Part 2. Biotechnology

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DEVELOPMENT OF DIFFERENTIATION METHOD FOR BOVINE HERPESVIRUS SEROTYPES (BHV-1, BHV-4, BHV-5) USING POLYMERASE CHAIN REACTION

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Summary. Infectious pneumoenteritis of cattle is etiologically caused by viruses of different families and species. Bovine herpesvirus type 1 — infectious rhinotracheitis virus — is the main and the most dangerous pathogen transmitted by cattle semen. At the same time, recently, according to European scientists' data, in addition to this pathogen, other herpesviruses have been circulating in cattle groups, in particular, bovine herpesvirused of types 4 and 5. Studies have been conducted using molecular-genetic and bioinformatic methods. Based on the analysis of the genomes of bovine herpesvirus of types 1 (IBR virus), 4 and 5 we developed primers BoHV-1 F/R, which flanks the DNA fragment of the IBR virus with the length of 204 bp, BoHV-4 F/R, which flanks the DNA fragment of bovine herpesvirus type 4 with the length of 615 bp, and BoHV-5 F/R for bovine herpesvirus type 5 DNA amplification with the formation of specific fragments 158 bp in length. The tests demonstrated that primers specific for bovine herpesvirus of types 1, 4 and 5 can be used in multiplex amplification format and hybridized only with specific DNA matrices of bovine herpesviruses. A standard operating procedure 'Indication of DNA of infectious bovine rhinotracheitis virus and bovine herpesviruses of types 4 and 5 by polymerase chain reaction' has been developed

Keywords: infectious bovine rhinotracheitis, infectious pneumoenteritis, cattle, primers

Introduction. The vertically transmitted infectious diseases of cattle are of great economic importance, they significantly hinder the development of the livestock industry, causing significant damage by reducing fertility, viability of young animals, productivity of the parent herd and the cost of veterinary measures. On the other hand, the detection of a number of infectious diseases of cattle is important for the development of international trade in animals and animal products.

One of the main factors in the transmission of infections and their spread among susceptible cattle is the semen of breeding bulls. There is a lot of information in the literature on the transmission of viral and chlamydial infections in cattle by semen (Muylkens et al, 2007; Sarangi et al, 2021; Roberts et al, 1981).

As the main object of international trade, the semen of breeding bulls needs close monitoring and comprehensive quality research, which is regulated by several European Union directives and the OIE Code (Edwards, 2007). From this point of view, the control of breeding semen is an important task of the veterinary service, as timely implementation of product screening for virus and chlamydia contamination can prevent economic losses, loss of productivity and ensure the efficiency of the industry, as well as high quality sperm and normal reproduction of farm animals.

The semen is considered suitable for artificial insemination if the following conditions are met: firstly, if the semen does not contain pathogenic and opportunistic microorganisms; secondly, if it is obtained from clinically healthy donor bulls that have negative results of laboratory tests performed in accordance with current veterinary regulations, for a number of infectious diseases included in the OIE list (infectious rhinotracheitis, chlamydia, viral diarrhea, *etc.*).

When screening of breeding animal semen for the presence of pathogens, high efficiency and reliability are demonstrated by molecular-genetic methods based on PCR. It allows the rapid direct detection of genetic material of a particular infectious agent. PCR, which is recommended by the OIE for the detection of viral contamination and in some countries for the detection of chlamydia, has significant advantages in semen testing compared to traditional virological tools due to, firstly, significantly higher sensitivity and specificity, and secondly, the toxicity of sperm to cell cultures and its possible contamination by microflora, which leads to a high probability of incorrect test results (Givens, 2018).

The main and most dangerous pathogen transmitted by cattle semen is bovine herpesvirus type 1 (BHV-1) — infectious rhinotracheitis virus (IBR virus). At the same time, recently, according to European scientists, in addition to this pathogen, other herpesviruses are circulating in cattle groups, in particular, bovine herpesvirus of types 4 (BHV-4) and 5 (BHV-5). They cause IBR-like clinic in newborn calves and serologically similar to IBR virus (Nefedchenko et al., 2019; Rapaliute et al., 2021).

The only effective mechanism for the differentiation of these viruses is molecular genetic testing. It is suitable for targeted identification of herpesviruses, which contributes to the accurate diagnosis and selection of effective treatment regimens and prevention of viral pneumoenteritis.

The **aim of our work** was to develop a method of PCR detection of bovine herpesviruses of different epizootically significant types.

Materials and methods. In order to perform computer analysis of the genes of IBR virus and other bovine herpeviruses from the databases DDBJ (https://www.ddbj.nig.ac.jp/ddbj) and GenBank (https://www.ncbi.nlm.nih.gov/genbank), the corresponding sequences of different lengths were obtained.

The resulting sequences were aligned using the ClustalX module to search for conserved sites in the DNA sequences of herpesvirus pathogens in cattle.

After conservative sites were identified, primer pairs were searched using AmplifX software.

The calculated primer sequences were synthesized, after which the amplification mode and parameters of the reaction mixture were selected using standard base kits: GenePack IsoGen (Denmark), Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X) (USA), and Fermentas MasterMix (Lithuania).

Matrix for annealing primers in the development of methods for the indication of BHV DNA were: DNA of IBR virus, strain Cooper (Friedrich Loeffler Institute, Germany) and strain Moldavsky (National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine (NSC 'IECVM'), Ukraine), DNA of BHV-4 and BHV-5 (İstanbul University — Cerrahpaşa, Turkey), Marek's disease virus, strain SB-1 and Aujeszky's disease virus, strain UNDIEV 18-B (NSC 'IECVM', Ukraine)

Mathematical analysis of the obtained data was performed using the Excel XP spreadsheet editor.

For the development and validation of methods we used the approaches previously developed in the NSC 'IECVM' (Stegniy and Gerilovych, 2014).

Results. At the first stage of research on the development of methods for differentiation of BHV-1, BHV-4, BHV-5 serotypes by PCR we conducted research on the selection of specific primers for amplification of DNA of pathogens.

At the beginning of this work, we analyzed the DNA sequences of herpesviruses in order to select target genes.

In order to study the polymorphism of nucleotide sequences of the *gC* gene, 12 sequences of BHV-1 isolates of different lengths obtained online from the GenBank database were studied.

The analysis showed that the nucleotide sequence of the studied gene has a length of 1,786 bp, and the mass is 1.09 MDa, G+C content is 72.32%, and A+T — 27.68%. The last parameter indicates the possibility of designing primer systems with a high melting point.

After analyzing the result of multiple alignments of *gC* gene sequences, we found that it contains 5 conservative nucleotide alignments with a length of 23–47 bp, the average entropy (Hx) of these segments was 0.000–0.001, which allowed them to be used to search for probable primer pairs. Subsequent bioinformatics analysis of the sequences of other types of herpesviruses showed that serotype-specific sites suitable for primer design, this gene does not contain.

After analyzing the sequences of *gC* and *gB* genes of different types of herpesviruses available in the GenBank database, we concluded that when creating primer systems to differentiate all three types of bovine herpesviruses that have epizootic significance, it is necessary to use DNA matrices of different genes as targets.

Thus, based on the analysis of multiple alignments obtained with BioEdit and the calculation of probable primer sequences, we identified oligonucleotides with AmplifX to detect different types of bovine herpesviruses (Table 1).

Table 1 — Oligonucleotide sequences for DNA detection of BHV-1, BHV-4, BHV-5

Agent	Primer system	Sequences 5'–3'	Annealing temperature, °C	Product size, bp
Bovine herpesvirus type 1 (IBR virus)	BoHV-1_F	GCGGGCCTGGTTGCGTACTAC	58	204
	BoHV-1_R	AGCAGATCTTCCGCGTTGATC		
	BoHV-1 gE_F	GCCAGCATCGACTGGTACTT	57	325
	BoHV-1 gE_R	GCACAAAGACGTAAAGCCCG		
Bovine herpesvirus type 4	BoHV-4_F	CCCTTCTTTACCACCACCTACA	58–61	615
	BoHV-4_R	TGCCATAGCAGAGAAACAATGA		
Bovine herpesvirus type 5	BoHV-5_F	CGGACGAGACGCCCTTGG	58–61	158
	BoHV-5_R	AGTGCACGTACAGCGGCTCG		

To facilitate the procedure of future amplification, all selected primer pairs were selected taking into account the divergence of the flanked areas to facilitate visual differentiation and with close 57–61°C hybridization temperatures to the matrix.

Thus, to detect IBR virus, we created BoHV-1 F/R primers suitable for amplification of a 204 bp *gB* region of the virus and BoHV-1 gE_F/R, complementary to the DNA sequence of the *gE* gene with a length of 325 bp. Both oligonucleotide systems had a theoretical annealing temperature of 57–58°C.

BoHV-4 primer set for the detection of BHV-4 DNA limited the region of the *gB* gene to 615 bp and were characterized by theoretical annealing temperature 58–61°C. Oligonucleotides BoHV-5 F/R flanked a region of the *gB* gene of BHV-5 with a length of 158 bp and were characterized by a similar theoretical annealing temperature.

Theoretical verification of the developed oligonucleotides showed their satisfactory qualities (absence of palindromes, GC content, proximity of melting temperatures), which was recorded at the limit of 87–94% of the maximum.

These primer sequences were synthesized and used for further research on PCR protocols.

Development of amplification reaction regulations was performed using strain Cooper of BHV-1 with a titer of 6.5 lg TCD_{50}/ml and strain Moldavsky as a positive control reaction. DNA of BHV-4 and BHV-5 from broods with virus titers of 6.7 lg TCD_{50}/ml and 5.5 lg TCD_{50}/ml, respectively.

Standard PCR parameters were tested for a total reaction volume of 30 µl containing 0.5 U of Taq polymerase, 2.0 mm Mg^{2+} ions, with the introduction of 5 µl of viral DNA solution isolated by sorbent method and from the obtained reference materials.

The first step in optimizing the reaction was to select the appropriate temperature regime. PCR was performed on three consecutive five-fold dilutions of standard DNA solution using 50 pM primers BoHV-1 F/R, BoHV-1 gE_F/R, BoHV-4 F/R and BoHV-5 F/R at temperatures of 50°C, 52°C, 54°C, 56°C, 58°C, and 60°C.

The results obtained during amplification showed the possibility of detecting the system BoHV-1 gE_F/R in five consecutive dilutions of viral DNA at 50°C and 56°C, however, together with a specific fragment in the tracks (325 bp), we observed bands of different lengths (approx. 150–200 bp and 700–800 bp), which made it impossible to properly account for the reaction, as specific, at the specified temperature.

The result of amplification at 60°C indicated an excessively high annealing temperature, as evidenced by the absence of specific bands in the third dilution.

Therefore, this technique, showing satisfactory sensitivity in five replicates at 58°C amplification, can be recommended for the detection and identification of viral isolates of IBR in titers 3–6 lg TCD_{50}/ml, which is a satisfactory analytical threshold for screening for virus from semen samples and other clinical materials, as well as in the study of samples of viral isolates.

Similar results were obtained using oligonucleotides BoHV-1 F/R. The 204 bp length DNA fragment amplified in all dilutions for use at a temperature of 58°C.

Subsequent studies of the primers of the BoHV-4 F/R system according to the scheme described above showed that the effective annealing temperature for these oligonucleotides is 56–58°C. Bands of amplicons of specific calculated length (615 bp) were observed in all tracks containing reaction products with specific DNA, which corresponded to the sensitivity threshold for the minimally detected virus titer of 2.0–2.5 lg TCD_{50}/ml. Due to the similarity of the lengths of the fragments amplified by BoHV-1 F/R and BoHV-4 F/R primers, it was decided to use the BoHV-1 primer system gE_F/R for the detection of BHV-1 DNA.

Optimization of the annealing regime of BoHV-5 F/R primers for the excretion of BHV-5 DNA also showed that annealing at temperatures of 58–60°C is optimal, and the detection threshold is 1.5–2.0 lg TCD_{50}/ml. Specific fragments of 158 bp in length were formed in the tracks of samples containing BHV-5 DNA.

At the next stage of the work, the influence of different concentrations of magnesium ions on the nature of amplicon formation and their contrast was tested. When checking the results of the reaction with concentrations of magnesium ions from 1.0 mm/µl to 4.0 mm/µl in steps of 1 mm/µl and then 0.5 mm/µl, it was found that the formation of a specific amplicon of maximum contrast was observed in the case adding 2.0–2.5 mm/µl of the reaction mixture of magnesium ions in the form of magnesium sulphate. At the same time, minimal comet tail and unspecific product formation was noted (visually detected only in 2 from 48 samples).

Analysis of the absence of cross-reactions with the genetic material of other alpha-herpesviruses (Marek's disease virus, strain SB-1; Aujeszky's disease virus, strain UNDIEV 18-B) and different types of bovine herpesviruses showed intraspecific specificity of primers of the developed systems. Thus, BoHV-1 primers gE_F/R amplified only IBR virus DNA, BoHV-4 F/R — only BHV-4 DNA, and BoHV-5 F/R — only BHV-5 DNA (Figs. 1–3).

To test the sensitivity and reproducibility of the developed protocol of IBR virus DNA amplification, the possibility of detecting different amounts of specific DNA (3–8 lg TCD_{50}/ml) was studied. IF-negative and IF-positive sperm samples were examined, as well as virus culture broods with different titers.

It was found that the proposed method is able to detect IBR virus DNA with a titers from 3 TCD_{50}/ml to 8 TCD_{50}/ml, so the sensitivity exceeds the sensitivity of IF in the twice amount. The studies were conducted three times, with repeated results reproduced (Fig. 4).

Thus, IBR virus DNA detection protocol based on amplification of 245 bp region of *gB* gene and 325 bp area of *gE* gene. In terms of sensitivity, the second method was more effective, which allowed to choose it as the basis for creating a diagnostic technique.

The testing of primers of BoHV-4 F/R and BoHV-5 F/R systems at the specified composition of the reaction mixture for annealing at a temperature of 58°C demonstrated the synthesis of the specific fragments with 615 bp and 158 bp length, respectively, that were formed in specific samples corresponding to the content of viral DNA viral titers of BoHV-4 broods — from 2 lg TCD_{50}/ml, and BoHV-5 — 2.5 lg TCD_{50}/ml.

Therefore, the developed amplification regulations were quite sensitive (analytical sensitivity 2–3 lg TCD_{50}/ml) and specific for cross-reactions with heterologous herpesviruses and animal DNA. This allowed us to move to the creation of methods for detecting and differentiating of bovine herpesviruses' different subtypes.

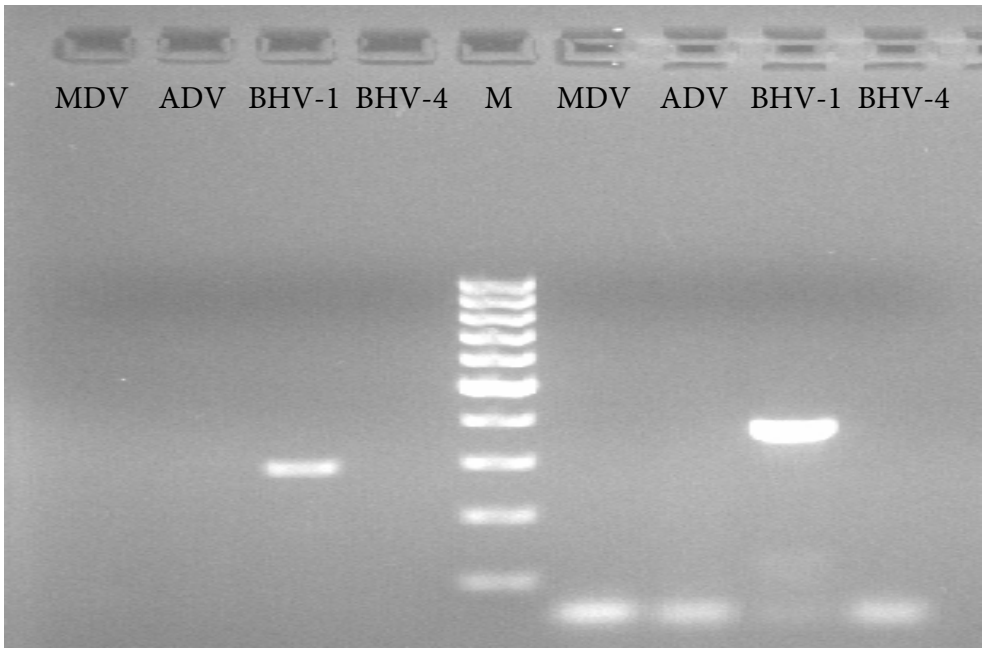

Figure 1. Amplification results for DNA of IBR virus and other herpesviruses with primers of BoHV-1 F/R (first 4 tracks) and BoHV-1 gE_F/R (4 tracks on the right, after the mass ladder) systems: M — ladder (100–1,000 bp); MDV — DNA of Marek's disease virus, strain SB-1; ADV — DNA of Aujeszky's disease virus; BHV-1 — DNA of IBR virus, Moldavsky strain, BHV-4 — DNA of BHV-4

To develop a diagnostic test system we optimized the amplification temperature and the number of cycles and selected the appropriate reaction mixture.

We optimized amplification cycles to detect small amounts of specific IBR virus DNA more effectively. PCR was performed using the components of the reaction mixture GenePack IsoGen, Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X), and Fermentas MasterMix.

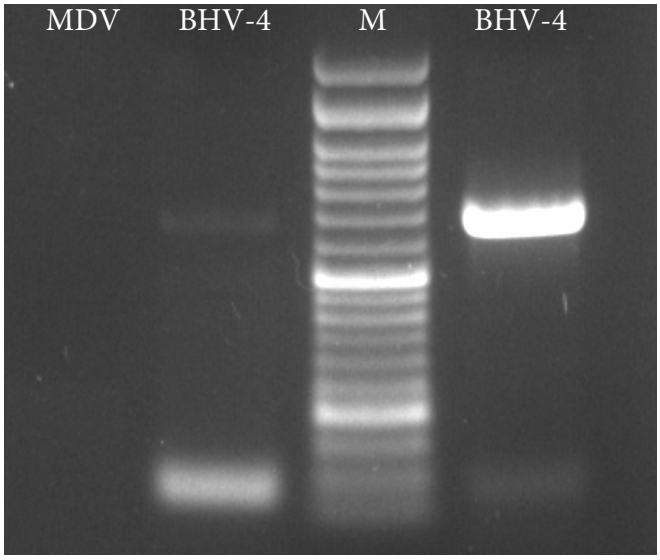

Figure 2. Amplification results for DNA of IBR virus and other herpesviruses (MDV) with primers of BoHV-4 F/R system: M — mass ladder (100-10,000 bp); MDV — DNA of Marek's disease virus, strain SB-1; BHV-4 — DNA of BHV-4 (2 lg TCD_{50}/ml and 5 lg TCD_{50}/ml)

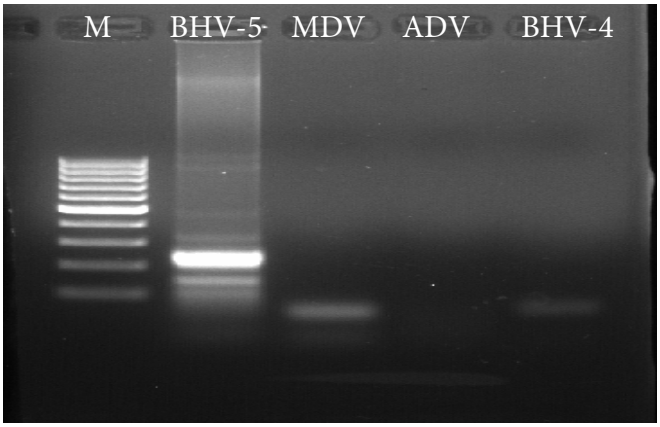

Figure 3. Amplification results for DNA of IBR virus and other herpesviruses with primers of BoHV-5 F/R system: M — mass ladder (50–1,000 bp); MDV — DNA of Marek's disease virus, strain SB-1; ADV — DNA of Aujeszky's disease virus; BHV-4 — DNA of BHV-4; BHV-5 — DNA of BHV-5

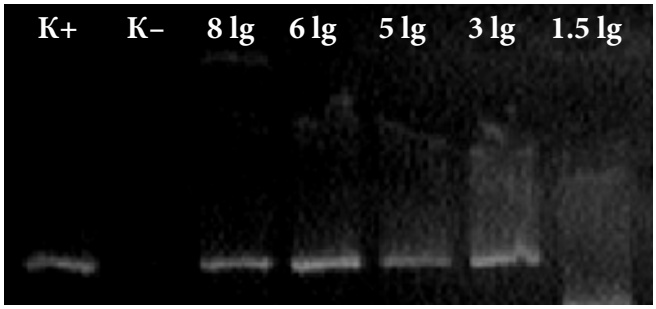

Figure 4. Sensitivity determination for the detection of 325 bp fragment of *gE* gene of IBR virus

According to the results of the experiments, it was found that the most contrasting strips of amplicons were formed using the reaction mixtures Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X) and GenePack IsoGen, but the latter option required increasing the annealing temperature of primers by 2–3°C, which led to a false negative result in the maximum dilutions of the virus (4 lg TCD_{50}/ml) and 3 lg TCD_{50}/ml) (Table 2).

Table 2 — The effectiveness of different reaction mixtures in the detection of IBR virus DNA

Master mix	Viral seed titer, lg TCD_{50}/ml							
	1.5	2.5	3.0	4.0	5.3	6.4	7.2	8.7
Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X)	–	+	++	++	++	++	++	++
GenePack IsoGen	–	±	+	++	++	++	++	++
Fermentas MasterMix	–	–	±	++	++	++	++	++

Thus, the optimal mixtures for amplification were recognized as basic sets of Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X), which became the basis of the amplification part of the method for detection of IBR virus DNA.

When using these mixtures, amplification should be performed using the 45-cycle protocol presented in Table 3.

Table 3 — Temperature cycles for the detection of IBR virus DNA by 325 bp fragment of the *gE* gene by PCR

No.	Temperature, °C	Time, min	Number of cycles
1	94	4	1
2	94	1	45
	58	1	
	72	1	
3	72	10	1
4	4	600	1

Similar results were obtained using BoHV-4 F/R and BoHV-5 F/R primer systems.

Therefore, Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X) reagents using specific primers at a concentration of 20 pM/μl proved to be the most effective as basic reagents for amplification of BHV-1, BHV-4, and BHV-5 DNA.

In order to verify the sensitivity, reproducibility of the results and the specificity of the methodology, laboratory tests were conducted.

To test the specificity and sensitivity, we used total DNA samples from virus-containing material from 10 IBR-infected animals, as well as dilution of BHV-4 and BHV-5 DNA, and 5 samples of sperm contaminated with IBR virus.

To check the taxonomic specificity of the test system were used broods of heterologous herpesviruses:

— Aujeszky's disease virus,

— Marek's disease virus,

— Infectious laryngotracheitis virus,

as well as eukaryotic DNA:

— from intact cattle (4 samples: lungs, kidneys, liver, blood and semen);

— from cell cultures of PT, TrT, PO-2.

In order to determine the reproducibility of the results obtained using PCR techniques, the study was performed three times.

Tests demonstrated, that primers specific for BHV-1, BHV-4, and BHV-5 hybridized only with specific matrices: BoHV-1 gE_F/R amplified only IBR virus DNA; BoHV-4 F/R — annealed only with samples containing BHV-4 DNA; BoHV-5 F/R — only with BHV-5 DNA; and does not hybridize to bovine and other herpesvirus DNA. The results were reproduced at repetitions (n = 3).

Based on the conducted study the standard operative procedure 'Indication of DNA of infectious bovine rhinotracheitis virus and bovine herpesviruses of types 4 and 5 by polymerase chain reaction' has been developed.

Conclusions. Based on the analysis of the genomes of bovine herpesviruses of types 1 (IBR virus), 4 and 5 developed primers BoHV-1 F/R, which flanks the DNA fragment of IBR virus with length of 204 bp, BoHV-4 F/R, which limits the DNA fragment of bovine herpesvirus type 4 with a length of 615 bp, and BoHV-5 F/R for excretion of bovine herpesvirus type 5 DNA with the formation of specific fragments of 158 bp.

It was shown that primers, specific for herpesviruses of types 1, 4 and 5 can be used in multiplex amplification format and hybridized only with specific matrices: BoHV-1 gE_F/R amplified only IBR virus DNA; BoHV-4 F/R — annealed only with samples containing BHV-4 DNA; BoHV-5 F/R — only with BHV-5 DNA; and does not hybridize to bovine and other herpesvirus DNA.

The standard operating procedure 'Indication of DNA of infectious bovine rhinotracheitis virus and bovine herpesviruses of types 4 and 5 by polymerase chain reaction' has been developed.

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