

UDC:616.993

DEVELOPMENT OF A PCR TEST SYSTEM FOR THE GENETIC DIAGNOSIS OF HEMORRHAGIC FEVER WITH RENAL SYNDROME

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ABSTRACT

Hemorrhagic fever with renal syndrome (HFRS) is an acute zoonotic disease caused by orthohantaviruses. Reliable molecular diagnostics are essential for early detection and epizootiological surveillance. This study aimed to develop and validate a reverse transcription quantitative real-time PCR (RT-qPCR) assay for detection of HFRS-associated orthohantavirus RNA. Species-specific primers (HV P1-F and HV P1-R) and a TaqMan probe (HV P1) targeting the nucleocapsid protein (N) gene within the S segment were designed. A plasmid positive control containing a 242-bp target fragment was constructed. Analytical sensitivity, specificity, reproducibility, and repeatability were evaluated. Field testing included samples from 343 small mammals collected in endemic areas of the West Kazakhstan Region. The assay consistently detected the plasmid control from 1 ng/μL to 1 fg/μL, with Ct values of 37.35 and 37.73 at 1 fg/μL. No specific amplification was observed at 100 ag/μL. A strong linear relationship between Ct values and template concentration was obtained ($R^2 = 0.998$). No false-positive reactions occurred with tick-borne encephalitis virus or Crimean-Congo hemorrhagic fever virus RNA. Reproducibility and repeatability showed 100% concordance of qualitative results. HFRS-associated orthohantavirus RNA was detected in 45 of 123 pooled field samples. The minimum infection prevalence was 13.1%, reaching 15.3% in *Myodes glareolus* and 13.3% in *Apodemus uralensis* and *Microtus arvalis*. The developed one-step RT-qPCR assay demonstrated high sensitivity, specificity, and reproducibility and is suitable for routine diagnostics and surveillance of natural HFRS foci.

Keywords: hemorrhagic fever with renal syndrome, RT-qPCR, molecular diagnosis, genetic diagnostics, primers, TaqMan probe, validation, hantavirus, orthohantavirus.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) remains one of the most significant natural focal viral infections in Eurasia, causing tens of thousands of cases annually, particularly in Asian and Eastern European countries [1–6]. Contemporary climate change, anthropogenic transformation of ecosystems, rodent migration, and urbanization contribute to the expansion of reservoir species ranges, thereby increasing the risk of the emergence of new natural foci of the infection [7–10].

Hemorrhagic fever with renal syndrome (HFRS) is a viral zoonotic natural focal disease characterized by pronounced intoxication, fever, specific vasculitis accompanied by hemorrhagic manifestations, and the development of renal inflammation with damage to the renal tubules and interstitium, resulting in impaired renal filtration and disruption of fluid and electrolyte homeostasis [11]. HFRS is caused by orthohantaviruses belonging to the family *Hantaviridae* and genus *Orthohantavirus* [12]. Orthohantaviruses exhibit considerable ecological plasticity, a broad range of natural reservoir hosts, and complex patterns of circulation within small-mammal populations [13–15].

The genome of orthohantaviruses consists of three segments of negative-sense single-stranded RNA: S, M, and L. The S segment encodes the nucleocapsid (N) protein, which binds viral RNA and participates in the formation of the ribonucleoprotein complex. N-gene sequences are widely used in the development of molecular assays for the detection of orthohantaviruses [16–18].

In the Republic of Kazakhstan, official surveillance and registration of HFRS cases have been conducted since 2000, with 302 cases recorded during the period from 2000 to 2024. The disease occurs both as sporadic cases and outbreaks, with pronounced seasonality and a predominance among rural populations [17, 19–21]. The seasonal peak in hospitalizations occurs from November to January, accounting for up to 87.6% of cases, with the highest incidence observed in November. This pattern is associated with the mass migration of rodents into human dwellings and agricultural structures during the cold season. In natural foci, the pathogen is maintained predominantly through circulation within rodent populations, including *Microtus arvalis*, *Myodes glareolus*, *Apodemus uralensis*, and other species [22, 23].

Conventional diagnostic methods for HFRS have substantial limitations. Classical virological investigations require propagation of the pathogen in specialized cell lines under BSL-3 containment conditions, which are available only in specialized virological laboratories. This process is labor-intensive and does not always yield successful results [24–26]. Serological methods may have insufficient specificity due to cross-reactivity with other orthohantaviruses and tick-borne pathogens and are also predominantly retrospective in nature. IgM antibodies against HFRS-associated viruses generally become detectable from 3–4 days after disease onset, whereas IgG antibodies typically appear by days 10–14 [11, 26–28].

In this context, RT-qPCR methods are considered a gold-standard approach for early diagnosis, providing high sensitivity, specificity, and rapid detection of viral RNA [29, 30]. International recommendations from the World Health

Organization (WHO), the United States Centers for Disease Control and Prevention (CDC), and the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines emphasize the need for validated RT-qPCR-based molecular platforms adapted to the genetic diversity and regional characteristics of circulating orthohantavirus strains [31–33]. Such approaches provide high analytical sensitivity, specificity, and reproducibility under the specific epidemiological and ecological conditions of individual natural foci.

The closest analogue to the proposed test system is the “OM-HFRS-RV” assay (Registration Certificate No. P3H 2021/15205, dated March 23, 2021, Russian Federation; “OM” product line developed by Syntol) for the detection of hantavirus RNA in biological specimens using real-time PCR. However, this assay has a limitation in that it requires a two-step reaction consisting of reverse transcription followed by amplification of the target RNA fragment. This increases the overall analysis time and reagent consumption [34].

The present study describes the design, development, and comprehensive validation of a novel one-step RT-qPCR test system for the molecular diagnosis of HFRS, including its application in field studies for the epizootiological surveillance of natural foci in Kazakhstan.

The technical outcome of the study is the development of specific oligonucleotides, establishment of positive controls, and detection of HFRS-associated viral RNA using real-time reverse transcription PCR.

MATERIALS AND METHODS

Oligonucleotide Design and Synthesis

An important step in the development of the PCR-based test system was the selection of an appropriate genetic target. A fragment of the *N* gene located within the *S* segment of the negative-sense single-stranded RNA genome was selected as the target. The target region was selected based on a comparative analysis of *S*-segment sequences of HFRS-associated orthohantaviruses available in the GenBank/NCBI databases, taking into account its suitability for primer and hydrolysis probe design [16, 35].

During oligonucleotide design, multiple sequence alignment of the *N* gene was performed. Sequence conservation within the proposed primer- and probe-binding regions, amplicon length, melting temperatures (*T_m*), GC content, and the potential for secondary structure formation were evaluated. The design process followed the recommendations of the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines and aimed to minimize the risk of nonspecific amplification [33, 36, 37].

The specificity of the selected primer and probe sequences was assessed *in silico* using BLAST and Primer-BLAST by comparison against sequences available in the NCBI GenBank database. Potential homology with non-target viral and bacterial genomes was also evaluated. The analysis revealed no significant sequence matches that could potentially result in nonspecific amplification of the target fragment.

The self-complementarity of the oligonucleotides and the potential formation of hairpin structures, homodimers, and heterodimers were additionally assessed using OligoAnalyzer

and AutoDimer software. Sequences with unfavorable predicted characteristics were excluded from further experimental evaluation [38, 39].

The following oligonucleotide sequences were selected for the detection of the *N* gene fragment within the *S* segment and are presented in Table 1.

Table 1. Oligonucleotide sequences for the detection of the HFRS-associated orthohantavirus *N* gene

Primer/Probe	Nucleotide sequence	Length	<i>T_m</i> , °C
HV P1-F	GATTGAAGATATT-GAGTCACC	21	52
HV P1-R	GTTGTATCCCCATT-GATTGTG	21	52
HV P1 probe	FAM- ATATGG-GTTTTTGCTGGAG-CACC-BHQ1	23	52

Oligonucleotide synthesis was performed at the National scientific center for especially dangerous infections (NSCEDI) using standard phosphoramidite chemistry on an H6 automated DNA/RNA synthesizer (K&A Laborgeraete GbR, Germany). Phosphoramidites from Glen Research Corporation (USA) and reagents from emp BIOTECH (Germany) were used. Purification of the aqueous primer solutions from synthesis by-products was performed using Centri•Pure N10 columns (emp BIOTECH GmbH, Germany).

The purified aqueous primer solutions were concentrated to dryness using a CentriVap concentrator (Labconco, USA) and subsequently resuspended in deionized water to a final concentration of 100 μM. Primer concentrations were determined using a NanoPhotometer® P-300 spectrophotometer (Implen, Germany). The probes were synthesized by BIGL (Novosibirsk, Russia). The lyophilized probes were reconstituted to a concentration of 100 μM.

To assess the diagnostic performance of the assay based on the developed oligonucleotide system, a positive control was constructed using a recombinant plasmid containing the synthesized target fragment of the *N* gene from the *S* segment of an HFRS-associated orthohantavirus. The nucleotide sequence of the target insert is presented below:

GATTGAAGATATTGAGTCACCATCATCGATATGGGTTTTTGCTGGAGCACCTGACCGTTGTCCACCAACATGCCTATTCATTGCAGGGATTGCTGAACCTTGAGCATTCTTTTCTATCCTGCAGGACATGCGGAATACAATTATGGCCTCAAAGACAGTGCGGAACATCTGAAGAAAAGTTACGAAAGAAATCATCATTCTATCAGTCTTATCTCAGGCGAACACAATCAATGGGGA-TACAAC (242 bp).

RT-qPCR optimization and amplification conditions

RT-qPCR conditions were optimized using the plasmid positive control and a negative control containing deionized water instead of the template. The annealing temperature was determined empirically based on the intensity of the specific amplification signal and the absence of amplification in the negative control. The amplification program is presented in Table 2.

Table 2. RT-qPCR amplification conditions

№	Stage	Temperature	Time	Number of Cycles
1	Temperature hold	50 °C	10 min	1
2	Initial denaturation	95°C	2 min	1
3	Denaturation	95°C	15 sec	5
	Annealing	60°C	30 sec	
	Extension	72 °C	15 sec	
4	Denaturation	95°C	15 sec	40
	Annealing	60°C	45 sec (fluorescence detection: Green, Yellow)	
	Extension	72 °C	sec	

RT-qPCR was performed using a Rotor-Gene 6000 real-time PCR cycler (Corbett Research, Australia), with fluorescence generated by the FAM reporter dye detected in the Green channel. The total reaction volume was 25 µL and consisted of 5.0 µL of 5× qPCR reaction mix, 1.0 µL of forward primer (20 pmol/µL), 1.0 µL of reverse primer (20 pmol/µL), 0.5 µL of probe (10 pmol/µL), 5.0 µL of sample RNA, and sterile deionized water to the final volume. The final concentrations of the primers and probe were 0.8 µM and 0.2 µM, respectively. A run was considered valid when a typical sigmoidal amplification curve was observed for the positive control and no increase in fluorescence signal was detected for the negative control. A sample was considered positive when the amplification curve crossed the predefined threshold within 45 cycles; in the absence of such a curve, the result was interpreted as negative.

PCR validation

To confirm the analytical performance characteristics of the developed reagent kit for the detection of HFRS-associated orthohantavirus RNA by reverse transcription quantitative real-time PCR (RT-qPCR), a validation study was performed. The validation study was designed to assess the analytical sensitivity, analytical specificity, reproducibility, and repeatability of the assay.

A preliminary assessment of the analytical sensitivity of the PCR component of the test system was performed using serial tenfold dilutions of the plasmid positive control in accordance with established general approaches to the analytical validation of molecular diagnostic methods [40, 41]. The lowest concentration consistently detected across replicates was determined, and the linearity of the relationship between Ct values and the logarithm of template concentration was assessed. A statistically validated limit of detection (LoD) for the complete RT-qPCR method was not established within the scope of the present study.

Analytical specificity was evaluated using a panel of heterologous viruses circulating in Kazakhstan, including tick-borne encephalitis virus and Crimean-Congo hemorrhagic fever virus, in accordance with the requirements for differential diagnosis of natural focal infections.

Interlaboratory testing was performed in three structural divisions of the NSCEDI: the Molecular Genetic Research Laboratory, the Laboratory of Natural Focal Viral Infections, and the OBTC-IL. Testing was conducted by different operators on different days using Rotor-Gene 6000 and QuantStudio 5 real-time PCR instruments, as well as auxiliary reagents

and consumables from different production lots.

RNA extraction and field study design

RNA was extracted from viral samples and field-material suspensions using the QIAamp RNA Mini Kit for 250 tests (QIAGEN, Germany), according to the manufacturer's instructions. The extracted RNA samples were subsequently used as templates to assess the specificity of the developed primer set.

The field study employed an observational descriptive-analytical design. Small mammals were trapped in August–September 2025 in HFRS-endemic districts of the West Kazakhstan Region, including Burlinsky, Shyngyrlau, Terektinsky, and Baiterek districts. Trapping was performed using “Gero” traps baited with standard bread bait and placed at 2-m intervals. Species identification was based on morphological characteristics.

Lung tissue samples were pooled by species and trapping location, resulting in 123 pooled samples derived from 343 individual animals. The minimum infection rate (MIR) was calculated as the number of positive pools divided by the total number of individuals tested and expressed as a percentage.

RESULTS

Analytical sensitivity of the test system

A preliminary assessment of the analytical sensitivity of the amplification component of the developed RT-qPCR test system was performed using serial tenfold dilutions of the positive plasmid control containing the target N gene fragment of the S segment of the orthohantavirus. Positive control concentrations ranging from 1 ng/µL to 100 ag/µL were tested. The plasmid DNA concentration was determined using the Quant-iT dsDNA HS Kit (Invitrogen, USA) on a Qubit fluorometer (Invitrogen, USA).

Each concentration was tested in duplicate. Serial tenfold dilution of the positive plasmid control resulted in a consistent increase in Ct values in the Green/FAM channel. Stable detection of the target fragment in both replicates was observed over the concentration range from 1 ng/µL to 1 fg/µL.

At a concentration of 1 fg/µL, a specific signal was detected in both replicates, with Ct values of 37.35 and 37.73, respectively, meeting the predefined criterion for a positive result ($Ct \leq 38$). The mean Ct value was 37.54 ± 0.27 . At a concentration of 100 ag/µL, no specific signal was detected in either replicate.

Thus, the lowest concentration of the positive plasmid

Table 3. Results of the analytical sensitivity assessment of the HV P1-F/HV P1-R primer system and HV P1 probe

Concentration	Green/FAM, Ct repeat 1	Green/FAM, Ct repeat 2	Mean Ct $\pm$ SD	Result
1 ng/ μ L	12,55	12,48	12,52 $\pm$ 0,05	2/2 positive
100 pg/ μ L	15,51	15,33	15,42 $\pm$ 0,13	2/2 positive
10 pg/ μ L	20,43	20,97	20,70 $\pm$ 0,38	2/2 positive
1 pg/ μ L	25,48	25,12	25,30 $\pm$ 0,25	2/2 positive
100 fg/ μ L	28,83	29,25	29,04 $\pm$ 0,30	2/2 positive
10 fg/ μ L	33,86	33,01	33,44 $\pm$ 0,60	2/2 positive
1 fg/ μ L	37,35	37,73	37,54 $\pm$ 0,27	2/2 positive
100 ag/ μ L	negative	negative	—	0/2
K–	negative	—	—	negative
K+	29,13	—	—	positive

Table 4. Results of the analytical specificity assessment

No.	Sample	Type	Green/FAM, Ct $\leq$ 38	Yellow/R6G
1	SARS-CoV-2 No. KA310	Sample	negatively	negatively
2	SARS-CoV-2 No. KA310	Sample	negatively	negatively
3	Tick-borne encephalitis virus No. 22	Sample	negatively	negatively
4	Tick-borne encephalitis virus No. 22	Sample	negatively	negatively
5	CCHF virus No. 20	Sample	negatively	negatively
6	CCHF virus No. 20	Sample	negatively	negatively
7	Rickettsia spp. No. 24	Sample	negatively	negatively
8	Rickettsia spp. No. 24	Sample	negatively	negatively
9	K–	Negative control	negatively	negatively
10	K+ (HFRS)	Positive control	15,63	16,35

control consistently detected in both replicates under the conditions of this experimental series was 1 fg/ μ L. Over the concentration range from 1 ng/ μ L to 1 fg/ μ L, a linear relationship was observed between the Ct value and the logarithm of the template concentration, with a coefficient of determination of $R^2 = 0.998$.

Since the positive control consisted of plasmid DNA and did not undergo the reverse transcription step, the obtained data primarily characterize the analytical sensitivity of the developed RT-qPCR test system. A statistically supported determination of the limit of detection (LoD) for the complete method requires additional studies using an RNA control and an increased number of replicates around the borderline concentrations. The results are presented in Table 3.

Analytical Specificity of the Test System

The analytical specificity of the developed test system was assessed based on the absence of cross-amplification when testing nucleic acids from heterologous pathogens. The test panel included SARS-CoV-2, tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus, and *Rickettsia* spp. A positive specific signal in the Green/FAM channel was expected in the presence of the target N gene fragment of the S segment of the HFRS-associated orthohantavirus.

No specific amplification in the Green/FAM channel was detected for any of the heterologous samples included in the test panel. The HFRS positive control demonstrated a specific signal with a Ct value of 15.63. No amplification was observed in the negative control.

Thus, no cross-reactivity of the developed primer and probe system was detected within the tested panel of heterologous pathogens. These findings demonstrate the analytical specificity of the test system with respect to the non-target pathogens included in the study. The results are presented in Table 4.

Reproducibility and repeatability

To assess reproducibility, different concentrations of the positive plasmid control (10 pg, 1 pg, and 100 fg) from the developed HFRS diagnostic kit were tested. The assays were performed in three different laboratories by three different researchers on different days, using Rotor-Gene 6000 and QuantStudio 5 real-time PCR instruments, as well as reagents and consumables from different lots. The results are presented in Table 5.

Table 5. Reproducibility control of PCR results obtained using the HV P1-F/HV P1-R primer system and HV P1 probe

Date, device, operator	10 pg	1 pg	100 fg
June 4, 2025, Rotor-Gene 6000, Operator 1	+	+	+
June 5, 2025, QuantStudio 5, Operator 2	+	+	+
June 6, 2025, Rotor-Gene 6000, Operator 3	+	+	+
Overall result	3/3 (100%)	3/3 (100%)	3/3 (100%)

The study demonstrated that RT-qPCR performed using the developed reagent kit for HFRS diagnosis in three different laboratories, by different researchers, and on different real-time PCR instruments produced identical amplification results. A consistent specific fluorescent signal was detected at all three concentrations tested.

Repeatability assessment. When PCR was performed using three concentrations of the positive plasmid control (10 pg, 1 pg, and 100 fg) under identical conditions by the same operator in duplicate, complete agreement of the results was observed. These findings confirm the high repeatability of the method when using the developed reagent kit for HFRS diagnosis.

Field study results

The performance of the developed system was additionally evaluated by RT-qPCR using rodent organ suspensions collected in the West Kazakhstan Region during the summer–autumn period of 2025. The material had previously been examined at the laboratory of natural focal viral infections of the NSCEDI. Testing of the samples using the developed PCR test system was performed in triplicate.

The field validation study included 343 small mammals captured in four districts of the West Kazakhstan Region: Burlinsky, Shyngyrlau, Terektinsky, and Baiterek. In terms of species composition, the Ural field mouse (*Apodemus uralensis*) predominated, accounting for 74.3% of all captured rodents. The second most abundant species was the bank vole (*Myodes glareolus*), accounting for 17.2%. The remaining species were captured less frequently: the common vole (*Microtus arvalis*) accounted for 4.4%, the striped field mouse (*Apodemus agrarius*) for 1.5%, while various species of shrews accounted for the remainder. Table 6 presents information on the captured animal species and the results of their PCR testing.

Table 6. Results of testing of field samples

Species	Individuals	Pools	PCR +	MIR (%)*
Wood mouse (<i>Apodemus uralensis</i>)	255	51	34	13.3
Bank vole (<i>Myodes glareolus</i>)	59	53	9	15.3
Common vole (<i>Microtus arvalis</i>)	15	11	2	13.3
Other species	14	8	0	0
Total	343	123	45	13.1

*MIR (Minimum Infection Rate), % — minimum infection rate.

Table 7. PCR data from field material with the HV P1-F/HV P1-R primer system and HV P1 probe.

Sample No.	Sample Type	Ct Value of Gene N	Result
1	Sample №1	27.27	Positive
2	Sample №2	29.25	Positive
3	Sample №3	32.44	Positive
4	Negative control	Negative	Negative
5	Positive control	29.67	Positive

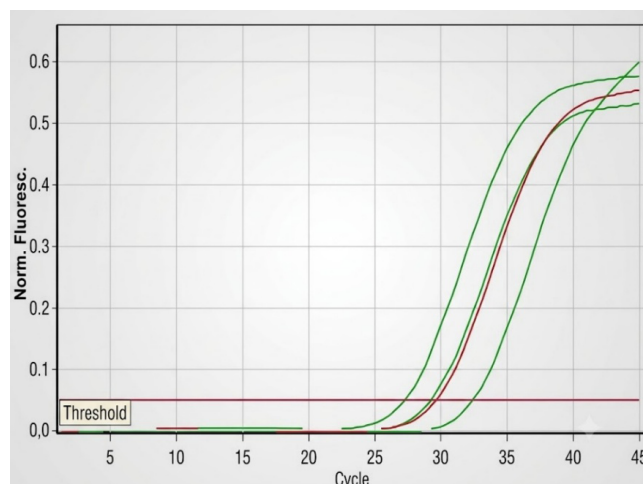


Figure 1. Results of PCR testing of field samples using the HV P1-F/HV P1-R primer system and HV P1 probe

The HFRS virus target gene was detected in three rodent species that were dominant among the captured animals: wood mice, bank voles, and common voles.

Table 7 and figure 1 present the positive results obtained from testing rodent organ suspensions by RT-qPCR using the HV P1-F/HV P1-R primers and HV P1 probe. Typical amplification curves crossing the threshold line were detected in the Green/FAM channel.

Analysis of the geographical distribution of infected rodents revealed a focal pattern of virus circulation. The highest number of positive samples was recorded in the Terektinsky (16 samples), Baiterek (13 samples), and Shyngyrlau (14 samples) districts, whereas lower activity was observed in the Burlinsky district (2 samples).

In 2025, 41 laboratory-confirmed HFRS cases were reported in the West Kazakhstan Region. The highest incidence was recorded in the Baiterek district (34.1%) and in the city of Uralsk (39.0%), which is surrounded by the territory of the Baiterek district.

DISCUSSION

The development and validation of molecular diagnostic tools for HFRS represent a significant advancement in the laboratory diagnosis of this important zoonotic infection [16, 42, 43]. Our study demonstrates the successful development of a highly specific and sensitive PCR test system that addresses the limitations of existing diagnostic approaches and provides substantial improvements over two-step protocols [18, 44].

The proposed HV P1-F/HV P1-R primer system and HV P1 hydrolysis probe are designed to detect a specific fragment of the N gene within the S segment of the orthohantavirus. A preliminary assessment of the analytical sensitivity of the PCR component of the test system using a plasmid positive control demonstrated consistent detection of the target sequence at concentrations down to and including 1 fg/μL. These performance characteristics are comparable to those reported for published RT-qPCR approaches for orthohantavirus detection [35, 45–47].

Statistical confirmation of the limit of detection (LoD) requires testing a larger number of replicates across the borderline concentration range. Since analytical sensitivity was

assessed using plasmid DNA, the obtained results primarily characterize the PCR component of the developed system and do not account for the efficiency of RNA extraction and reverse transcription.

The analytical specificity of the test system was assessed using nucleic acids from heterologous pathogens, including SARS-CoV-2, tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus, and bacteria of the genus *Rickettsia*. Each sample was tested in duplicate. No specific FAM signal was detected in any sample from the heterologous panel. These findings demonstrate the absence of cross-amplification within the tested panel and are consistent with the results of the *in silico* specificity assessment of the primers and probe.

The one-step RT-qPCR format combines reverse transcription and amplification in a single reaction tube, reducing the overall assay time and the number of handling steps while also decreasing the risk of contamination. These features facilitate the application of the test system in routine laboratory and epizootiological investigations [45, 48].

The validation results demonstrated excellent reproducibility and repeatability, which are critical characteristics for routine diagnostic application. Complete (100%) agreement of results obtained with different operators, instruments, and reagent lots indicates high method stability under different laboratory conditions.

The field study confirmed active circulation of HFRS-associated orthohantavirus in natural foci of the West Kazakhstan Region. The overall minimum infection rate (MIR) of 13.1% indicates moderate to high viral activity, with Ural field mice (*Apodemus uralensis*) and bank voles (*Myodes glareolus*) playing an important role in maintaining the infection. The observed focal distribution pattern is consistent with the ecology of hantavirus transmission and highlights the need for targeted surveillance in areas at increased risk.

The one-step RT-qPCR format provides significant advantages over two-step protocols, including reduced assay time, lower contamination risk, and improved workflow efficiency [18]. This addresses a key limitation of the closest analogue, the OM-HFRS-RV kit, which requires a two-stage reaction, thereby increasing analysis time and reagent consumption.

Improved efficiency and analytical sensitivity in the molecular testing of field samples by RT-qPCR expand the informational capacity of laboratory surveillance while simplifying and shortening the analytical process. The developed primer set also provides a basis for local production of the test system, enabling broader application of RT-qPCR for routine epizootiological surveillance of HFRS-endemic areas while potentially reducing diagnostic costs.

Global climate change and the associated overall warming trend may contribute to further expansion of HFRS through changes in the geographic ranges of vectors and viral reservoirs. The developed test system may therefore be recommended not only for pathogen identification but also for screening field samples during routine surveillance of endemic and potentially emerging natural foci [11, 50, 51].

The development of a domestic test system also has strategic importance for strengthening the independence of regional laboratory diagnostic services in Kazakhstan, reducing costs, and enhancing the national biological safety system [52]. In

the future, the system could be integrated into comprehensive molecular surveillance programs, including the genotyping of circulating orthohantavirus strains.

CONCLUSION

As a result of this study, a novel RT-qPCR test system, “NSCEDI HFRS Virus RT-qPCR,” was developed and validated for the detection of orthohantavirus RNA, the causative agent of hemorrhagic fever with renal syndrome (HFRS). The HV P1-F/HV P1-R primer system and HV P1 probe provided specific amplification of a fragment of the N gene within the S segment of the orthohantavirus genome.

When serial dilutions of the plasmid positive control were tested, the test system enabled detection of the target fragment down to a concentration of 1 fg/μL. The lowest concentration consistently detected in all replicates within the experimental series was 1 fg/μL.

No nonspecific amplification was observed within the tested panel of heterologous viruses. Testing under different laboratory conditions, by different operators, and using different real-time PCR instruments resulted in 100% qualitative agreement of the results. Repeat testing performed by the same operator under identical conditions also demonstrated complete agreement of qualitative results.

The performance of the test system was further demonstrated using field material, specifically organ suspensions from small mammals collected in natural foci in the West Kazakhstan Region. Testing of 343 animals represented by 123 pooled samples resulted in the detection of orthohantavirus RNA in 45 pools. These findings indicate active circulation of the HFRS-associated orthohantavirus among small-mammal populations in the surveyed area and confirm the suitability of the developed system for the epizootiological monitoring of natural foci of infection.

The one-step RT-qPCR format reduces the overall assay time and the number of manipulations involving the reaction mixture, thereby contributing to a lower risk of contamination. The developed test system can be used for field-sample screening, routine epizootiological investigations, and surveillance of orthohantavirus circulation in endemic areas.

Thus, the developed RT-qPCR test system represents a specific and practically relevant tool for the detection of orthohantavirus RNA in field material. Further studies using a representative set of clinical specimens are required to substantiate its application in the clinical diagnosis of HFRS in humans, including an expanded assessment of diagnostic sensitivity and specificity.

AUTHOR CONTRIBUTIONS

Conceptualization, A.K.R., N.B.T. and N.A.T.; methodology, A.K.R., Z.B.Z., D.T.Y., D.B.K. and A.K.S.; validation, A.K.R., B.Z.A., A.A.A. and N.B.T.; formal analysis, A.K.R., Z.B.Z. and D.T.Y.; investigation, A.K.R., Z.B.Z., D.T.Y., D.B.K., A.K.S., B.Z.A., A.A.A., S.K.U., G.Z.T. and B.A.I.; resources, N.A.T., N.U.K. and N.B.T.; data curation, A.K.R., Z.B.Z. and D.T.Y.; writing—original draft preparation, A.K.R. and N.B.T.; writing—review and editing, A.K.R., N.B.T., N.A.T. and N.U.K.; visualization, A.K.R., D.T.Y. and Z.B.Z.; supervision, N.A.T., N.B.T. and N.U.K.; project ad-

ministration, N.A.T. and N.B.T.; funding acquisition, N.A.T. and N.U.K. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was conducted within the framework of the scientific and technical program BR25293296, “Methodological Foundations for the Development and Implementation of Diagnostic Products for Especially Dangerous Infections to Ensure Biological Safety” (2024–2026), funded by the Ministry of Health of the Republic of Kazakhstan.

REFERENCES

- Jiang H., Zheng X., Wang L., Du H., Wang P., Bai X. Hantavirus infection: a global zoonotic challenge // *Virologica Sinica*. – 2017. – № 32(1). – P. 32–43. <https://doi.org/10.1007/s12250-016-3899-x>
- Xiao Y., Li Y., Li Y., Yu C., Bai Y., Wang L., Wang Y. Estimating the Long-Term Epidemiological Trends and Seasonality of Hemorrhagic Fever with Renal Syndrome in China // *Infection and Drug Resistance*. – 2021. – № 14. – P. 3849–3862. <https://doi.org/10.2147/IDR.S325787>
- Savitskaya T.A., Ivanova A.V., Chumachkova E.A., Pospelov M.V., Isaeva G.Sh., Reshetnikova I.D., Kabve E., Davidyuk Yu.N., Trifonov V.A., Ziatdinov V.B., Serova I.V. Overview of Hantavirus Infections in the World, the Epidemiological Situation on Hemorrhagic Fever with Renal Syndrome in the Russian Federation in 2021, and Forecast for 2022 // *Problems of Particularly Dangerous Infections*. – 2022. – № 2. – P. 54–63. <https://doi.org/10.21055/0370-1069-2022-2-54-63>
- Jiang H., Zheng X., Wang L., Du H., Wang P., Bai X. Hantavirus infection: a global zoonotic challenge // *Virologica Sinica*. – 2017. – № 32(1). – P. 32–43. <https://doi.org/10.1007/s12250-016-3899-x>
- Avšič-Županc T., Saksida A., Korva M. Hantavirus infections // *Clinical Microbiology and Infection*. – 2019. – № 21S. – P. e6–e16. <https://doi.org/10.1111/1469-0691.12291>
- European Centre for Disease Prevention and Control. Hantavirus infection // *Annual Epidemiological Report for 2023*. – Stockholm: ECDC, 2025. – URL: <https://www.ecdc.europa.eu/en/publications-data/hantavirus-infection-annual-epidemiological-report-2023>
- Klempa B. Hantaviruses and climate change // *Clinical Microbiology and Infection*. – 2009. – № 15(6). – P. 518–523. <https://doi.org/10.1111/j.1469-0691.2009.02848.x>
- Hofmann J., Heuser E., Weiss S., Tenner B., Schoppmeyer K., Esser J., Klier C., Drewes S., Ulrich R.G., Kruger D.H. Autochthonous ratborne Seoul virus infection in woman with acute kidney injury // *Emerging Infectious Diseases*. – 2020. – № 26(12). – P. 3096–3099. <https://doi.org/10.3201/eid2612.200708>
- Roda Gracia J., Schumann B., Seidler A. Climate Variability and the Occurrence of Human Puumala Hantavirus Infections in Europe: A Systematic Review // *Zoonoses and Public Health*. – 2015. – № 62(6). – P. 465–478. <https://doi.org/10.1111/zph.12175>
- Hjelle B., Torres-Pérez F. Hantaviruses in the Americas and their role as emerging pathogens // *Viruses*. – 2010. – № 2(12). – P. 2559–2586. <https://doi.org/10.3390/v2122559>
- Tariq M., Kim D.M. Hemorrhagic Fever with Renal Syndrome: Literature Review, Epidemiology, Clinical Picture and Pathogenesis // *Infection & Chemotherapy*. – 2022. – № 54(1). – P. 1–19. <https://doi.org/10.3947/ic.2021.0148>
- Krautkrämer E., Peintner L., Essbauer S. Hantaviruses in a Global Perspective // *Zoonoses: Infections Affecting Humans and Animals*. – Cham: Springer, 2022. https://doi.org/10.1007/978-3-030-85877-3_30-1
- Kabwe E., Davidyuk Y., Shamsutdinov A., Garanina E., Martynova E., Kitaeva K., Malisheni M., Isaeva G., Savitskaya T., Urbanowicz R.A., et al. Orthohantaviruses, Emerging Zoonotic Pathogens // *Pathogens*. – 2020. – № 9(9). – Article 775. <https://doi.org/10.3390/pathogens9090775>
- Fabbri D., Mirolo M., Tagliapietra V., Ludlow M., Osterhaus A., Beraldo P. Ecological determinants driving orthohantavirus prevalence in small mammals of Europe: a systematic review // *One Health Outlook*. – 2025. – № 7(1). – Article 15. <https://doi.org/10.1186/s42522-025-00136-w>
- Savitskaya T.A., Ivanova A.V., Chumachkova E.A., Pospelov M.V., Isaeva G.Sh., Reshetnikova I.D., Kabve E., Davidyuk Yu.N., Trifonov V.A., Ziatdinov V.B., Serova I.V. Overview of Hantavirus Infections in the World, the Epidemiological Situation on Hemorrhagic Fever with Renal Syndrome in the Russian Federation in 2021, and Forecast for 2022 // *Problems of Particularly Dangerous Infections*. – 2022. – № 2. – P. 54–63. <https://doi.org/10.21055/0370-1069-2022-2-54-63>
- Nunes B.T.D., de Mendonça M.H.R., Simith D.B., et al. Development of RT-qPCR and semi-nested RT-PCR assays for molecular diagnosis of hantavirus pulmonary syndrome // *PLoS Neglected Tropical Diseases*. – 2019. – № 13(12). – Article e0007884. <https://doi.org/10.1371/journal.pntd.0007884>
- Tukhanova N., Shin A., Turebekov N., et al. Molecular Characterisation and Phylogeny of Tula Virus in Kazakhstan // *Viruses*. – 2022. – № 14(6). – Article 1258. <https://doi.org/10.3390/v14061258>
- Machado A.M., de Souza W.M., de Pádua M., da Silva Rodrigues Machado A.R., Figueiredo L.T. Development of a one-step SYBR Green I real-time RT-PCR assay for the detection and quantitation of Araraquara and Rio Mamore hantavirus // *Viruses*. – 2013. – № 5(9). – P. 2272–2281. <https://doi.org/10.3390/v5092272>
- Grazhdanov A.K., Bidashko F.G., Zakharov A.V., Ayazbayev T.Z., Belonozhkina L.B., Andryushchenko A.V. Results of a ten-year study of hemorrhagic fever with renal syndrome in Kazakhstan // *Infection and Immunity: Proceedings of the 10th Congress of the All-Russian Scientific and Practical Society of Epidemiologists, Microbiologists, and Parasitologists*. – 2012. – № 2(1–2). – P. 134.
- Yegemberdiyeva R.A. Hemorrhagic fever with renal syndrome in Kazakhstan // *Medicine of Kyrgyzstan*. – 2011. – № 5. – P. 35–37.
- Maikanov N.S., Kanatkalieva Zh.A., Ramazanova S.I., Surov V.V., Tanitovsky V.A. Natural infection of small mammals with the HFRS virus in the West Kazakhstan region during the period 2001–2023 // *Steppes of Northern Eurasia*:

Proceedings of the 10th International Symposium. – 2024. – № 10. – P. 802–807. <https://doi.org/10.24412/cl-37200-2024-802-807>

22. Gubareva U., Horth R., Nabirova D., et al. Hantavirus antibody seroprevalence and risk factors among adults in West Kazakhstan, 2023 // *Frontiers in Public Health*. – 2025. – № 12. – Article 1519117. <https://doi.org/10.3389/fpubh.2024.1519117>

23. Tukhanova N., Shin A., Bakuli A., et al. Characterisation of Orthohantavirus Serotypes in Human Infections in Kazakhstan // *Viruses*. – 2025. – № 17(7). – Article 925. <https://doi.org/10.3390/v17070925>

24. Afzal S., Ali L., Batool A., et al. Hantavirus: an overview and advancements in therapeutic approaches for infection // *Frontiers in Microbiology*. – 2023. – № 14. – Article 1233433. <https://doi.org/10.3389/fmicb.2023.1233433>

25. Gu S.H., Kumar M., Sikorska B., et al. Isolation and partial characterization of a highly divergent lineage of hantavirus from the European mole (*Talpa europaea*) // *Scientific Reports*. – 2016. – № 6. – Article 21119. <https://doi.org/10.1038/srep21119>

26. Lagerqvist N., Hagström Å., Lundahl M., Nilsson E., Juremalm M., Larsson I., Alm E., Bucht G., Ahlm C., Klingström J. Molecular Diagnosis of Hemorrhagic Fever with Renal Syndrome Caused by Puumala Virus // *Journal of Clinical Microbiology*. – 2016. – № 54(5). – P. 1335–1339. <https://doi.org/10.1128/JCM.00113-16>

27. Rout P., Anjum F. Hemorrhagic Fever Renal Syndrome // *StatPearls*. – Treasure Island (FL): StatPearls Publishing, 2023.

28. Jiang H., Du H., Wang L.M., Wang P.Z., Bai X.F. Hemorrhagic Fever with Renal Syndrome: Pathogenesis and Clinical Picture // *Frontiers in Cellular and Infection Microbiology*. – 2016. – № 6. – Article 1. <https://doi.org/10.3389/fcimb.2016.00001>

29. Xue X., Liu Y., Song C., Liu T., Liu Z., Niu W., Jiang Z., Xu Y., Zhang Y., Lin L., Chen Z. Development of an in-house dual RT-qPCR assay for detecting SFTSV and Hantaan virus simultaneously // *Biosafety and Health*. – 2025. – № 7(2). – P. 110–116. <https://doi.org/10.1016/j.bsheal.2025.03.007>

30. Heuser E., Drewes S., Trimpert J., Kunec D., Mehl C., de Cock M.P., de Vries A., Klier C., Oskamp M., Tenhaken P., Hashemi F., Heinz D., Nascimento M., Boelhaue M., Petraityte-Burneikiene R., Raafat D., Maas M., Krüger D.H., Latz A., et al. Pet Rats as the Likely Reservoir for Human Seoul Orthohantavirus Infection // *Viruses*. – 2023. – № 15(2). – Article 467. <https://doi.org/10.3390/v15020467>

31. World Health Organization. Hantavirus [Electronic resource]. – Geneva: WHO, 2026. – URL: <https://www.who.int/news-room/fact-sheets/detail/hantavirus>

32. Centers for Disease Control and Prevention. Clinician Brief: Hemorrhagic Fever with Renal Syndrome (HFRS) [Electronic resource]. – Atlanta: CDC, 2024. – URL: <https://www.cdc.gov/hantavirus/hcp/clinical-overview/hfrs.html>

33. Bustin S.A., Ruijter J.M., van den Hoff M.J.B., Kubista M., Pfaffl M.W., Shipley G.L., Tran N., Rödiger S., Untergasser A., Mueller R., Nolan T., Milavec M., Burns M.J.,

Huggett J.F., Vandesompele J., Wittwer C.T. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments // *Clinical Chemistry*. – 2025. – № 71(6). – P. 634–651. <https://doi.org/10.1093/clinchem/hvaf043>

34. Scientific Research Company Syntol LLC. PCR test systems for detection of DNA/RNA of pathogens of particularly dangerous infections [Electronic resource]. – URL: <https://www.syntol.ru/catalog/dlya-viyavleniya-dnk-rnk-voz-buditeley-osobo-opasnykh-infektsiy-metodom-pcr-v-real-nom-vremeni/>

35. Romeo M.A., Tofani S., Lapa D., Mija C., Maggi F., Scicluna M.T., Nardini R. Orthohantaviruses: An Overview of the Current Status of Diagnostics and Surveillance // *Viruses*. – 2025. – № 17(5). – Article 622. <https://doi.org/10.3390/v17050622>

36. Rodríguez A., Rodríguez M., Córdoba J.J., Andrade M.J. Design of Primers and Probes for Quantitative Real-Time PCR Methods // *PCR Primer Design. Methods in Molecular Biology*. – New York: Humana Press, 2015. – Vol. 1275. https://doi.org/10.1007/978-1-4939-2365-6_3

37. Guo J., Starr D., Guo H. Classification and review of free PCR primer design software // *Bioinformatics*. – 2020. – № 36(22–23). – P. 5263–5268. <https://doi.org/10.1093/bioinformatics/btaa910>

38. Ye J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S., Madden T.L. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction // *BMC Bioinformatics*. – 2012. – № 13. – Article 134. <https://doi.org/10.1186/1471-2105-13-134>

39. Owczarzy R., Tataurov A.V., Wu Y., et al. IDT SciTools: a suite for analysis and design of nucleic acid oligomers // *Nucleic Acids Research*. – 2008. – № 36(Web Server issue). – P. W163–W169. <https://doi.org/10.1093/nar/gkn198>

40. Al Faifi K.H., Zoeir A.R., Musawa H.H., Albalawi W.F., Alqhtani M.S., Altemani S.S., Albalawi K.F., Alsulami B.M., Alahmari M.M., Aljohani O.M., Alyami M.A., Almahri H.S., Alatawi K.A., Alatawi M.A., Almalki W.A. Quality Assurance and External Quality Assessment in Molecular Diagnostics: Standards, Challenges, and Best Practices for Clinical Laboratories // *Journal of Clinical Practice and Medical Research*. – 2025. – № 1(3). – P. 143–149. [https://doi.org/10.59324/jcpmr.2025.1\(3\).21](https://doi.org/10.59324/jcpmr.2025.1(3).21)

41. U.S. Food and Drug Administration. Bioanalytical Method Validation. Guidance for Industry [Electronic resource]. – 2018. – URL: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>

42. Xue X., Liu Y., Song C., et al. Development of an in-house dual RT-qPCR assay for detecting SFTSV and Hantaan virus simultaneously // *Biosafety and Health*. – 2025. – № 7(2). – P. 110–116. <https://doi.org/10.1016/j.bsheal.2025.03.007>

43. Goodfellow S.M., Nofchissey R.A., Ye C., Dunnum J.L., Cook J.A., Bradfute S.B. Use of a Novel Detection Tool to Survey Orthohantaviruses in Wild-Caught Rodent Populations // *Viruses*. – 2022. – № 14(4). – Article 682. <https://doi.org/10.3390/v14040682>

44. Mohamed N., Nilsson E., Johansson P., et al. Devel-

opment and evaluation of a broad reacting SYBR-green based quantitative real-time PCR for the detection of different hantaviruses // *Journal of Clinical Virology*. – 2013. – № 56(4). – P. 280–285. <https://doi.org/10.1016/j.jcv.2012.12.001>

45. Kramski M., Meisel H., Klempa B., Krüger D.H., Pauli G., Nitsche A. Detection and typing of human pathogenic hantaviruses by real-time reverse transcription-PCR and pyrosequencing // *Clinical Chemistry*. – 2007. – № 53(11). – P. 1899–1905. <https://doi.org/10.1373/clinchem.2007.093245>

46. Kim W.K., Cho S., Lee S.H., et al. Genomic Epidemiology and Active Surveillance to Investigate Outbreaks of Hantaviruses // *Frontiers in Cellular and Infection Microbiology*. – 2021. – № 10. – Article 532388. <https://doi.org/10.3389/fcimb.2020.532388>

47. No J.S., Kim W.K., Kim J.A., et al. Detection of Hantaan virus RNA from anti-Hantaan virus IgG seronegative rodents in an area of high endemicity in Republic of Korea // *Microbiology and Immunology*. – 2016. – № 60(4). – P. 268–271. <https://doi.org/10.1111/1348-0421.12370>

48. Mackay I.M. Real-time PCR in the microbiology

laboratory // *Clinical Microbiology and Infection*. – 2004. – № 10(3). – P. 190–212. <https://doi.org/10.1111/j.1198-743x.2004.00722.x>

Jiang W., Yu H.T., Zhao K., et al. Quantification of Hantaan virus with a SYBR green I-based one-step qRT-PCR assay // *PLoS ONE*. – 2013. – № 8(11). – Article e81525. <https://doi.org/10.1371/journal.pone.0081525>

Tkachenko E., Dzagurova T., Galieva G., et al. Clinical Manifestations of Hemorrhagic Fever with Renal Syndrome, Various Nosologic Forms and Issues of Hantavirus Infections Terminology // *Viruses*. – 2025. – № 17(4). – Article 578. <https://doi.org/10.3390/v17040578>

Lu W., Kuang L., Hu Y., Shi J., Li Q., Tian W. Epidemiological and clinical characteristics of death from hemorrhagic fever with renal syndrome: a meta-analysis // *Frontiers in Microbiology*. – 2024. – № 15. – Article 1329683. <https://doi.org/10.3389/fmicb.2024.1329683>

Law of the Republic of Kazakhstan “On Biological Safety” No. 122-VII, dated May 21, 2022.