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Multiplex primers employment for detection of rinderpest virus RNA by PCR

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Abstract. Given the high contagiousness and rapid spread of the rinderpest virus, timely and accurate diagnosis plays a key role in preventing epidemics and taking measures to control the disease. The study aims to evaluate the efficiency of using multiplex primers in the polymerase chain reaction method for the detection of rinderpest virus ribonucleic acid. The study included the analysis of samples such as blood serum and conjunctival swabs from 50 animals with clinical manifestations

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of the disease. The experiment involved the collection of clinical samples such as blood serum and conjunctival washings. The results demonstrate the high specificity of the developed primers. These primers stand out because they use two pairs of the same gene region with different variable sequences that are specific for all strains of the rinderpest virus. In the polymerase chain reaction, both pairs of primers are used simultaneously at equal concentrations and under the same conditions. An additional polymerase chain reaction performed using these primers at the optimal annealing temperature confirmed the successful amplification and specificity of the primers. The absence of dimers and nonspecific products in the negative control confirmed the purity and reliability of the results. Thus, these results demonstrate that the use of these multiplex polymerase chain reaction primers allows for the efficient detection of the ribonucleic acid of the rinderpest virus of different strains. The developed multiplex primers represent an innovative method for the diagnosis of rinderpest virus with the potential for use in veterinary practice and animal disease control

Keywords: oligonucleotides; specificity; annealing; diagnostic efficiency; biological samples; amplification

INTRODUCTION

In today's world, which includes globalisation, climate change, large-scale breeding and increasing numbers of pets, animal diseases are becoming an increasing problem. These conditions favour the spread of infections, affecting not only the welfare of pets. Scientists face the challenge of effectively controlling and preventing epidemics, as the rapid spread of infections can have a serious impact on various sectors of society. Therefore, the development and implementation of accurate diagnostic methods are critical for the timely detection and control of diseases and the minimisation of their adverse effects, hence the research.

A. Safarov *et al.* (2021), canine distemper is a serious viral disease occurring in puppies and adult dogs. Common manifestations include increased body temperature, digestive disturbances and diarrhoea, sudden vomiting, lethargy, depression, loss of appetite, and symptoms involving the respiratory, eye, skin, or nervous system. In some cases, complications may occur in the form of pneumonia, which can be fatal, or paralysis. K. Altay *et al.* (2023) and K. Beus *et al.* (2023) state that accurate detection of the disease plays an important role in controlling and preventing the spread of the disease. The most effective method for diagnosing rinderpest today is polymerase chain reaction (PCR). This method detects the pathogen at early stages, even when there are no obvious signs of the disease. PCR is characterised by the possibility of multiple amplification of genetic material and visualisation using fluorescent dyes, which makes it an effective tool for early detection and accurate diagnosis. In the context of this method, a range of disagreements and opinions arise among researchers focusing on its efficiency, cost-effectiveness and comparative sensitivity compared to alternative approaches. The innovativeness of multiplex primers is manifested in their ability to amplify multiple RNA sites simultaneously. This increases diagnostic efficiency, speeding up the detection of rinderpest virus and providing a wider range of information about the presence of infection.

R. Uakhit *et al.* (2022) also note that the use of multiplex primers also involves saving resources such as reagents and time. A single reaction can simultaneously cover multiple genes. However, for all their efficiency, optimising multiplex PCRs can be challenging. The diversity of plague virus genes requires careful selection of primers to avoid competition between them, which may affect the reliability of the results. Y. Yamamoto *et al.* (2021) raised concerns about the sensitivity of multiplex PCR. Possible interactions between primers may reduce the sensitivity of the method, which requires careful validation of the results and comparison with alternative techniques. To ensure the validity of the results, additional tests and data verification are critical. This is particularly important in the case of multiplex assays, where it is potentially difficult to interpret the results. Given the advances in molecular biology, the use of PCR and multiplex primers represents a promising avenue in the development of diagnostic methods that allow more accurate and rapid detection of the causative agent.

Thus, the use of multiplex primers in PCR represents an innovative approach that can significantly improve diagnostic accuracy and efficiency. Such technological developments are of great importance in modern medical practice. The study aims to review and evaluate the efficacy of using multiplex primers in a polymerase chain reaction method for the detection of RNA of rinderpest virus. The main focus is to identify the advantages of this method compared to conventional approaches for the diagnosis of this highly contagious virus. Key objectives of the study include: selection of primers and analysis of genetic sequences; specificity assessment and development of diagnostic primers; evaluation of the sensitivity of the PCR method.

MATERIALS AND METHODS

The research was carried out between 2022 and 2023 at the Kyrgyz Research Institute of Veterinary Medicine named after Arstanbek Duisheyev и Kyrgyz National Agrarian University named after K.I. Skryabin laboratory.

The National Centre for Biotechnology Information (NCBI) bioinformation database was used to obtain genetic data of the plague virus. Oligonucleotide sequences were analysed in GenBank. Carnivore plague genes were analysed using the BLAST programme. The selection of primer parameters was performed using the MEGA 10 software package, which includes the Clustal program, as well as manual selection. As part of the experiment, samples such as blood serum and eye conjunctiva washes were collected from 50 animals with clinical manifestations of the disease. The dogs ranged in age from 2 months to 7 years.

In the preparation of biological materials for research, blood serum was used, which was obtained from the blood of animals, and collected in special vacuum tubes. The tubes with blood were left in the refrigerator at +4°C for 1-2 hours for complete clot formation. The serum was then transferred to a smaller tube and stored at -20°C until the time of testing. Special sterile cotton swabs were used to obtain flushes from animals. The sticks were used to wipe the mucous membrane and then placed in a tube with a physiological solution. After vortexing and centrifugation, the material was stored at -20°C. For the study, the methodology included the following steps:

1. RNA isolation of viruses using a commercial RIBO-sorb kit (AmpliSens) according to the instructions for use. These steps provided high-quality RNA for subsequent analysis steps.

2. PCR using selected primers: after obtaining RNA from the samples, a polymerase chain reaction was performed using selected primers. PCR parameters were optimised for maximum specificity and amplification efficiency.

3. Validation of PCR results: the PCR products obtained were subjected to agarose gel analysis to verify their size and purity.

4. Vaccine strain experiment: RNA from the Onderstepoort vaccine strain was used to optimise PCR conditions and validate the methodology. This step provided additional data for the correct interpretation of the results and determination of the sensitivity of the method.

5. Reverse transcription: reverse transcription processes were performed using a commercial Qiagen kit (Qiagen, Germany) according to the instructions for use.

This integrated approach to methodology allows for the reliable acquisition and analysis of genetic data from the carnivore plague virus and maintains the accuracy and validity of the study results. The results were processed for reliability using multivariate analysis of variance MANOVA using Microsoft Excel software and the Statistica 10 software package. Differences in the obtained results are possible at a significance level of $p \leqslant 0.05$ using the Student's criterion. No animals were harmed during sampling.

RESULTS

Rinderpest virus undergoes an evolutionary development that can be traced at the phylogenetic level down to its older roots. Over time, under the influence of a variety of environmental factors, the rinderpest virus is periodically infected, resulting in the acquisition of new genetic properties. These changes are accompanied by nucleotide mutations, and as a result, the carnivore plague virus is currently represented by several genetic lineages (Trogu *et al.*, 2021). It is important to consider the variability of the virus gene when making a diagnosis. In this study, primers were selected to account for the different variations of the carnivore plague pathogen. After processing all data using Clustal software, an aligned gene was obtained. The study allowed the selection of a relatively conserved region suitable for primer design. However, the gene alignment showed differences in nucleotide sequence between strains of rinderpest virus. This makes the detection of identical gene regions a challenge. The most similar gene regions of this virus were adenine (A) and thymine (T) rich, which precluded their use. As a result, specific gene regions were selected, and appropriate primers were designed (Table 1).

Table 1. *Nucleotide sequences of direct primers for the carnivore plague virus gene*

Primer	Oligonucleotide sequence	GenBank position	Length of the resulting product in nucleotide pairs
D-1	5'-TCGTTCCTCCTATCCCTCCT-3'	3,519-3,539	681
D-2	5'-GGCTCATACCCCAAGTCAGA-3'	4,180-4,200	

Source: *compiled by the authors*

For diagnostic use, the primers selected should have maximum specificity to specific regions of the sample. The low specificity of primers used in diagnostics may cause false negatives. For this reason, diagnostic primers are subject to specificity requirements to avoid errors in the results. To develop diagnostic primers that would apply to all strains of rinderpest virus, their specificity was tested using computer programs. Genetic information on different strains of rinderpest virus was extracted from GenBank (Fig. 1 and 2). From the studies presented in

Figures 1 and 2, it can be seen that the selected primers were not found to be specific for all strains of the tested virus. The forward primer was identical for strains 1-3, 4, 7, 10 and 13, but did not show specificity to some strains at three sites: C (cytosine)→A, A→G (guanine) and C→T. Similar to the forward primer, the reverse primer was found to be specific for certain strains of carnivore plague virus. It perfectly matches strains of lines 1-5, 9-11, and 15-19, but has differences in the sequence of nitrogenous bases at two and three sites.

0	G	G	C	T	C	A	T	A	C	C	C	C	A	A	G	T	C	A	G	A
1	-	-	-	-	A	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	A	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-
9	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	A	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-
15	-	-	-	-	A	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-
16	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-

***Figure 1**. Evaluation of the forward primer specificity*
***Note:** 0 – primer sequence; 1-21 – different strains of rinderpest virus strains*
***Source:** compiled by the authors*

0	T	C	G	T	T	C	C	T	C	C	T	A	T	C	C	C	T	C	C	T
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
3	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
4	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
10	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
11	-	G	-	-	-	-	-	C	-	-	-	-	-	T	-	-	-	-	-	-
12	-	G	-	-	-	-	-	C	-	-	-	-	-	T	-	-	-	-	-	-
13	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
16	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

***Figure 2**. Results of reverse primer specificity testing*
***Note:** 0 – primer sequence; 1-21 – different strains of rinderpest virus strains*
***Source:** compiled by the authors*

Other variants of primer selection for gene sections without variations were also tested in the course of the work. However, it proved difficult to select primers with absolute (100%) specificity when examining other gene sections. Some sections of the plague virus gene remained unchanged but contained excess adenine and

thymine or were too short. When selecting primers, their length and the ratio of nitrogenous bases play an important role, which significantly affects the accuracy of the polymerase chain reaction. For example, if adenine and thymine predominate, the annealing temperature may be low, which increases the risk of decreasing the specificity of primers and thus the quality of PCR analysis. However, too high guanine and cytosine content also affect the quality of PCR analysis, as excess of these bases can lead to the formation of secondary product structure and slow down the activity of DNA polymerase (Alfano *et al.*, 2020).

Under the influence of these factors, the sensitivity of the PCR method may be reduced, which may lead to false negatives. With this in mind, primers rich in G and C nitrogenous bases are favoured when selecting primers. By analysing all possible variants, another pair of primers was designed considering the sequence differences between 3,519-3,539 and 4,180-4,200 using the multiplex PCR principle, in this case, two pairs of primers are involved in the analysis. When multiplex PCR is used to diagnose several types of diseases, it is necessary to provide different lengths of PCR products for each species. When designing primers for multiplex polymerase chain reactions, it is important to avoid mutual complementarity. In this case, the size of the polymerase chain reaction products will retain homogeneity since the goal is not to differentiate between strains of rinderpest virus but to identify the pathogen itself. Considering previous results, the design of primers was modified to make them universal for multiplex PCR. Thus, two primer pairs were obtained instead of one (Fig. 3).

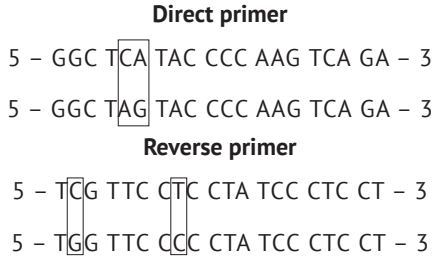

Figure 3. *Multiplex primer pairs with modified sequences*
Source: *compiled by the authors*

To adapt to the variability of the study site, the nitrogenous bases of the primers were modified. The forward primer was modified by replacing two sites (C→A, A→G), and the reverse primer was modified by replacing two sites (C→G and T→C). In subsequent studies, all four primers were applied simultaneously using multiplex PCR, which increases the probability of interaction between them. However, increasing the concentration of primers may favour the formation of dimers, which affects the specificity of the reaction. Dimerization results in the formation of a variety of PCR products, and false products may compete with the formation of the product under investigation. These problems are exacerbated when low-temperature annealing and excessive annealing times are used. Dimers have a significant impact on real-time polymerase chain reaction (PCR-RV) results, especially when using an asymmetric cyanine dye (SYBR Green I). When this dye is used, any product obtained during the reaction is considered a positive result. Therefore, dimers contribute to false positives when SYBR Green I is used in PCR-RV.

The primers proposed in the study, although almost identical, have slight differences in sequence, which provides a better diagnosis of the different genetic lineages of the rinderpest virus. The main principle determining specificity lies in the 3'-end of the primers. This end, according to the principle of complementarity, should be at least 4 nucleotide pairs closer to the tested material. The complementarity of the 3'-end of the primer with the test material significantly affects the sensitivity of PCR. Therefore, this feature should be considered when selecting primers. Another characteristic feature is the fact that when annealing 3'-ends to each other, primers practically lose their efficiency, unable to interact correctly with the material under study. In such situations, the polymerase chain reaction may demonstrate a negative result. Taking all these features into account, the first two primers were analysed using the developed validation method. The results of this test allowed us to evaluate the specificity of the primers against different strains of rinderpest virus.

To develop the design of primers used in multiplex PCR, a grouping of rinderpest virus strains based on the similarity of nitrogenous bases was carried out. This allowed the design of four primers, two for each of the forward and reverse directions corresponding to each strain group. Genetic data from GenBank were used to form a group of 20 strains of rinderpest virus representing different genetic lineages of the virus. Before forming this group, many variants of the carnivore plague gene were analysed using BLAST software. From the entire dataset, 20 plague virus sequences representing different genetic lineages of the virus were selected and divided into four groups for subsequent specificity analysis using each primer.

The results of the reverse primer specificity study of variant No. 1 revealed that for one line of carnivore plague virus strains, complete specificity was not observed when nucleotide C was replaced by T (Fig. 4). However, in the remaining cases, a complete match was achieved. Similar results were obtained when analysing the second variant of the reverse primer (Fig. 5). The specificity of the forward primer was also analysed, and the results showed that the first variant of the forward primer had mismatches at one site where C is replaced by T (Fig. 6). The second variant of the forward primer achieved complete similarity in all cases (Fig. 7).

0	T	G	G	T	T	C	C	C	C	C	T	A	T	C	C	C	T	C	C	T
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4. *Specificity of reverse primer, variant No. 1*
Note: *0 – primer sequence; 1-13 – different strains of rinderpest virus strains*
Source: *compiled by the authors*

0	T	C	G	T	T	C	C	T	C	C	T	A	T	C	C	C	T	C	C	T
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 5. *Specificity of reverse primer, variant No. 2*
Note: *0 – primer sequence; 1-7 – different strains of rinderpest virus strains*
Source: *compiled by the authors*

0	G	G	C	T	A	G	T	A	C	C	C	C	A	A	G	T	C	A	G	A
1	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 6. *Specificity of the forward primer, variant No. 1*
Note: *0 – primer sequence; 1-7 – different strains of rinderpest virus strains*
Source: *compiled by the authors*

0	G	G	C	T	C	A	T	A	C	C	C	C	A	A	G	T	C	A	G	A
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 7. *Specificity of forward primer variant No. 2*
Note: *0 – primer sequence; 1-7 – different strains of rinderpest virus strains*
Source: *compiled by the authors*

In two cases, the primers obtained had some differences in nucleotide sequences, but these changes should not significantly affect PCR sensitivity. It is important to note that such small substitutions in the middle of the primer usually do not adversely affect PCR sensitivity. However, mismatches at the 3' end should be avoided during primer selection. Currently, a wide range of computer programs is available that greatly simplify the primer selection process. These programmes facilitate the experimental stage of primer design, allowing researchers to quickly select the necessary primers, determine annealing conditions for PCR reactions, and detect variability in gene regions based on sequenced gene variants. This information significantly reduces the time spent on experiments in laboratories.

Despite the abundance of available computer programs capable of identifying potential primers, their efficiency and performance can only be assessed through laboratory experiments. In the subsequent stage of the work, the selected primers were repeatedly processed using computer programs to establish the annealing temperature and melting temperature. For the initial determination of the annealing temperature, a gradient temperature regime is often used. Given the participation of two primer pairs in the reaction, the possibility of dimer and hairpin formation increases significantly. The appearance of dimers significantly affects the sensitivity of the polymerase chain reaction. In addition, another important factor affecting the formation of dimers is the presence of the enzyme revertase. Revertase used in reverse transcription can promote dimer formation. To exclude this factor, revertase was inactivated after the reverse transcription reaction by incubating for 3 minutes at 95°C. Inactivated samples were used to perform gradient PCR. The selected temperature regime included the following values from 53°C to 57°C.

For polymerase chain reaction using the selected primers, a 20 µl reagent mixture was prepared with a total volume of 20 µl, including the following components: 4 µl of 5x buffer, 5.2 µl of deionised water, 3 µl of CDV-1 primer mixture (0.6 µM concentration), 3 µl of CDV-2 primer mixture (0.6 µM concentration), 0.4 µl of 10x dNTP mix, 1.2 µl of 25 mM $MgCl_2$, 3 µl of cDNA with a concentration of 25 ng/µl, 0.2 µl of Taq DNA polymerase. This configuration of the reaction mixture was used for subsequent PCR to amplify the gene under study. Reverse primers were mixed with reverse primers and forward primers were mixed with forward primers, the mixing ratio being 50:50. During PCR, annealing was performed at temperatures of 53°C, 54°C, 55°C, 55°C, 56°C and 57°C for 40 seconds, elongation at 72°C continued for 45 seconds and denaturation at 95°C for 30 seconds. These conditions were used to perform PCR using positive samples known to contain the causative agent of carnivore plague (Fig. 8).

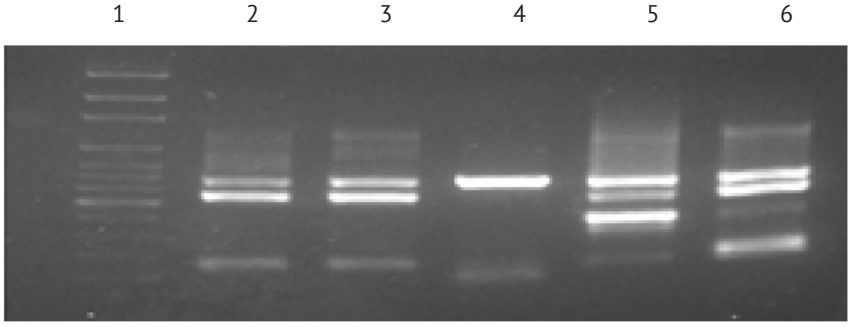

Figure 8. *Spectra of amplification products at different temperature regimes*
Note: *1 – 100 bp molecular weight marker; 2 – 53°C; 3 – 54°C; 4 – 55°C; 5 – 56°C; 6 – 57°C*
Source: *compiled by the authors*

Different primer annealing temperatures were found to produce PCR products, but the optimum temperature to obtain a clear and specific result was 55°C. Different annealing temperatures (53°C, 54°C, 56°C, and 57°C) lead to the appearance of non-specific products, as shown by the luminescent bands at the level of 500-600 bp relative to the marker. The annealing temperature of 55°C is optimal because at this temperature there are no non-specific products and the product obtained is at 681 bp, indicating the high specificity of the primer used. To confirm these results, an additional PCR was performed using newly selected primers and 55°C annealing temperature (Fig. 9).

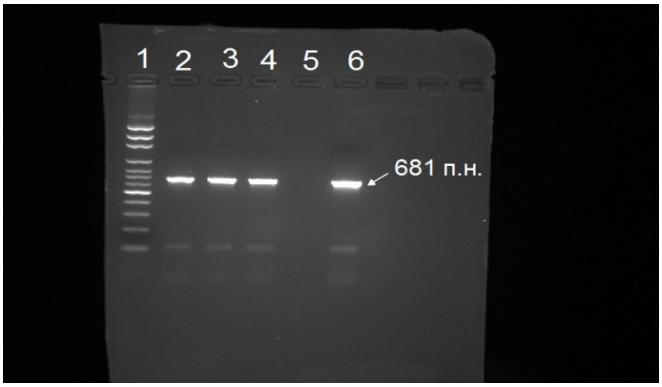

Figure 9. *Results of PCR product amplification of 681 nucleotide pairs in length*
Note: *Line 1 – molecular weight marker, lines 2 to 4 – known samples in advance, line 5 – negative control, line 6 – positive control*
Source: *compiled by the authors*

Thus, the selected primers were successfully optimised for multiplex PCR. This is confirmed by the fact that the PCR products obtained are at the expected nucleotide length of 681 bp. No dimers or other non-specific products were detected during multiplex PCR. This is important because their presence can negatively affect the final results and accuracy of the assay. The negative control (sample No. 5) confirms the purity of the results. The absence of timers and dimers in the negative control indicates that the selected primers do not possess self-sparking nucleotide sites. It was experimentally confirmed that the selected primer is successfully used in the polymerase chain reaction for the detection of the causative agent of plague in carnivores. Thus, the approach to selecting and optimising primers for multiplex PCR is successful and the results demonstrate the specificity and analysis purity.

DISCUSSION

Carnivore plague is a disease that has historically had a significant impact on domestic dogs. First described in ancient times, this infection still poses a serious threat to animal health, especially in regions with inadequate sanitary infrastructure. Rapid and accurate detection of the presence of virus in infected samples is essential to effectively control the spread of rinderpest (Uakhit *et al.,* 2022; Takeda *et al.*, 2020; Tan *et al.,* 2011). Carnivore plague is characterised by rapid spread in the carnivore population and remains a major challenge for veterinary medicine, requiring continuous improvement of diagnostic and prophylactic methods to control and prevent the consequences of epidemics. The contagiousness of the virus emphasises the need for rapid control measures in the event of outbreaks. Effective prevention, including vaccination and quarantine strategies, also plays an important role in managing the risks and limiting the negative consequences of rinderpest epidemics.

Studies conducted by authors such as K. Ergunay *et al.* (2022) and Y. Yamamoto *et al.* (2021) emphasise the difficulties in the treatment of carnivore plague. The peculiarities of this disease are the involvement of the central nervous system, which makes it difficult to treat. To date, no effective therapy exists, making carnivore plague a serious threat to animals. One of the factors hampering the development of effective therapies is the peculiarities of the reproductive cycle of the virus that causes the disease. These peculiarities create difficulties in the search for approaches capable of targeting the virus and interrupting its life cycle. In addition, differential diagnosis of rinderpest is important, given that many other diseases show similar clinical signs, such as parvovirus enteritis and other diseases. Distinguishing between these diseases is key to accurate diagnosis and the subsequent provision of appropriate treatment, as this study also confirms.

M. Karki *et al.* (2022) noted that the key to controlling rinderpest is early and accurate detection of the presence of the virus before it crosses the blood-brain barrier. This is of particular importance to pet owners and veterinary professionals who care for sick animals. Treatment depends on the form of the disease

(respiratory, intestinal, or cutaneous), so early and accurate diagnosis becomes crucial. Traditional methods for diagnosing rinderpest virus can have disadvantages such as low specificity or limited ability to detect different strains. Multiplex primer testing seeks to overcome these limitations and represents a promising and effective approach, providing more accurate and rapid detection of the virus while minimising the disadvantages of traditional methods.

The multiplex primer-based methodology for the diagnosis of rinderpest virus represents an advanced approach in veterinary medicine. Multiplex primers, short nucleotide sequences, are designed to detect multiple RNA variants of the virus, providing efficient detection and differentiation of different strains. This specificity for different strains is a key advantage of the method, allowing more accurate characterisation of the virus and targeted control measures. K. Beus *et al.* (2023) confirm the focus on optimising PCR reaction conditions. Thus, during PCR using multiplex primers, virus RNA is amplified, generating sufficient material for subsequent analysis. Experimental steps involve optimising reaction conditions, such as annealing temperature and PCR time, to ensure maximum specificity and efficiency. The resulting products are subjected to confirmation assays such as electrophoresis, using control samples to verify the results.

K. Altay *et al.* (2023) and R.M. Nayak *et al.* (2020), focused on innovative methods, and support efforts in the use of multiplex primers. The authors believe that polymerase chain reaction, as a high-tech method, allows not only the diagnosis of infection but also the differential analysis of the genetic material of the virus. The intervention of multiplex primers in the PCR process represents an innovative approach that allows simultaneous consideration of multiple sites of the virus RNA, which significantly increases the efficiency of testing. Z. Lanszki *et al.* (2021) and Y. Gedik *et al.* (2021), emphasising multiplex methods and their advantages, are also consistent with the discussion. Multiplex primers are designed to specifically amplify multiple genetic sites, making the method more sensitive and specific. This approach is particularly useful when the virus may manifest in different variants or when multiple strains need to be detected simultaneously. The use of multiplex primers also shortens the analysis time and reduces the likelihood of false positives, which is essential for rapid diagnostic measures, as also noted in the study conducted.

Polymerase chain reaction in the laboratory diagnosis of infections is characterised by high efficiency due to its rapidity, unrivalled sensitivity, and high specificity. It allows the detection of microorganisms even at very low concentrations, e.g. 1-10 pathogens in a sample. RNA of infectious agents is successfully extracted from various biological fluids, tissues, and from the environment such as soil, water, and food. PCR is effective in detecting bacteria, fungi, parasites, and viruses (Saltik & Kale, 2020; Worsley-Tonks *et al.,* 2021). R. Uakhit *et al.* (2022) state that the PCR method is characterised by a sensitivity sufficient to detect 10-100 pg of viral RNA in a sample. This method allows rapid and quantitative detection of RNA even in unconcentrated samples. It also enables the examination of various surfaces (animal bodies, care items, soil, feed) for the presence of viruses. However, the author notes that the hypersensitivity of the method can sometimes lead to false positives.

F. Alfano *et al.* (2022), D.K. Yang *et al.* (2020), and A. Beineke *et al.* (2009) applied polymerase chain reaction to detect viral RNA in biological samples. According to the authors, the application of PCR is in the works to become a powerful tool for determining the presence and type of virus in a variety of biological samples, including tissues, fluids, and the environment. The results of their research propel PCR into the role of a key technique in the diagnosis of viral diseases, emphasising its importance in modern medical science and veterinary medicine. They open new horizons in the understanding of viral pathologies and provide the basis for the development of more effective treatment and prevention methods. I. Ricci *et al.* (2021) also believe that the use of multiplex primers in PCR represents a modern diagnostic method with improved specificity and sensitivity. The use of multiplex primers in the polymerase chain reaction method for the detection of RNA of rinderpest virus is an important method in veterinary diagnostics, providing effective detection and identification of different strains of the virus, which also confirms the conducted study.

All studies reviewed, including the present study, emphasise the need for effective control of rinderpest, and detection of the virus before central nervous system involvement, and encourage the use of innovative diagnostic methods. However, in comparison to the authors' results, the study conducted adds to the existing body of knowledge by bringing innovation in diagnostic methods for carnivore plague. Understanding the commonalities and differences helps to create a more complete picture of the issues, which is an important contribution to the field of veterinary medicine. Thus, the polymerase chain reaction method is a powerful tool for detecting and analysing the genetic material of microorganisms, including rinderpest virus. However, the efficiency of PCR directly depends on the use of specific and highly efficient primers. Therefore, multiplex primers represent a promising approach for the simultaneous detection of several virus genes, which increases the sensitivity and accuracy of diagnosis.

CONCLUSIONS

The carnivore plague virus exhibits phylogenetic branching due to the effects of various environmental factors. This variability is manifested through

nucleotide mutations, resulting in the formation of different genetic lineages of the virus. The study aimed to select multiplex primer pairs for the detection of RNA of carnivore plague virus by PCR represents a significant step in the field of veterinary diagnostics and animal disease control.

As a result of laboratory experiments, multiplex pairs of primers were selected for the detection of plague virus RNA using PCR in biological materials such as blood serum and eye conjunctiva washes containing the virus. A distinctive feature of the use of these primers is the use of two pairs of the same gene region with different variable sequences. These sequences are specific for all strains of carnivore plague virus. Both pairs of primers are applied simultaneously at equal concentrations and under the same conditions in the polymerase chain reaction, and the results obtained demonstrate the high specificity of the designed primers. Additional PCR performed using the selected primers and optimal annealing temperature confirmed the successful amplification of specific sites and the high specificity of the applied primers. The absence of dimers and non-specific products in the negative control was observed, reinforcing the purity and reliability of the results obtained. Consequently, the results convince us that these primers used in multiplex PCR can effectively detect RNA of rinderpest virus of different strains circulating worldwide.

The developed multiplex primers represent an innovative and practically important method for the diagnosis of rinderpest virus, with great potential for application in veterinary practice and animal disease control. Further research on this issue should focus on the interaction of the rinderpest virus with other infections that may affect diagnosis and epidemiology.

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None.

CONFLICT OF INTEREST

None.

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Використання мультиплексних праймерів для виявлення РНК вірусу чуми м'ясоїдних методом ПЛР

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Анотація. З урахуванням високої контагіозності та швидкого поширення вірусу чуми м'ясоїдних, своєчасна та точна діагностика відіграє ключову роль у запобіганні епідеміям та вжиття заходів щодо контролю за захворюванням. Мета дослідження – провести оцінку ефективності використання мультиплексних праймерів у методі полімеразної ланцюгової реакції для виявлення рибонуклеїнової кислоти вірусу чуми м'ясоїдних. Дослідження включало аналіз зразків, таких як сироватка крові і змив з кон'юнктиви очей у 50 тварин з клінічними проявами захворювання. В рамках експерименту проводився збір клінічних зразків, таких як сироватка крові та змив з кон'юнктиви очей. Отримані результати демонструють високу специфічність розроблених праймерів. Ці праймери виділяються тим, що вони використовують дві пари однієї й тієї ж ділянки гена з різними варіабельними послідовностями, які є специфічними для всіх штамів вірусу чуми м'ясоїдних. У процесі полімеразної ланцюгової реакції обидві пари праймерів застосовуються одночасно за рівних концентрацій та однакових умов. Додаткова полімеразна ланцюгова реакція, проведена з використанням цих праймерів при оптимальній температурі відпалу, підтвердила успішну ампліфікацію та специфічність праймерів. Відсутність димерів та неспецифічних продуктів у негативному контролі підтверджує чистоту та надійність отриманих результатів. Таким чином, дані результати переконують у тому, що використання цих мультиплексних праймерів полімеразної ланцюгової реакції дозволяє ефективно виявляти рибонуклеїнову кислоту вірусу чуми м'ясоїдних різних штамів. Розроблені мультиплексні праймери представляють інноваційний метод для діагностики вірусу чуми м'ясоїдних з потенціалом для застосування у ветеринарній практиці та контролю захворювань тварин

Ключові слова: олігонуклеотиди; специфічність; відпал; ефективність діагностики; біологічні зразки; ампліфікація