viruses, involved the following samples:

extra amniotic fluid (EAF) of intact chicken embryos;

EAF of chicken embryos, infected with the strain of influenza A virus/chicken/Sivash/02/06/H5N1 with the titer in RGA 1:16, 1:256 and 1:512;

EAF of chicken embryos, infected with the strain of influenza A virus/tern/South Africa/61/H5N3, heterological regarding neuraminidase,

EAF of chicken embryos, infected with strains of influenza A virus, heterological regarding hemagglutinin and neuraminidase: A/duck/Alberta/60/70/H12N5,

A/chicken/Russia/5/87/H7N1,

A/chicken/Germany/N/49/H10N7,

A/duck/Czech Republic/56/H4N6,

A/pig/Iowa/H1N1;

heterological causative agents:

Newcastle disease virus, Gumboro disease virus, samples of which are kept in the Department of Poultry Disease Study.

The specificity was determined by the number of determined negative samples, and the sensitivity – by the number of positive samples, determined correctly. The index of reaction reproducibility was the reproduction of the obtained results twice with the matches in all the samples.

After the amplification we found the presence of specific amplicons in three samples of EAF from chicken embryos, infected with influenza virus subtype H5N1, and in the track of the positive control sample (total RNA from the inactivated EAF). RNA samples from the material of intact embryos did not form the amplicons after the amplification.

The samples of total RNA of heterogeneous controls did not form specific amplicons after the amplification.

The relative regulatory standards for the test-system have been executed; the interdepartmental approbation of diagnosticum is in progress in SSCIBSM. The elaborated method was protected by the Declaration patent of Ukraine. Patent for Utility Model No. 18727 Ukraine, IPC 2006 C12N7 / 00. Method of identification of cDNA strains of highly pathogenic avian influenza H5 by amplification of specific areas of HA-gene [Text] / Gerilovich A. P., Stegniy B. T.; NSC «IECVM». – No. u 200605945; appl. May 29, 2006; publ. Nov. 15, 2006. Bul. No. 11. −6 p.

The amplification of the 425 bp long region of *NA* gene with the system of primers AivN1 with positive and intact samples demonstrated the presence of specific amplicons in 5 investigated samples of EAF of embryos, infected with HPAI, as well as in the track of the positive control sample of cDNA of HPAI virus (living virus of the strain of influenza virus A/chicken/Sivash/02/06/H5N1, as the neuraminidase gene of formalin-inactivated virus is not amplified contrary to the gene of hemagglutinin). The samples of cDNA from the clinical material of intact embryos did not form the amplicons after the amplification. The specificity of developed primers was proven by the absence of the formation of PCR-products during the study of the panel of heterological samples.

The design of specific primers for the indication of influenza A virus involved the analysis of 28 sequences of M gene, which was the target gene in the study of the method. The primers obtained were called IV_A_M_f and r. It was established that they are specific regarding cDNA of influenza A virus of seven subtypes (H1, H2, H4, H5 (N1 and N3), H7, H10), flanking the region of 560 bp. The specificity of the primer system and the method, elaborated on its basis, were proven by the determination of cDNA region of the calculated length in the samples of seven subtypes of influenza A virus and its absence in the samples of genetic material of influenza B virus, Newcastle disease and infectious bursal disease.

The final stage of our studies was the determination of promising sequences and the elaboration of PCR-method to identify RNA of Newcastle disease virus.

The analysis of all the investigated sequences after the alignment demonstrated the presence of numerous small (3−15 bp) and five large (over 15 bp) conservative regions in the structure of *F* gene of Newcastle disease virus. The average entropy of the investigated regions was in the range of 0.024–0.058.

The analysis of these conservative regions demonstrated that two of the identified regions have the length, minimal for the primer design. The results of the screening revealed a high degree of palindrome nature of some conservative regions for the nucleotide sequence of *F* gene, which is the reason of probable formation of secondary non-canonic structures (including two variants of hairpin-formation with a large loop of 7−8 bp).

Taking into consideration the presence of only one promising region for the search of primers, where theoretically secondary structures are negligible, we decided to have the design only on complete sequences of *F* gene, involving the degenerated zones. The analysis of conservative regions of the gene allowed revealing 8 primer pairs, among which we selected the most promising pair that is flanking the 345 bp long region of *F* gene of Newcastle disease virus. This pair (NDV fusion FF/RR) has high parameters of PCR-quality regarding the dimerization and self-hybridization, does not form any secondary non-canonic structures, and is remarkable for high ratio of GC-pairs and the melting temperature of 64 °C.

The empirical selection of the parameters demonstrated that the method retains its maximal sensitivity and specificity at the annealing temperature of 62 °C and the concentration of magnesium ions in the reaction mixture at the level of 2.5–3.0 mM/ml.

The analysis of the method specificity demonstrated the ability to amplify only for cDNA of Newcastle disease virus and the inability to react with cDNA of influenza A and B viruses as well as cDNA of Gumboro disease virus (Fig. 2).

The elaborated methods were tested using about 500 samples of fowl and poultry, demonstrating 32 samples, positive for influenza A virus, 12 of which were positive for subtype H5N1, and 6 identified isolates of Newcastle disease virus. The genotyping of the mentioned viruses regarding the elaborated amplicons revealed that the influenza viruses belong to the European and Asian genetic lines, while

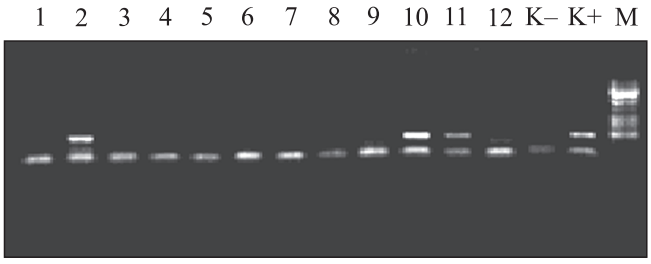
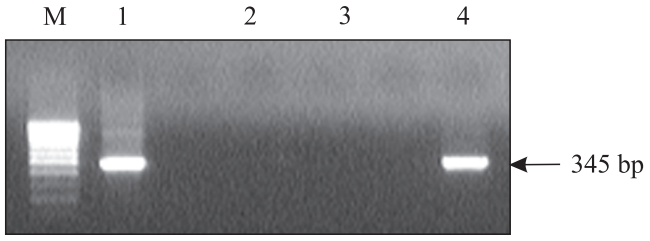


Fig. 1. Electrophoregram of PCR results: 1 − EAF of intact chicken embryos; 2, 10, 11 − cultivation of strain A/chicken/Sivash/02/06/H5N1 in dilutions 1:16; 1:256 and 1:512, respectively; 3 – RNA of cultivated strain of influenza A virus /tern/South Africa/61/H5N3, 4 − RNA of cultivated influenza A virus/duck/Alberta/60/70/H12N5; 5 − A/chicken/Russia/5/87/H7N1; 6 − A/pig/Iowa/H1N1; 7 − A/chicken/Germany/N/49/H10N7; 8 − A/duck/Czech Republic/56/H4N6; 9 − Newcastle disease virus; 12 − Gumboro disease virus



K+ – LaSofa 1–3 – influenza viruses

4 – newcastle disease virus

Fig. 2. Results of testing the specificity of primers to *F* gene of Newcastle disease virus

Newcastle disease virus belongs to genotypes 2, 4 and 5.

CONCLUSIONS

Primers AivH5, AivN1, IV-A-M, remarkable for high indices of PCR-quality and flanking highly variable regions of genes *HA*, *NA* and M of influenza virus, 487, 425 and 560 bp, respectively, have been developed. They are suggested for the diagnostics of influenza and identification of isolates N1- and H5-subtypes.

The developed primers are suitable for the identification of RNA of Newcastle disease virus regarding *F* gene (345 bp long region).

The approbation of the methods using the samples of the genetic material of homologous and heterological viruses of poultry demonstrated high specificity of the elaborated methods. The study of 200 samples of the clinical material of fowl and poultry allowed identifying RNA of influenza virus A − in 12 samples, includ-

ing subtype H5 – in 7, as well as RNA of Newcastle disease virus – in 6.

We plan on further application of the developed tests for wide surveillance studies and on enhancing their molecular and epizootic relevance by deeper elaboration of the methods of analyzing sequences (the study of polymorphism of the site of cutting *HA*-gene of HPAI and the hypervariable region of *F* gene of Newcastle disease virus) on their basis.

Створення засобів моніторингу високопатогенного грипу птиці та ньюкаслської хвороби на основі молекулярно-генетичних технологій

Б. Т. Стегній,
А. П. Герілович, А. Б. Стегній

e-mail: antger@vet.kharkov.ua

Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини»

Вул. Пушкінська, 83, Харків, Україна, 61023

Високопатогенний грип птиці та ньюкаслська хвороба представляють значну небезпеку для країн з розвиненим птахівництвом, особливо за умов сучасної складної епізоотичної ситуації щодо цих захворювань в усьому світі. **Мета.** Створення системи методик з виявлення РНК вірусів грипу та ньюкаслської хвороби на основі ПЛР-аналізу. **Методи**. Використано методи молекулярної діагностики та біоінформатики. **Результати.** Запропоновано праймери і розроблено методики виявлення РНК вірусу грипу А (за М-геном), вірусу високопатогенного грипу птиці субтипу H5N1 за генами НА та NA, вірусу ньюкаслської хвороби за ампліфікацією високоваріабельної ділянки гена F. **Висновки.** Створені методики виявились ефективними як при дослідженні референтних, так і польових зразків.

Ключові слова: вірус, високопатогенний грип, ньюкаслська хвороба, полімеразна ланцюгова реакція.

Создание средств мониторинга высокопатогенного гриппа птицы и ньюкаслской болезни на основе молекулярно-генетических технологий

Б. Т. Стегний, А. П. Герилович, А. Б. Стегний

e-mail: antger@vet.kharkov.ua

Высокопатогенный грипп птицы и ньюкаслская болезнь представляют значительную опасность для стран с развитым птицеводством, особенно в условиях современной сложной эпизоотической ситуации по этим заболеваниям во всем мире. **Цель.** Создание системы методик по выявлению РНК вирусов гриппа и ньюкаслской болезни на основе ПЦР-анализа **Методы.** Использованы методы молекулярной диагностики и биоинформатики. **Результаты.** Предложены системы праймеров и созданы методики выявления РНК вируса гриппа А (по М-гену), вируса высокопатогенного гриппа птицы субтипа H5N1 по генам *HA* и *N*A, вируса ньюкаслской болезни с амплификацией высоковариабельного участка гена F. **Выводы.** Созданные методики оказались эффективными как при исследовании референтных, так и полевых образцов.

Ключевые слова: вирус, высокопатогенный грипп, ньюкаслская болезнь, полимеразная цепная реакция

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