У наступних дослідженнях рекомендовано використовувати метод ізотермічної ампліфікації нуклеїнових кислот у польових умовах. Це дасть змогу своєчасно контролювати занесення збудника на територію країни.

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Test of method of isothermal amplification of nucleic acids of virus of bird flu of H5N1

Abstract. *The determination of sensitivity, specificity, and reproducibility of the method of isothermal nucleic acid amplification avian influenza virus subtype H5N1 was made. Found that these indicators are high. Substantiated that the promising is the implementation in practice of veterinary medicine laboratories of Ukraine RT - LAMP as express – method of avian influenza diagnosis.*

Key words: *avian influenza diagnosis, isothermal nucleic acid amplification, specificity, sensitivity, reproducibility.*

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Diagnosis of bird flu conducted comprehensively considering epizootic data, clinical, pathological changes, and laboratory studies [1-3]. According to OIE recommendations HAIV laboratory diagnosis is based on virus isolation from pathological material (tracheal and cloacal swabs of live poultry or organ samples from dead birds' material) on chicken embryos with its subsequent identification; molecular genetic methods; determining intravenous pathogenicity index; serological tests - available for the detection of specific antibodies

The specificity of LAMP primers for identifying different subtypes of the virus and bird flu strains

No.	Name	Result
1	AI influenza, subtype H_1N_1	+
2	AI influenza, subtype H_3N_2	+
3	AI influenza, subtype H_5N_1	+
4	AI influenza, subtype H_5N_2	+
5	AI influenza, subtype H_7N_1	+
6	AI influenza, subtype H_9N_3	+
7	AI influenza, subtype H_5N_1, UA strain A/hen/SivashBay/02/05	+
8	AI influenza, subtype H_5N_1, UA strain A/hen/Primorsky/02/06	+
9	AI influenza, subtype H_5N_1, UA strain A/hen/Krasnohvardysky/58/08	+
10	AI influenza, subtype H_5N_1, UA strain A/hen/Krasnohvardysky/59/08	+
11	AI influenza subtype, H_5N_1, UA strain A/hen/Krasnohvardysky/60/08	+
12	Negative control: Newcastle disease virus, strain La Sota	-
13	Negative control: Infectious bronchitis virus, strain Masachusetts H120	-
14	Negative control: Infectious laryngotracheitis virus, strain VNIIBB-U	-
15	Negative control: Infectious bursal disease virus, strain BER-93	-
16	Negative control: Egg drop syndrome virus, strain L-497	-
17	Negative control: Salmonella gallinarum pulorum	-
18	Negative control: Mycoplasma gallisepticum	-
19	Negative Control: no DNA	-

to influenza virus type A: ELISA, diffuse precipitation reaction, delayed hemagglutination reaction.

The important place occupied by rapid methods because there is a need of quick research results. Among them wide practical importance is polymerase chain reaction (PCR). Unfortunately, PCR analysis requires the use of expensive equipment and reagents and therefore not always available to laboratories that have resource constraints. Unfortunately, PCR analysis requires the use of expensive equipment and reagents and therefore not always available to laboratories that have resource constraints. Therefore important is the development of simple and sensitive rapid methods of diagnosis of avian influenza, adapted to local conditions. One of these is a new method that is based on isothermal nucleic acid amplification (LAMP) [9, 11].

We have previously elaborated and chosen conditions for LAMP [4]. But for wide implementation in practice it is necessary to determine the sensitivity, specificity and reproducibility of this method. The purpose of this study is to determine the sensitivity, specificity and reproducibility of isothermal nucleic acid amplification method of avian influenza virus subtype H5N1.

Materials and Methods

In studies were used a previously developed LAMP method for identification of avian influenza subtype N5N1. The composition of the reaction mixture and reaction conditions were described earlier [4]. For determination specificity of comparative aspect analyzed a set of different avian influenza virus subtypes – H1N1, H3N2, H5N1, H5N2, H7N1, H9N3, and heterologous strains of –Newcastle disease virus, infectious bronchitis of chickens, infectious laryngotracheitis, Gumboro disease, reducing egg syndrome, salmonella, mycoplasma halisektikum and pathological material from healthy poultry (Table 1).

National Scientific Center « Institute of Experimental and Clinical Veterinary Medicine», (Kharkiv) given cDNA reference strain of avian flu virus subtype H5N1 with different concentrations from 0,01to 10 ng per sample for determination sensitivity of RT-LAMP (Fig. 1,2). Reproducibility of LAMP method was determined by triplicate using reference strains of avian influenza virus subtype H5N1 in concentrations of 0.1 and 0.01 ng per sample (Figure 1). For negative sample was selected DNA of pathological material from healthy birds.

Research results

The results of specificity determining of the LAMP method are presented in Table. The data table shows that positive amplification was noted in samples positive reference strains of different subtypes – H1N1, H3N2, H5N1, H5N2, H7N1, H9N3. In the other samples that accordance to another (heterologous) strains and clinical material from healthy poultry amplification result was negative, indicating the specificity of the proposed method.

After determining the specificity of the method lamps were tested to determine the sensitivity and reproducibility of this method. The results of determining of these parameters presented in Figure 1.2.

The data presented in Figure 1 show that the sensitivity of this method is 0.1 ng per sample. The most accurate coloring observed at breeding bird flu virus cDNA from 10 to 0.1 ng per sample. Coloring was barely noticeable in other breeding cDNA reference strain. This figure corresponds to the world standards. The literature shows that the detection sensitivity of LAMP in the avian flu subtype H5 and H7 equal to 0.1 ng per sample [8]. To confirm the conclusion, the visual detection materials of LAMP were electrophoretically examined (Figure 2). As seen in Figure 2, the most clear strips on specific transilyuminatorisposterihaly at breeding cDNA reference strain of bird flu from 10 to 0.1 ng (track 1-4).

To confirm the our conclusion, we visual detection of LAMP Materials electrophoretically examined (Figure 2). As seen in Figure 2, the most clear strips on specific transilyuminator observed at dilutions of cDNA reference strain of bird flu from 10 to 0.1 ng (track 1-4). However, as seen in Fig. 2, reference samples with a concentration of 0.01 ng per sample (track 5-7) may give a negative result. We can confidently noted that LAMP sensitivity ranges from 0.01 to 0.1 ng per sample. From the literature it is known that most closely approximates the sensitivity of the method for isothermal amplification of nucleic acids is a measure of the sensitivity of polymerase chain reaction in real time. It is in the range of 0.01 to 0.1 ng per sample [7].

As you can see in Figure 2 in determining the reproducibility of this method in three repetitions in three positive samples reference material with a defined concentration of 0.01 ng, fluorescence intensity were the same and are identified similarly positive control (reference strains HAIV).

The results of these studies proved high sensitivity, specificity and reproducibility of the isothermal nucleic acid amplification method of avian influenza virus LAMP.

The conclusions

1. We have developed a reaction isothermal nucleic acid amplification to determine the subtype of bird flu virus H5N1.

2. Found that for specificity it is generic.

3. Sensitivity within the limits of 0,01-0.1 ng per sample.

4. In these studies recommended method for isothermal amplification of nucleic acids in the field conditions. This will enable timely control of pathogen entry into the country.

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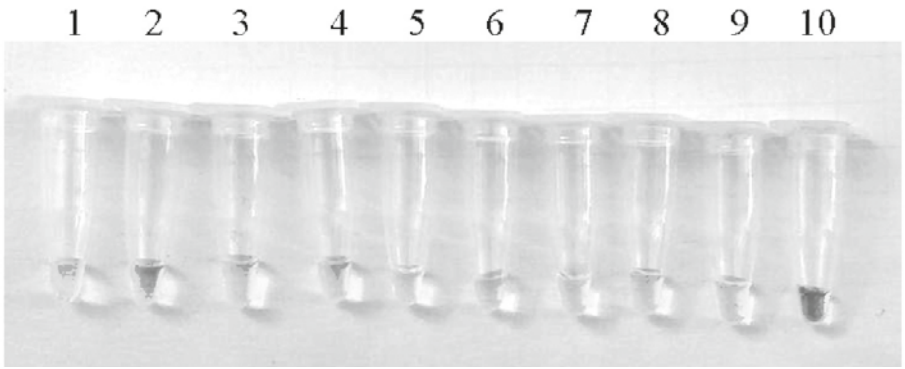
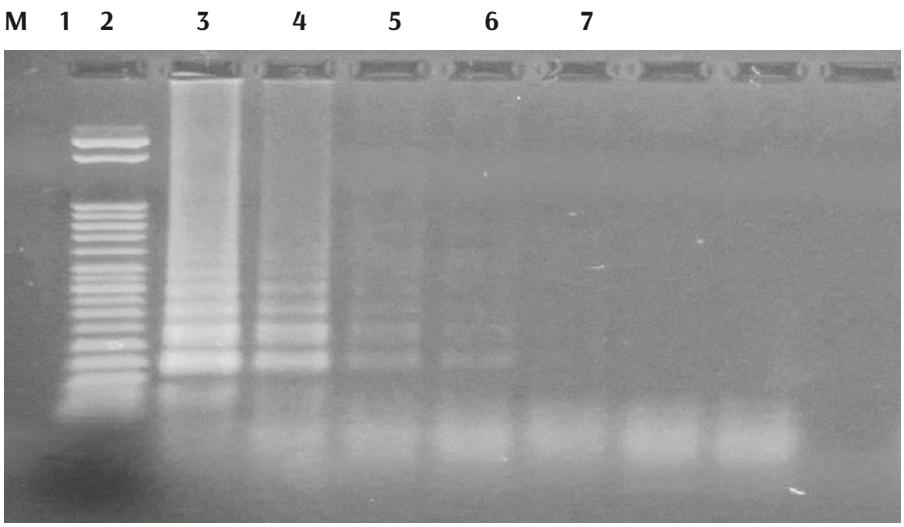


Figure 1. Visual detection of RT - LAMP products, made with different concentrations of bird flu virus cDNA (ng the experimental sample):
1 – 10; 2 – 5; 0, 3 – 1,0; 4 – 0,1; , 5 – 7 - 0,01; 8-9 -0,1; 10- negative



F*ig.2 Electrophoretic detection of RT - LAMP products, made with different concentrations of bird flu virus cDNA (ng the experimental samples):*
M- molecular weight marker 1 – 10,0, 2 – 5,0, 3 – 1,0, 4 – 0,1, 5-7 – 0,01

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