2. ВЕТЕРИНАРНА ВІРУСОЛОГІЯ ТА МІКРОБІОЛОГІЯ

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The article is dedicated to the memory of Doctor of Sciences, Professor, Academician of Ukrainian Academy of Agrarian Sciences Polina Pavlivna Fuks, former Director of the Institute of Experimental and Clinical Veterinary Medicine of Ukrainian Academy of Agrarian Sciences

DESIGN OF PRIMER AND LINEAR PROBE SETS FOR SWINE AND WILD BOAR HEV DETECTION BY PCR

Lymanska O. Yu.
National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine", Kharkiv, Ukraine, e-mail: olgaliman@ukr.net

Hepatitis E virus (HEV) infects humans and several mammals and it has 8 genotypes (HEV1-HEV8). HEV1-4 is the causative agent of Hepatitis E in humans. HEV5-6 was detected only in wild boar in Japan, HEV7-8 was detected in camels. HEV3-4 is characterized by zoonotic potential. Swines and wild boars are main natural reservoirs for this virus. Besides, HEV-3 was detected in deers, dolphins, rabbits, cattle, goats that is additional risk for virus interspecies transmission from domestic animals to humans. Availability of mismatched nucleotides in the complexes of primer/probe with viral targets was applied for estimation of primer sets. Based on determined conserved fragments two sets with LNA-containing primer and linear LNA-containing probe without mismatched nucleotides in the complexes of primer/probe with single-stranded amplicon for real-time reverse transcriptase PCR detection of swine and wild boar HEV-3 and HEV-4 isolates were designed. Primer sets may be used for HEV detection by standard PCR

Ключові слова: *Hepatitis E virus, HEV, real-time PCR, LNA, linear probe*

Hepatitis E is a liver disease and one of the five known forms of human hepatitis. As a result of its widespread distribution throughout the world, the high incidence rate (20 million infection cases annually), the possibility of developing acute hepatitis and a significant number of deaths, the World Health Organization (WHO) considers Hepatitis E as a significant public health problem that requires constant attention [1, 2].

The causative agent of Hepatitis E is highly variable non-enveloped virus, the genome of which is represented by a positive single-stranded RNA molecule with length of 7100–7300 nucleotides (nt) [3]. Hepatitis E virus (HEV) was initially assigned to the *Caliciviridae* family [4]. However, in the future, according to the results of research on the HEV genome structure, the taxonomy of the virus was officially changed, and according to the modern classification, HEV belongs to the *Hepeviridae* family, which contains two genera: *Orthohepevirus* (which includes 4 species — *Orthohepevirus A*, *B*, *C*, *D*) and *Piscihepevirus* [5]. HEV, which infects humans and some mammals [6], belongs to one of the most studied *Orthohepevirus A* species and has 8 genotypes — HEV1-HEV8, of which HEV1-4 are the causative agents of Hepatitis E in humans, HEV5-6 is detected only in wild boars in Japan [7, 8], HEV7-8 — in camels [9].

HEV1-2 is the cause of outbreaks of acute hepatitis in the countries of Asia, Africa, and Latin America because of water pollution and a low level of sanitation [10]. Cases of Hepatitis E in developing countries, as well as in Europe, the United States, and the People's Republic of China, are associated with HEV genotypes 3 and 4. HEV3-4 is characterized by zoonotic potential, the main natural reservoirs of this virus are pigs and wild boars [11–15]. In addition, HEV-3 was detected in deer, dolphins, as well as rabbits, small (sheep, goat) and cattle, which is an additional risk factor of interspecies transmission of the virus to people from domestic animals [16–21].

The European Association for the Study of the Liver (EASL) offers a number of methods for HEV detection, in particular, those based on the amplification of specific fragments of the viral genomic RNA by number of PCR formats [2], which are also indispensable for the detection of genomic of HEV material in pig production and, therefore, its quality estimation [22].

A sensitive, accurate, and reproducible method for the detection and quantification of all four main HEV genotypes based on quantitative real-time RT-PCR was developed in [23] using probes and primer set that flank a fragment of the overlapping HEV open reading frames ORF2/ORF3, which are the targets for the primers and probes.

In the article [24], an experimental comparison of kits for the detection and differentiation of four HEV genotypes by RT-PCR have shown that one of the kits allows detection of 96 % of HEV RNA from fecal samples experimentally inoculated with swine HEV genotype 3 and 67 % for field samples.

In the current study, based on the computer analysis of the ORF2/ORF3 fragments of the HEV genome we have designed two primer and linear probe sets with LNA-modified nucleotides for real-time PCR detection of swine and wild boar HEV-3 and HEV-4. These primers and probes have no mismatched nucleotides in the complexes of primer/probe with viral targets for known of swine and wild boar HEV-3 and HEV-4 isolates with complete genome.

Materials and Methods. Nucleotide sequences of 111 isolates of HEV RNA were obtained in 2022 by search of taxonomy ID (txid) 291484 in the GenBank database of the National Center for Biotechnology Information (USA). Additionally, 6 HEV isolates with complete genome were captured in 2024.

Multiple alignment was performed on the basis of computer analysis of nucleotide sequences of ORF2/ORF3 by ClustalW software built into the MEGA 6.0 package [25]. Thermodynamic analysis of primers and probes was performed by MeltCalc [26] and Oligo (version 3.0) softwares [27]. Primers and probes melting temperature was determined for the following parameters: oligonucleotide concentration — 0.1 μM and 0.25 μM, ionic strength — 60 mM Na^+.

Results. A number of primer and probe sets have been developed for the detection of HEV RNA in various samples (serum, feces, from the environment) by real-time PCR, taking into account the heterogeneity of HEV strains. The sensitivity of real-time PCR analysis can vary from 10 to 1000 times when samples are testing simultaneously in the same laboratory [24].

The sensitivity of the PCR assay can vary significantly depending on the selected primer and probe targets, as well as on the HEV genotype. Preliminary comparison of sets of primers and probes for the detection of HEV3-4 RNA by real-time PCR have shown that the selection of the conserved region ORF2/ORF3 as a target is more reliable compared to the applying mismatched primers and probes that target less conserved fragments of the genome than ORF2/ORF3.

In this study, several primer and probe sets for real-time reverse transcriptase PCR detection of swine and wild boar HEV-3 and HEV-4 were designed. One of the sets without mismatched nucleotides in the complexes of primer with single-stranded amplicon for known swine and wild boar HEV-3 and HEV-4 isolates is represented in Table 1.

Table 1 — Primers and probe set for real-time PCR detection of swine and wild boar HEV-3 and HEV-4 (without mismatched nucleotides for primer (probe) complex with single-stranded amplicon). HEVfor, HEVrev, $HEVprobe^{LNA}$ are forward primer, reverse primer and linear probe respectively. R = G/A, Y = C/T, W = A/T, [+G], [+C] — LNA-modified G and C. FAM is a xanthene series fluorescent dye O,O'-dipivaloyl-5-carboxy fluorescein phosphoramide; BHQ — fluorescence quencher. Positions are shown for AF060669 HEV isolate. Reverse primer $HEVrev^{LNA}$ was corrected in 2024 comparing with its sequence in 2022.

Primer/ probe	Sequence	Melting temperature, ^0C	Concentration, μM
HEVfor (5237–5256)	5′-TGCCTATGYTGCCCGCGCCA-3'	66.7	0.1
$HEVrev^{LNA}$ (5379–5354)	5′- GGGGTTGGYTG[+G]ATGAATATAGGGGA-3′ 2022 5′- GGGGTTGGYTG[+G]ATGAA**W**ATAGGGGA-3′ **2024**	67.0	0.25
$HEVprobe^{LNA}$ (5310–5328)	5′- FAM CRGTG[+G]TTT[+C]YGGG[+G]TGAC-BHQ-3′	66.6	0.25

Primers and probes for PCR HEV detection were designed in 2022 on the basis of 111 swine and wild boar HEV isolates and characterized by absence of mismatched nucleotides for primer (probe) complex with single-stranded amplicon. But up to the September, 2024 6 new HEV isolates were deposited in GenBank [28]. Taking in the account this information we have checked our primers and probes. 100 % identity was proved for primers targeting corresponding fragments of 117 HEV isolates including 6 new HEV isolates. 1 mismatch was identified for HEVrev^{LNA} reverse primer (for LC794352 isolate). For HEVprobe 100 % identity was found for 116 from 117 HEV isolates but 11 mismatches were determined for OQ286030 *Sus scrofa* HEV isolate.

Corrected sequence of HEVprobe in 2024 comparing with its sequence in 2022 (T→ **W**) has 99,1 % accuracy. HEVfor — HEVrev^{LNA} primer set has 100 % accuracy for all known HEV isolates and it may be used for swine and wild boar HEV detection by standard PCR.

The second primer set consists of from HEVfor (5237–5256), HEVrev2 (5523–5506) and HEVprobe (5354–5379).Amplicon length is 143 nt for the first primer set and 287 nt for the second one.

Reverse primer HEVrev^{LNA} and probe HEVprobe^{LNA} contain one and three locked nucleic acid monomers (LNA), respectively.

LNA monomers are analogs of nucleotides differing in that they contain an additional methylene bridge between 2' oxygen and 4' carbon atoms in thefuranose ring. This bridge locks the 3' endo conformation in the sugar (Fig. 1).

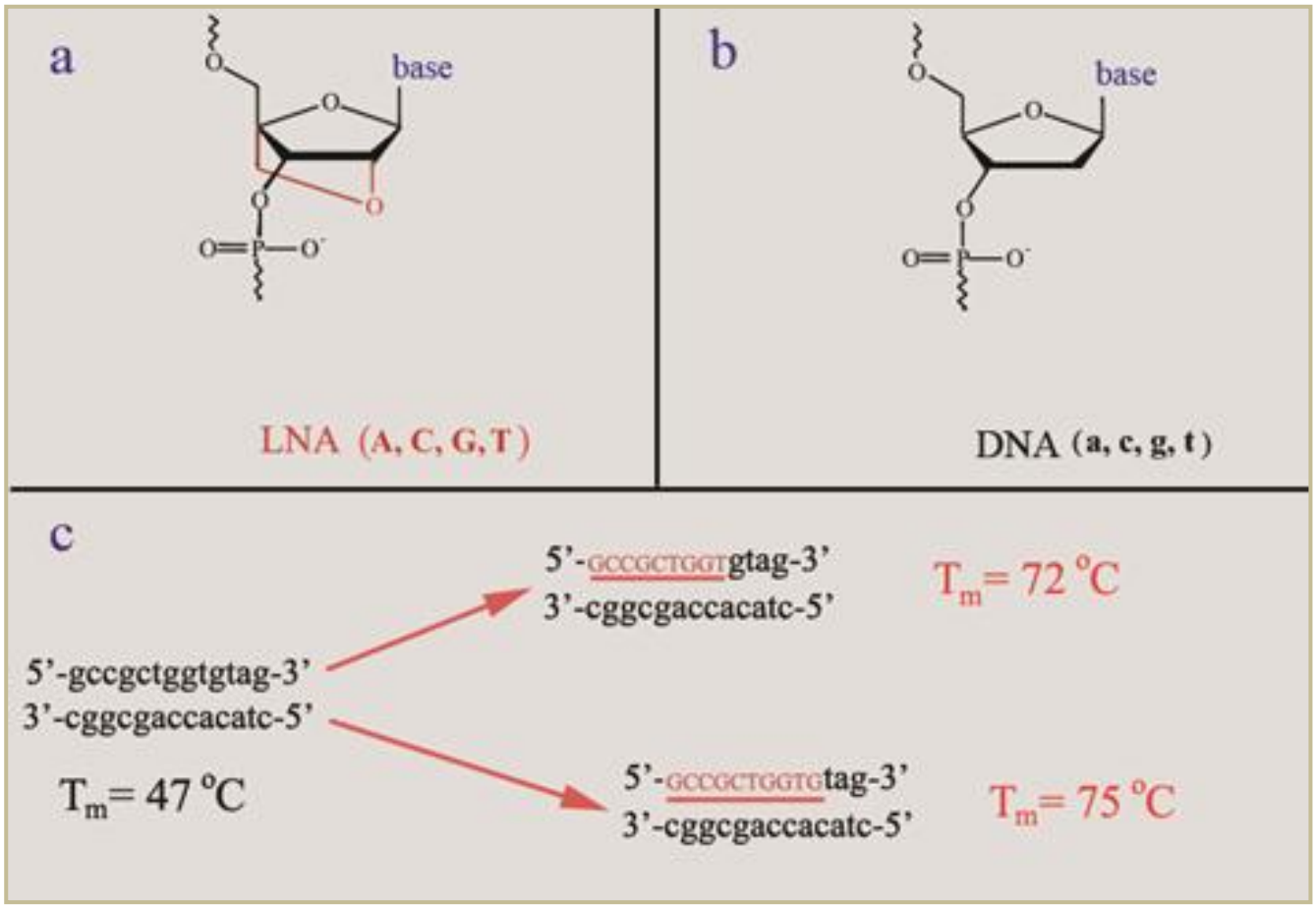

Fig. 1. A locked nucleic acid (LNA) monomer **(a)** can replace nucleotides in DNA **(b)** and RNA molecules [29]. Increasing melting temperatures from 47 ^{0}C up to 72 ^{0}C for 13 bp oligonucleotide with 9 LNA-modified nucleotides and up to 75 ^{0}C for 13 bp oligonucleotide with 10 LNA-modified nucleotides **(c)**.

Discussion. Duplexes with LNA monomers are extremely thermostable [30, 31]. Insertion of one LNA-modified nucleotide to one strand of a 13 bp duplex increases its T_m by 3–4 ^{0}C. Nine LNA monomers increase it by 25 ^{0}C, and ten increase it by 28 ^{0}C. Thus, the contribution of one LNA-containing nucleotide to T_m increasing is 3 ^{0}C at the same ionic strength.

Unique hybridization properties, increased resistance of LNA-containing oligonucleotides to the action of endonucleases [32, 33] and high discriminating ability contribute to their applying for diagnostic screening of mutations, differentiation and identification of pathogens. The main principle of the technique used to solve such problems is to use the difference in melting temperatures of duplexes, which are formed during the hybridization of wild-type or mutant DNA with an oligonucleotide probe containing an LNA-modified nucleotide in the mutation site and which is complementary to wild type or mutant DNA. The presence of a mutation results in a significant decreasing the melting temperature of an imperfect duplex, which is formed during the hybridization of mutant DNA with an LNA probe that is non-complementary at the DNA mutation site, compared to the melting temperature of a perfect duplex, which is the result of hybridization of a DNA matrix with a probe whose LNA nucleotide is complementary to the corresponding nucleotide in the DNA mutation site. The effectiveness of LNA-modified primers largely depends on the choice of positions for modified nucleotides, their number and PCR conditions.

It was demonstrated that the hairpin probe is characterized by a higher fluorescence-quenching efficiency as compared with the linear one, which leads to a lower background fluorescence level when performing real-time PCR [34]. Designed linear probes can be modified into hairpin probes by attaching selfcomplementary oligonucleotides with length of 4-5nt to 5'end and 3'end of our linear probes.

Applying primers and hairpin probe targeting different fragments of the HEV genome with a high level of identity can increase accuracy of HEV detection. A comparison of the sets have demonstrated the possibility of design of primers and different probes sets that do not contain mismatched nucleotides in the complex of primer (probe) with single-stranded PCR product for HEV isolates.

Conclusions. 1. In the present study, primers and linear probe sets with LNA-modified nucleotides without mismatched nucleotides in the complexes of primer with single-stranded amplicon for real-time PCR detection of swine and wild boar HEV-3 and HEV-4 were designed.

2. Designed primer sets may be used for swine and wild boar HEV detection by standard PCR.

3. Designed in 2022 the primers and probes based on swine and wild boar HEV isolates available in the GenBank database were perfect at the design time, as they did not contain non-complementary nucleotides in complex with the viral target.

4. Designed in 2022 primers, including the reverse primer after correction, have remained perfect for all known swine and wild boar HEV isolates from GenBank in spite of appearance of new isolates in 2024.

5. Real-time PCR probe have remained perfect for 116 from 117 swine and wild boar HEV isolates too. However 11 mismatches were found for one HEV isolate (from *Sus scrofa*) that confirms the thesis of the need for periodic testing of primers and probes for PCR detection of pathogens due to the appearance of new isolates over time.

Prospects for using the obtained results. Theory without practice is dead, practice without theory is blind. Therefore, the sets of primers and probes created in the current paper await verification for implementation in the practice of diagnostic laboratories.

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РОЗРОБКА НАБОРІВ ПРАЙМЕРІВ ТА ЛІНІЙИХ ПРОБ ДЛЯ ДЕТЕКЦІЇ ВІРУСУ ГЕПАТИТУ Е СВИНІ ТА ДИКОГО КАБАНА ЗА ДОПОМОГОЮ ПЛР

Лиманська О. Ю.
Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини», Харків, Україна

Метою роботи було створення наборів праймерів та зондів, які не містять некомплементарні нуклеотиди в комплексі олігонуклеотид–вірусна мішень, для детекції вірусу гепатиту Е (ВГЕ) свині та дикого кабана третього та четвертого типів за допомогою стандартної ПЛР та ПЛР у реальному часі. Множинне вирівнювання проводили на основі комп'ютерного аналізу нуклеотидних послідовностей фрагментів відкритих рамок зчитування ORF2/ORF3, що перекриваються, для 117 ізолятів геномної РНК ВГЕ з бази даних GenBank за допомогою програми MEGA. Термодинамічний аналіз проведено за допомогою програми MeltCalc. Створено два набори праймерів та проб, що містять декілька модифікованих нуклеотидів (із замкненою нуклеїновою кислотою, ЗНК), які не містять неспарені нуклеотиди в комплексі праймер–однонитковий амплікон, для детекції вірусу гепатиту Е свині та дикого кабана за допомогою зворотньотранскриптазної ПЛР у реальному часі. Створені в 2022 році праймери та проби на підставі наявних в базі даних GenBank ізолятів були досконалими на час створення, оскільки не містили некомплементарні нуклеотиди в комплексі з вірусною мішенню. З появою нових ізолятів у 2024 році створені праймери, в тому числі, зворотний праймер після корекції, залишилися досконалими для всіх ізолятів ВГЕ свині та дикого кабана в GenBank. Зонд для ПЛР у реальному часі також залишився досконалим для 116 з 117 ізолятів ВГЕ свині та дикого кабана. Проте для одного ізоляту (від Sus scrofa) було виявлено 11 помилкових нуклеотидів, що підтверджує тезу про необхідність періодичної перевірки праймерів та проб для ПЛР детекції патогенів через появу з часом нових ізолятів. Створені набори праймерів можна застосовувати для детекції вірус гепатиту Е свині та дикого кабана за допомогою стандартної ПЛР

Keywords: *вірус гепатиту Е, ВГЕ, ПЛР у реальному часі, ЗНК, лінійний зонд*