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COMPARATIVE ANALYSIS OF THE METHODS OF MOLECULAR DETECTION OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1

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Summary. The article presents results of monitoring investigation avian influenza in birds of various kinds: synanthropic, wildlife, zoo, private sector and poultry farms during 2013. A comparative analysis of the molecular detection of avian influenza virus by polymerase chain reaction and isothermal nucleic acid amplification (RT-LAMP) conducted. Proved that RT-LAMP has a promising implementation in practice of veterinary medicine laboratories of Ukraine as express-method of diagnosis of avian influenza virus.

Keywords: avian influenza virus, diagnostics, epizootic situation, monitoring investigation, the method of isothermal nucleic acid amplification

Introduction. Avian influenza — a highly contagious viral disease that is characterized by high birds mortality (up to 100%). Etiologic agent that causes the disease — RNA-containing virus which belongs to the genus Influenzavirus, family Ortomyxoviridae. It is mainly spherical vibrios diameter of 80–120 nm polymorphic. The virus is a type A has 16 subtypes by subtypes of hemagglutinin and 9 — by neuraminidase. For most pathogenic avian subtypes are H5 and H7 (Pryskoka et al., 2014; Bakulov et al., 2002; Capua et al., 2003).

In Ukraine, the diagnosis of avian influenza carried out comprehensively considering epizootic data, clinical, and laboratory pathologists change research.

Influenza must be differentiated from other diseases of birds such as laryngotracheitis, Newcastle disease, respiratory disease (Golovko, 2007; Belousova, Trotsenko, Preobrazhenskaya, 2006).

Therefore, the method of diagnosis avian influenza virus put forward a number of requirements in indicators of specificity, sensitivity, reproducibility and duration analysis (Pryskoka et al., 2014; Golovko, 2007).

In laboratory diagnostics occupies a special place highly sensitive method of polymerase chain reaction. This method is based on the amplification of specific sections of the genome of a certain type of pathogen. High sensitivity, specificity and short time analysis make it promising in the diagnosis of avian influenza virus. Unfortunately, polymerase chain reaction analysis requires the use of expensive equipment and reagents and therefore not always available to laboratories that have resource constraints (Sidoti et al., 2009; OIE, 2012).

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 approach that is based on isothermal nucleic acid amplification**.** In combination with reverse transcription LAMP suitable for amplification of RNA-matrix (RT-LAMP) (Ji et al., 2010; Shivakoti et al., 2010). We previously chosen reaction mixture and the reaction conditions optimized RT-LAMP for the diagnosis of avian influenza subtype H5N1 (Postoienko et al., 2013).

The aim of this work is testing in monitoring studies of our proposed method a RT-LAMP and comparative sensitivity analysis and the detection results of avian influenza by polymerase chain reaction in real time (PCR-RT) and RT-LAMP.

Materials and methods. In conducting a comparative analysis of results of detection of avian influenza virus subtype H5N1 by PCR-RT, RT-LAMP using experimental data obtained at the State Research Institute of Laboratory Diagnostics and Veterinary Expertise in the study of pathological material from birds, which arrived from all regions of Ukraine monitoring under the State Plan for 2013.

During PCR-RT were used commercial kits both domestic and foreign manufacturers, namely 'Bird-Influenza-PCR' (Ukrzoovetprompostach, Ukraine) and 'Quageen' (USA). Terms of amplification reactions and parameters given in the guidelines for use kits.

Terms RT-LAMP described earlier. In work applies the optimum temperature and reaction time — 59°C and 60 min (Postoienko et al., 2013).

The sensitivity of the diagnostic kit 'Bird Hrypp-PCR' and our proposed RT-LAMP determined by examining the cDNA reference strain of avian influenza H5N1, which provided National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) in the concentration range from 10.0 to 0,01 ng per sample.

Results. For 2013 we studied 1,943 samples of material from birds by PCR-RT and RT-LAMP (Table 1).

Table 1 – Monitoring of avian influenza in Ukraine in 2013

Material type	The number of investigations by PCR-RT	The number of investigations by RT–LAMP
Pathological material from birds	692	692
Tracheal and cloacal wash	33	33
Sand	1	1
Birds's manure	1217	1217
Total	1943	1943

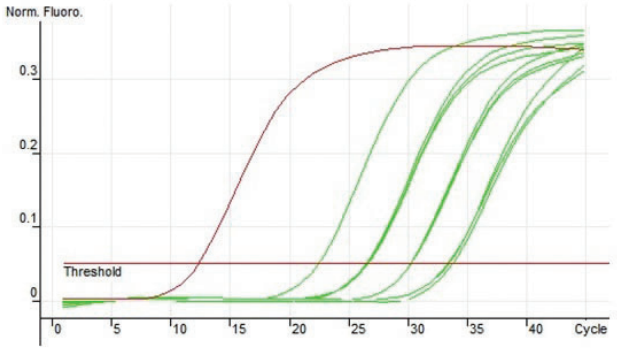
Monitoring of avian influenza among birds made of various kinds: synanthropic, wild, zoo, private sector and industrial poultry farms. The experimental data showed lack of avian influenza in studied samples. Also found that both methods use molecular detection of avian influenza virus, namely PCR-RT and RT-LAMP gives comparable results. But the practical use of RT-LAMP compared to PCR-RT has some significant advantages such as significant reduction of the duration of the analysis, the applicability of this approach in the field and lack of expensive equipment. Along with this, one of the important characteristics of a method is its sensitivity.

The indicator for PCR-RT was determined using a set of 'Bird-Influenza-PCR' (Table 2, Figure 1).

Table 2 – The sensitivity of PCR-RT test system 'Bird-Influenza-PCR'

QuantitycDNA, ng per sample	Type	Cross threshold	
		FAM	JOE
10	unknown	26.05	22.61
1	unknown	30.73	26.78
1	unknown	30.36	26.73
1	unknown	30.26	26.66
0.1	unknown	34.05	30.41
0.1	unknown	34.99	30.40
0.1	unknown	34.10	33.90
0.01	unknown	43.37	33.90
0.01	unknown	42.33	33.59
0.01	unknown	36.68	33.52
–	negative control	—	—
+	positive control	11.45	12.50

The number of cycles in the amplification channel FAM



The number of cycles in the amplification channel JOE

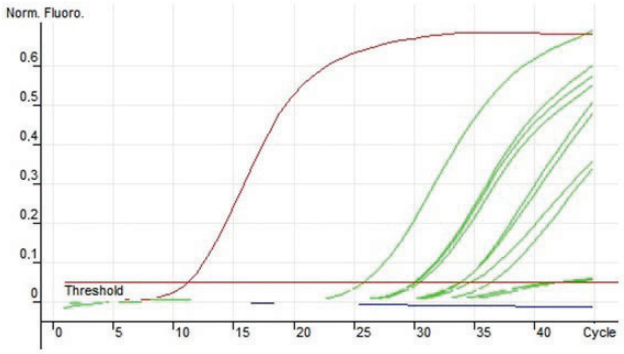


Figure 1. The sensitivity of PCR-RT test system 'Bird-Influenza-PCR'

The main criterion for evaluation of the results is to determine the threshold cycle (Ct), characterizing a stage PCR-RT, which observed a statistically significant increase in fluorescence compared to the background level. When using a diagnostic kit 'Bird Hrypp-PCR' samples were considered positive if the value of Ct through the channel FAM or where less or equal to 40 (St≤40), indicating the presence of gene amplification of influenza virus H5N1 found fragments respectively. Found that sensitivity of PCR-RT dial 'Bird-Influenza-PCR' is equal to 0.01 ng (FAM) and 0.1 ng (JOE) to test cDNA avian influenza virus type A subtype H5N1.

Sensitivity set developed by us during the research RT-LAMP method is 0.1 ng in the sample (Figure 2).

Slightly lower sensitivity of RT-LAMP is because in this method using visual detection reaction products. However, this figure corresponds to the world standards. The literature shows that the detection sensitivity of LAMP in avian influenza subtypes H5 and H7 equal to 0.1 ng per sample (Sidoti et al., 2009).

Found that sensitivity of both methods is high. Comparative analysis suggests a lower figure for LAMP, which may explain the ability of visual detection of the reaction products.

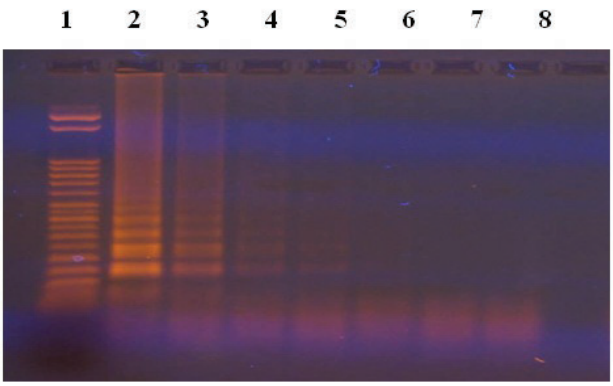


Figure 2. Electrophoretic detection products RT-LAMP, made with different concentrations of avian influenza virus DNA (ng prototypes): 1 — molecular weight marker, 2 — 10.0, 3 — 5.0, 4 — 1.0, 5 — 0.1, 6–8 — 0.01

Conclusions. 1. Comparative analysis found that using of both methods molecular detection of avian influenza virus namely PCR-RT and RT-LAMP gives comparable results confirming the promising method of rapid diagnosis of infectious animal diseases.

2. It is proved that the sensitivity of both methods of molecular detection of avian influenza virus meet international requirements.

3. Implementation RT-LAMP in the practice of veterinary medicine will enable timely control the entry of pathogens into the country.

4. Viability using RT-LAMP in the practice of veterinary medicine substantiated obtaining a number of significant advantages: short time of analysis, the applicability of this approach in the field without the use of expensive equipment.

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