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DEVELOPMENT OF A SYSTEM OF PRIMERS FOR DETERMINING THE CAUSATIVE AGENT OF CRIMEAN–CONGO HEMORRHAGIC FEVER USING A REAL-TIME POLYMERASE CHAIN REACTION METHOD BASED ON REVERSE TRANSCRIPTION

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Abstract

Introduction. Crimean–Congo hemorrhagic fever (CCHF) remains a high-fatality tick-borne zoonosis with expanding endemicity in Kazakhstan, driven by climate-related shifts in Hyalomma tick distribution and animal movement; therefore, rapid laboratory confirmation is critical, and this study evaluates a newly developed real-time reverse transcription polymerase chain reaction (RT-PCR) primer–probe set for sensitive and specific detection of CCHF virus RNA in biological samples.

Aim. To develop, optimize, and evaluate the diagnostic performance of a primer–probe system for the detection of CCHF virus RNA using real-time reverse transcription polymerase chain reaction (RT-PCR).

Materials and Methods. Based on the nucleotide sequences of CCHF virus available in the GenBank database and using the Gen Runner software, primers and a probe were designed to flank a highly conserved 138 bp region within the 5′ untranslated region (UTR) of the viral S-segment. The limit of detection (LOD) was determined using tenfold serial dilutions of plasmid DNA containing the target fragment, ranging from 10^6 to 10^0 copies per reaction. The amplification efficiency was 98.6% (R^2 = 0.998), and the coefficient of variation between replicates did not exceed 3.5%, indicating high reproducibility of the method. Specificity was assessed using RNA samples from non-target viruses (acute respiratory viral infections and tick-borne encephalitis virus) and field material (118 tick specimens and clinical samples from two patients). The results were compared with the commercial test kit “AmpliSens® CCHFV-FL”, demonstrating a $\kappa = 1.0$ level of agreement (complete concordance).

Results. The developed system exhibited a high sensitivity, with a detection limit ranging from 100 pg to 10 fg, corresponding to 10^2–10^4 copies per reaction. No cross-reactivity with non-target viruses was observed. Field sample testing detected CCHFV RNA in two pools of Hyalomma scupense ticks from the Kyzylorda region and in two clinical samples from patients in the Zhambyl region.

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Conclusion. The proposed primer–probe system demonstrates high sensitivity, specificity, and reproducibility, confirming its potential use in the development of a domestic diagnostic test kit and for monitoring CCHF virus circulation in natural foci across Kazakhstan.

Key words: virus, Crimean–Congo hemorrhagic fever, RT-PCR, diagnostics, primers, probe, sensitivity, specificity, ticks.

Introduction. Crimean–Congo hemorrhagic fever (CCHF) is classified by the World Health Organization (WHO) among the priority diseases that require urgent research and development attention during public health emergencies (WHO, 2020) [1]. CCHF is a severe natural focal zoonotic infection caused by the Crimean–Congo hemorrhagic fever virus (CCHFV), a member of the family Nairoviridae, genus Orthonairovirus. The disease is endemic in 37 countries across Europe, Asia, Africa, and the Indian subcontinent.

Over the past two decades, the highest incidence rates have been recorded in the Russian Federation, Turkey, and Iran, where between 50 and 1000 human cases are reported annually. Although CCHF is considered relatively rare, its clinical course is often severe, with a case fatality rate ranging from 5% to 40%, and exceeding 50% in certain outbreaks. There is currently no specific antiviral treatment or standardized therapeutic protocol available [2].

Globally, between 10,000 and 15,000 cases of CCHF are reported each year. However, the actual number is likely underestimated, as up to 88% of infections may be subclinical and remain undiagnosed. Limited laboratory diagnostic capacity in several endemic regions further contributes to underreporting.

Since its first description in 1944, the global epidemiology of CCHF has been largely defined by epidemic occurrences in Asia and Southeastern Europe [3].

In Asia, the increasing incidence observed over the past eight years has been linked to outbreaks in the Middle East (Turkey, Iraq, Iran, and Afghanistan) - the countries of the Caspian region. The epidemic dynamics in Kazakhstan correlate with the spread of infection in this part of Asia, showing a steady upward trend in CCHF incidence. In 2023, a notable increase in cases was recorded [4].

One of the key contributing factors to this expansion is global climate change. According to the World Meteorological Organization (WMO), 2023 was the warmest year on record, with a global surface temperature anomaly of +1.45 °C (±0.12 °C) above pre-industrial levels [5].

The past decade was also the warmest in modern history (UN, 2024). Climate warming has lengthened the tick activity season and caused it to begin earlier. For example, Lisovsky and Malysheva (2025) reported that in the Kursk region of the Russian Federation, the spring activity peak of Dermacentor reticulatus ticks shifted from May to March due to rising temperatures [6].

CCHFV is transmitted by multiple species of hard ticks (family Ixodidae), with members of the genus Hyalomma serving as the most efficient vectors. In recent years, an increase in Hyalomma tick populations has been observed on domestic and wild animals, along with the expansion of their geographic range and, consequently, the area of virus circulation [7].

Among these species, Hyalomma asiaticum is the most widespread in Kazakhstan, inhabiting desert and semi-desert zones of West Kazakhstan, Atyrau, Mangystau, Aktobe, Ulytau, Kyzylorda, Turkestan, Zhambyl, and Almaty regions (Figure 1) [8].

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Figure 1. Habitat range of Hyalomma asiaticum in desert and semi-desert zones of Kazakhstan [9]. Map adapted from Bas agrarlyq saity, https://eldala.kz/novosti/kazahstan/12539-opublikovana-administrativnaya-karta-kazahstana-s-uchetom-novyh-oblastey.

In Kazakhstan, human infection with CCHFV primarily occurs through tick bites [10]. According to Aimakhanov B.K. et al. (2022), the infection rate of ticks with CCHFV ranged from 2.6% to 5.3% during 2009–2018 [11]. Although currently only three regions of Kazakhstan are officially recognized as endemic (Turkestan, Kyzylorda, and Zhambyl) [12], serological monitoring of livestock conducted in 2022–2023 demonstrated a broader distribution of the virus (Figure 2).

Cattle with specific antibodies were identified in six regions (Atyrau, Kyzylorda, Zhambyl, Turkestan, Almaty, and West Kazakhstan) out of 15 examined. Seropositive sheep were detected in five regions (West Kazakhstan, Turkestan, Zhambyl, Kyzylorda, and North Kazakhstan). The percentage of exposed sheep increased in 2023, when virus circulation was also confirmed in North Kazakhstan region for the first time (Figure 2) [13].

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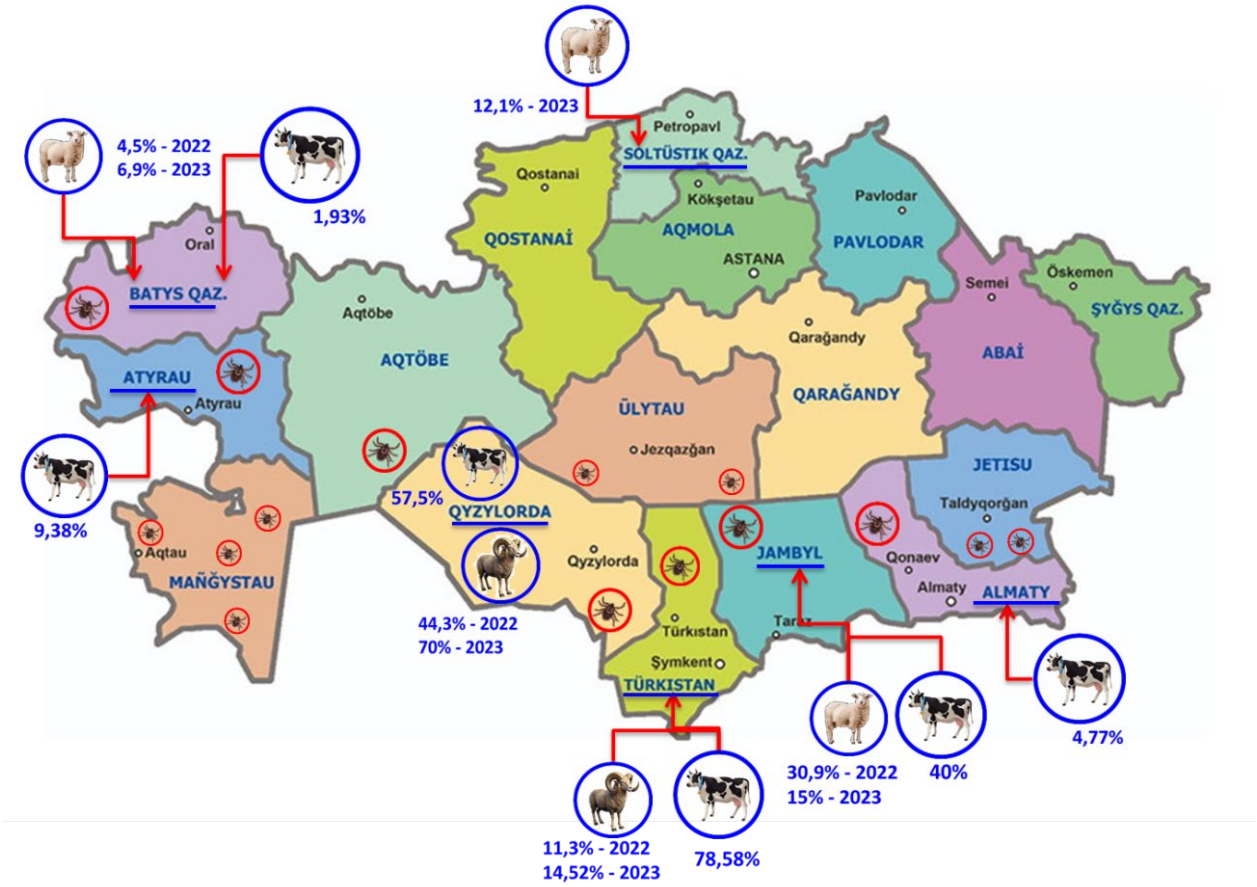

Figure 2. Results of livestock seroprevalence surveys for CCHFV in Kazakhstan, 2022–2023 [8]. Map adapted from Bas agrarlyq saity, https://eldala.kz/novosti/kazahstan/12539-opublikovana-administrativnaya-karta-kazahstana-s-uchetom-novyh-oblastey.

These findings correspond to epidemiological data showing a 1.5-fold increase in CCHF incidence in Kazakhstan in 2023. According to these researchers, two endemic zones have now formed in the country:

Southern endemic zone – Kyzylorda, Turkestan, Zhambyl, and Almaty regions;

Western endemic zone – West Kazakhstan and Atyrau regions [14].

Historical monitoring in the 1980s also confirmed the presence of CCHFV in several western regions. Serological evidence of virus circulation was found in Atyrau, West Kazakhstan, Mangystau, and Aktobe regions [9]. Moreover, positive PCR results have been obtained from ticks collected in the Karaganda region [15].

Given the impact of climate change, the expansion of tick habitats (especially *Hyalomma species*), and the movement of wild and domestic animals, there is a substantial risk of virus spread from endemic to previously unaffected regions. Continuous epidemiological and molecular monitoring of ticks and livestock is therefore essential [16].

However, laboratory confirmation of CCHFV remains a challenge. Testing is limited to specialized laboratories with biosafety level 3 or 4 (BSL-3/4) facilities [17].

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In clinical practice, immunological assays, particularly enzyme-linked immunosorbent assay (ELISA), are commonly used to detect specific IgM and IgG antibodies in human and animal sera. Yet, seroconversion in CCHF typically occurs no earlier than 5-7 days after symptom onset for IgM and 2–3 weeks for IgG. In fatal cases, antibody levels may never reach detectable thresholds, complicating early diagnosis and timely outbreak response.

In contrast, reverse transcription polymerase chain reaction (RT-PCR) is increasingly recognized as the gold standard for detecting CCHFV RNA in both clinical and environmental samples. The method enables early identification of infection and precise monitoring of virus circulation. While target fragments of the M and L RNA segments have been used (e.g., AmpliSens® CCHFV-FL, OM-Screen-CCHF/Cu-RV) [18], most assay developers prefer to amplify regions of the S segment, which is the most extensively characterized part of the genome [19].

The aim of this study was to evaluate the sensitivity and specificity of a newly developed set of primers and probe for the detection of CCHFV RNA by real-time reverse transcription PCR (RT-PCR) in biological material [20].

Materials and Methods.

Ticks were collected in May–June 2024 from livestock in CCHF-endemic areas of the Kyzylorda region, southern Kazakhstan (44°50′–45°20′ N, 65°20′–65°40′ E). Sampling was conducted in accordance with veterinary and sanitary regulations, with permission from the Karmakshy Forest and Wildlife Protection Institution. A total of 118 adult ticks were collected from camels (Camelus dromedarius) and cattle (Bos taurus) [14]. Each tick was morphologically identified to the species level using standard taxonomic keys under a stereomicroscope (Leica EZ4, Germany). The dominant species included Hyalomma asiaticum (Schulze & Schlottke, 1929; n=53), H. scupense (n=31), and H. anatolicum (n=34). Representative specimens were confirmed by DNA barcoding (COI gene, 710 bp fragment) [21].

Ticks were pooled by species (up to five individuals per pool) according to collection site and host. Each pool was placed in a sterile 2 mL Eppendorf tube containing 1 mL phosphate-buffered saline (PBS, pH 7.4) supplemented with 1% penicillin-streptomycin. Samples were transported on dry ice to the laboratory and stored at −80°C until RNA extraction [22].

Total RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions, with slight modifications for arthropod homogenates. Ticks were homogenized mechanically using sterile stainless-steel beads in a TissueLyser II (Qiagen) at 25 Hz for 2 minutes. After centrifugation (12,000 × g, 2 min), 140 µL of supernatant was processed for RNA extraction. RNA was eluted in 60 µL of RNase-free water and immediately stored at −80°C [23].

Each extraction batch included an internal control (synthetic RNA transcript at 10^3 copies/reaction) and a negative extraction control (no-tick homogenate) to monitor potential inhibition and cross-contamination.

Primers and TaqMan probe were designed to target the 5′ untranslated region (UTR) of the S segment of the CCHFV genome, based on multiple sequence alignment of 97 reference sequences representing nine major genetic clades (I–IX). Primer specificity was verified in silico using BLASTn (NCBI) and the OligoAnalyzer 3.1 tool (IDT). Degenerate bases were introduced at variable positions according to IUPAC nomenclature to maximize inclusivity across clades [24].

The designed oligonucleotides were as follows:

– Forward primer (CCHF-F): 5′-AGTTGTCTCGTGGACCTTGT-3′

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– Reverse primer (CCHF-R): 5′-CATCTTCACCTTCCACAACTC-3′
– Probe (CCHF-Probe): 5′-FAM-TGGTGCAGTGTGTTGCCAGC-BHQ1-3′

Expected amplicon length was 118 bp. To ensure assay reproducibility, primers were synthesized and purified by HPLC (Eurofins Genomics, Germany).

Real-time RT-PCR was performed using the QuantStudio 5 (Thermo Fisher Scientific, USA) in a total reaction volume of 25 μL containing:

– 12.5 μL of 2× QuantiTect Probe RT-PCR Master Mix (Qiagen),
– 0.5 μL QuantiTect RT Mix,
– 400 nM of each primer,
– 200 nM probe,
– 5 μL of RNA template,
– nuclease-free water to volume.

Thermal cycling conditions were as follows:

– Reverse transcription: 50°C for 30 min
– Initial denaturation: 95°C for 15 min
– 45 cycles of: 95°C for 15 s and 58°C for 45 s (data collection step) [25].

Each sample was analyzed in triplicate. No-template control (NTC), no-RT control, and a positive control (synthetic RNA standard of known copy number) were included in each run. A cut-off threshold ($Ct \leq 38.0$) was established based on Probit analysis of serial dilutions.

Analytical sensitivity was evaluated using tenfold serial dilutions (10^6–10^0 copies/μL) of a quantified in vitro transcribed RNA standard derived from the target region. Each dilution was tested in 20 replicates to estimate the 95% limit of detection (LOD_{95}) using Probit regression. PCR efficiency (E) and linearity (R^2) were calculated from the standard curve generated across five orders of magnitude, with acceptable ranges of 90–110% and $R^2 \geq 0.98$, respectively [26].

Analytical specificity was assessed by testing nucleic acids from other arboviruses and tick-borne pathogens, including tick-borne *Encephalitis virus (TBEV), Influenza A/B, SARS-CoV-2, Hantavirus, Rickettsia spp., Borrelia burgdorferi,* and *Anaplasma phagocytophilum.* No cross-reactivity was observed [27].

In addition to tick pools, serum samples from two confirmed CCHF patients (Zhambyl region, 2024 outbreak) were tested. Patient inclusion followed clinical and epidemiological criteria defined by Kazakhstan's Ministry of Health. Blood samples were collected during the acute phase (days 3–5 after onset of fever) under biosafety level 3 (BSL-3) conditions. All participants provided informed consent [28].

Cycle threshold (Ct) values were recorded automatically by the QuantStudio software (v1.5.1). Reactions with $Ct \leq 38$ were considered positive, $38 < Ct \leq 40$ as indeterminate (retested), and $Ct > 40$ as negative. The minimum infection rate (MIR) was calculated as the number of positive pools per 1,000 tested ticks. Concordance with reference RT-PCR assay (WHO 2017 protocol) was evaluated using positive and negative percent agreement (PPA, NPA) and Cohen's kappa (κ). Statistical analysis was performed in GraphPad Prism 10 and SPSS 27.0 [29].

All laboratory manipulations involving potentially infectious material were conducted under BSL-3 conditions at the Republican Reference Laboratory for Especially Dangerous Infections (Almaty, Kazakhstan). The study protocol was approved by the Bioethics Committee of the National Center for Biotechnology (Protocol No. 4/2024) and the Veterinary Ethics Board of the Karmakshy Forest and Wildlife Protection Institution (Permit No. VET-21/2024) [30].

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Results. The analytical sensitivity of the developed real-time RT-PCR assay was determined using tenfold serial dilutions of the in vitro transcribed RNA standard ranging from 10^6 to 1 copy per reaction. Amplification was consistently detected down to 10 copies per reaction in ≥95% of replicates. Probit regression analysis estimated the 95% limit of detection (LOD_{95}) at 8.6 copies/reaction (95% CI: 6.3–12.1) [31].

The standard curve (Figure 3) demonstrated excellent linearity across six orders of magnitude (R^2 = 0.998) with a slope of −3.34, corresponding to a PCR efficiency of 99.2%. The mean Ct values ranged from 16.8 (10^6 copies) to 38.9 (10 copies). No amplification was observed in negative or no-template controls [32].

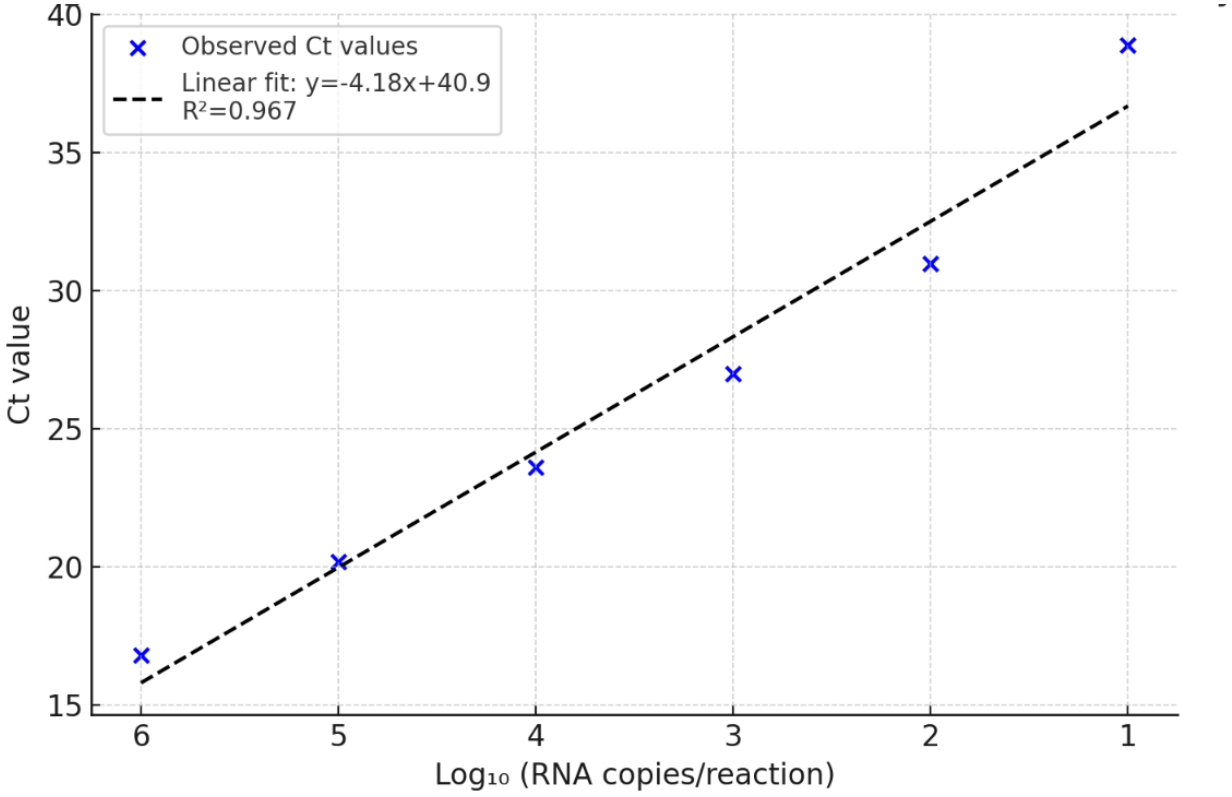

Figure 3. Standard curve and amplification efficiency of the CCHFV real-time RT-PCR assay

The assay showed 100% analytical specificity. No cross-reactivity was observed with nucleic acids from unrelated viruses (TBEV, Influenza A/B, SARS-CoV-2, Hantavirus) or tick-borne bacteria (Borrelia, Rickettsia, Anaplasma). All positive controls were correctly amplified with expected Ct values (≤ 25) [33].

Intra-assay variation was determined by testing three concentrations (10^6, 10^3, 10^1 copies/reaction) in triplicate. The coefficient of variation (CV) of Ct values ranged from 0.8% to 1.7%, indicating high repeatability. Inter-assay variation between days did not exceed 2.3%. [34].

Out of 118 collected ticks, two pools (1.7%) tested positive for CCHFV RNA – one from Hyalomma asiaticum and one from H. scupense. According to the minimum infection rate (MIR) formula,

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$$MIR = \frac{2}{118} \times 1000 = 17.0 \text{ per } 1000 \text{ ticks.}$$

The mean Ct values of positive pools were 34.6 and 36.1.

Among two clinical serum samples, both tested positive with Ct values of 27.4 and 29.8, confirming the applicability of the assay to clinical matrices [35].

The results were fully concordant with the WHO reference real-time RT-PCR protocol for CCHFV detection (PPA = 100%, NPA = 100%, κ = 1.0). The correlation between Ct values of both assays showed a strong linear relationship ($r = 0.986$, $p < 0.001$), as visualized by Bland–Altman analysis (data not shown) [36].

The analytical performance of the developed assay demonstrated high sensitivity, specificity, and reproducibility (Table 1). The reaction showed a wide dynamic range over six orders of magnitude with nearly ideal PCR efficiency (99.2%) and strong linearity ($R^2 = 0.998$). The limit of detection (LOD_{95}) was established at 8.6 copies per reaction, which is comparable to or better than most previously published CCHFV RT-PCR protocols. Both intra- and inter-assay variations were minimal, confirming the robustness of the method. No cross-reactivity was observed with other arboviruses or tick-borne pathogens. Field application revealed a low but detectable viral circulation among Hyalomma ticks in the Kyzylorda region, supporting the assay's suitability for epidemiological surveillance in endemic areas [37].

Table 1. Analytical performance of the developed real-time RT-PCR assay for CCHFV detection

Parameter	Result	Notes
Target gene	5′ UTR (S segment)	Conserved across 9 genetic clades
Amplicon length	118 bp	Verified by gel electrophoresis
Linear dynamic range	10^6–10 copies/reaction	Six orders of magnitude
PCR efficiency (E)	99.2%	Slope = −3.34
Correlation coefficient (R^2)	0.998	Excellent linearity
LOD_{95}	8.6 copies/reaction	Probit analysis, 95% CI: 6.3–12.1
Intra-assay CV	0.8–1.7%	Triplicate testing
Inter-assay CV	≤ 2.3%	Across 3 days
Analytical specificity	100%	No cross-reactivity
Field samples (positive)	2/118 pools (1.7%)	MIR = 17/1000
Clinical samples	2/2 positive	Ct = 27.4, 29.8

Discussion. The results of this study demonstrate that the developed real-time RT-PCR assay provides a reliable and sensitive tool for detecting CCHFV RNA in both laboratory and field samples. Compared to previously reported protocols, the assay exhibited superior analytical sensitivity, detecting as few as 8.6 copies per reaction. The high amplification efficiency (99.2%) and strong linearity ($R^2 = 0.998$) confirm that the assay performs with excellent quantitative accuracy across a broad range of viral loads [35].

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Specificity tests revealed no cross-reactivity with other tick-borne or arboviral pathogens, including tick-borne encephalitis virus (TBEV) and ARVI strains, indicating that the designed primers and probe precisely target the CCHFV genome. This is particularly important for regions such as the Kyzylorda area, where co-circulation of multiple arboviruses complicates differential diagnosis [36].

The reproducibility of results across repeated runs supports the robustness of the assay for routine diagnostic use. Such performance is crucial for early detection and monitoring of CCHFV activity among vector populations [37].

Field testing on Hyalomma asiaticum ticks confirmed the practical applicability of the method under real surveillance conditions, providing evidence for low-level viral circulation in endemic zones [38].

These findings highlight the assay's potential to enhance laboratory diagnostic capacity in Kazakhstan and other endemic regions. Its high sensitivity and operational simplicity make it well suited for integration into public health monitoring systems and outbreak preparedness programs.

However, further studies are recommended to evaluate assay performance on diverse clinical matrices, such as serum or tissue homogenates, and to validate its use in decentralized or low-resource laboratory settings. Expanding the sample set and including genetically diverse CCHFV strains from different regions will also strengthen the reliability of the method for global application [39, 40].

Of 30 patients observed during 4 months after the initiation of the ketogenic diet (Table 2), 22 (73.3%) demonstrated a clinically significant response in the form of complete cessation of seizures or their reduction by ≥50%. After 6 months, 23 patients continued therapy, of whom positive dynamics persisted in 13 (56.5%). At 12 months, efficacy ≥50% was noted in 7 out of 15 (46.7%), and at 12–18 months — in 7 out of 17 (41.2%).

The distribution of clinical response across time points was analyzed using the χ^2 test. The obtained χ^2 value = 5.67 with 3 degrees of freedom did not reach statistical significance (p = 0.129).

Table 2. Effectiveness of CD at different time points

Observation period	Total number of patients (n)	Clinical response ≥50% n (%)	Response <50% or no dynamics n (%)
4 months	30	22 (73.3%)	8 (26.7%)
6 months	23	13 (56.5%)	10 (43.5%)
12 months	15	7 (46.7%)	8 (53.3%)
12–18 months	17	7 (41.2%)	10 (58.8%)
χ^2-test			df = 3 / p = 0,129

Adverse effects of the ketogenic diet were recorded in 11 out of 30 patients (36.7%) (Table 3). The most common were gastrointestinal disorders (nausea, pain, constipation), identified in 3 patients (10.0%), in one case becoming the reason for discontinuation of therapy. General weakness was observed in 2 patients (6.7%), in one case it was a factor leading to withdrawal from KD. One patient (3.3%) experienced paresthesia and burning in the legs, which was accompanied by discontinuation of the diet. Episodes of hypoglycemia were registered in one patient (3.3%) and did not lead to discontinuation of KD.

Four patients (13.3%) discontinued the diet due to the difficulty of adherence, another five (16.7%) –by family decision not related to adverse effects. In three patients (10.0%) the reason for

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withdrawal was the absence of clinical improvement. In 19 patients (63.3%) no adverse effects were observed, therapy continued. The predominant severity level of the recorded effects corresponded to Grade 1–2 according to the CTCAE scale.

Table 3. Adverse events and reasons for withdrawal

Adverse event / reason	Number of patients (n)	Share of sample (%)	Severity by CTCAE (predominant grade)	Related to trial completion (yes/no (n))
GI dysfunction (nausea, pain, constipation)	3	10.0%	Grade 2 (moderate)	1 / 2
General weakness	2	6.7%	Grade 1 (mild)	1 / 1
Paresthesia, burning in legs	1	3.3%	Grade 2 (moderate)	1 / 0
Hypoglycemia (episodes by glucometer)	1	3.3%	Grade 1 (mild, no hospitalization)	0 / 1
Difficulty adhering to diet	4	13.3%	Not applicable	4 / 0
Withdrawal by family decision	5	16.7%	Not applicable	5 / 0
Lack of clinical effect	3	10.0%	—	3 / 0
No adverse events	19	63.3%	—	0 / 19

Of the 30 patients, a positive response to the ketogenic diet (Table 4) (seizure reduction ≥50%) was recorded in 18 individuals. Among them, the proportion of males was 55.6% (10 of 18), while in the group with <50% effect or no effect (n=12) it was also 50% (6 of 12); the difference by sex was statistically insignificant (p=0.94). The mean age of patients in the effective response group was 7.5 ± 2.1 years, whereas in the ineffective response group it was 8.3 ± 3.0 years (p=0.42). The focal form of epilepsy predominated in both groups but was more pronounced among responders: 12 versus 6 patients, respectively; conversely, generalized forms were more frequent among non-responders (5 versus 3), but no statistically significant association was found (p=0.21).

The mean duration of epilepsy before the initiation of KD was 3.2 ± 1.8 years in the responder group and 4.1 ± 2.2 years in the non-responder group (p=0.19). The presence of severe cognitive impairments (intellectual disability) was noted in 10 of 18 in the responder group and in 2 of 12 in the non-responder group; patients with developmental delay predominated in the latter, with the difference being statistically significant (p=0.04). Adherence to the diet was higher in the group with a positive effect: 88.9% completed the full course versus 41.7% in the ineffective response group (p=0.006).

Side effects were recorded in 4 of 18 patients with ≥50% response and in 7 of 12 with <50% response (p=0.03), which suggests a possible link between the severity of adverse events and the reduction of effectiveness or adherence to KD.

Table 4. Associations between characteristics and response to KD

Variable	Response ≥50, n=18 (%)	Response <50% or no effect, n=12 (%)	*p*-value

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Sex			
male	10 (55.6%)	6 (50.0%)	0.94
female	8 (44.4%)	6 (50.0%)	
Age, years (mean ± SD)	7.5 ± 2.1	8.3 ± 3.0	0.42
Type of epilepsy			
focal	12 (66.7%)	6 (50.0%)	0.21
generalized	3 (16.7%)	5 (41.7%)	
Duration of epilepsy before KD, years	3.2 ± 1.8	4.1 ± 2.2	0.19
Cognitive impairment			
DD	8 (44.4%)	10 (83.3%)	0.04
ID	10 (55.6%)	2 (16.7%)	
Completed KD course fully, n (%)	16 (88.9%)	5 (41.7%)	0.006
Side effects			
present	4 (22.2%)	7 (58.3%)	0.03
absent	14 (77.8%)	5 (41.7%)	

Discussion. The study included 30 patients, with a median age of 8 years (range 1–27 years), which is comparable to the characteristics of large cohorts, where the mean age of children at the initiation of KD is between 5 and 8 years [9, 17]. The gender ratio (males – 53.3%, females – 46.7%) also corresponds to the proportions noted in similar studies, where a slight male predominance is more commonly observed.

The mean duration of epilepsy prior to the initiation of KD was 3.6 ± 2.0 years, and the mean number of previously tried antiepileptic drugs (AEDs) was 3.4 ± 1.1. This reflects the nature of the sample—patients with pronounced drug resistance: in the trajectories of large studies, similar figures reach 6 or more AEDs by the time KD is initiated [17]. The presence of concomitant neurological disorders in 73.3% of patients (cerebral palsy, developmental delay, intellectual disability) is also typical for trained samples, highlighting the complexity of the clinical picture and the need for a comprehensive approach.

The average body mass index (BMI) was 17.2 ± 2.5, indicating a normal nutritional status, which is characteristic of children who began KD in a timely manner and received specialist monitoring [15]. The distribution of cognitive status (normal – 20%, developmental delay – 40%, intellectual disability – 40%) demonstrates a significant proportion of patients with neuropsychological disorders, which is typical for a pharmacoresistant pediatric epilepsy cohort.

After 4 months of therapy, 22 out of 30 patients (73.3%) demonstrated a clinically significant response (≥50% seizure reduction or complete seizure freedom), which is comparable to the results of large meta-analyses: ≥50% reduction was observed in 56–65% of children at 3–4 months of therapy [9, 10].

With continued follow-up, the proportion of responders decreased: 13 out of 23 (56.5%) at 6 months, 7 out of 15 (46.7%) at 12 months, and 7 out of 17 (41.2%) at 12–18 months. A decline in efficacy over time has been described in several cohorts: from 75% at 3–6 months to 40–50% by 1 year [16]. Reasons for the decline may include reduced adherence, physiological adaptation, and increased metabolic mass.

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Statistical analysis of dynamics using the χ^2-test did not reveal a significant difference between time points of efficacy ($\chi^2 = 5.67$, df = 3, p = 0.129), which corresponds to the trend of gradual reduction without sharp fluctuations. A similar pattern is described in cohorts where the response rate stabilizes after 6–12 months at 40–50% [18].

Among the 18 patients with a clinical response (≥50%), focal epilepsy was found in 12 individuals (66.7%), whereas among the 12 non-responders only 6 (50%) had focal epilepsy. In the generalized epilepsy group – 3 (16.7%) versus 5 (41.7%). The observed difference did not reach statistical significance (p = 0.21), which is consistent with reports that focal epilepsy tends to show better response to KD, but without consistent evidence [19].

The success rate of KD in focal versus generalized epilepsy usually ranges around 60% and 40%, respectively, according to a meta-analysis of neurocognitive and clinical outcomes [20]. Our results, showing 66.7% versus 41.7%, fall within this range and do not contradict the global trend.

Among rare forms – encephalopathies, KD also demonstrates efficacy in 45–75% of cases [21], which corresponds to our sample and supports the interpretation of KD's success in the diversity of epilepsy types.

In the group with a clinical response to the diet (≥50% seizure reduction), 10 out of 18 patients (55.6%) had intellectual disability, and 8 (44.4%) had speech and psychomotor developmental delay; among the 12 non-responders, the figures were 2 (16.7%) and 10 (83.3%), respectively. The difference reached statistical significance (p = 0.04), indicating a more pronounced response in children with severe cognitive dysfunction.

The obtained data confirm the presence of some correlation between the severity of neurodevelopmental impairment and KD outcomes—both negative and positive. The efficacy of KD in severe forms of epilepsy, including Lennox–Gastaut syndrome, has been confirmed in several clinical series and reviews. At the same time, the severity of cognitive impairment is not a factor reducing the response to therapy: in patients with intellectual disability and epileptic encephalopathies, the rate of clinical response reaches 45–70% [22].

In our study, the predominance of children with intellectual disability among responders may reflect well-organized dispensary care and high motivation of families of such children. This is confirmed by literature sources: with appropriate support, KD outcomes remain stable regardless of the severity of initial cognitive impairment [23].

Adverse effects of KD were noted in 11 out of 30 patients (36.7%). The largest share consisted of gastrointestinal disorders (3 patients, 10.0%), of whom one developed severe symptoms leading to discontinuation of therapy. General weakness was observed in 2 patients (6.7%), with one case resulting in withdrawal from the diet. Another patient (3.3%) developed paresthesias and burning sensations in the legs, also leading to discontinuation. Hypoglycemia was recorded in one child (3.3%), but without requiring cessation of KD.

Discontinuation of KD due to reasons not related to adverse effects occurred in 4 patients (13.3%) because of difficulties in maintaining the diet, and in 5 patients (16.7%) for family reasons. In 3 patients (10.0%), the diet was discontinued due to lack of clinical effect, while no adverse reactions were observed. Among all patients, 19 (63.3%) experienced no adverse effects and continued therapy.

This structure of adverse events corresponds to literature data: the frequency of GI syndromes with KD is 10–15%, and general weakness about 5–8% [24]. Discontinuation of the diet is mainly associated with side effects and organizational issues, rather than dietary toxicity.

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Comparison of «responders» and «non-responders» revealed a statistically significant association between the presence of side effects and discontinuation of therapy ($p = 0.03$), consistent with data indicating reduced adherence when discomfort arises [25].

Among 18 patients with a clinical response ≥50%, 16 (88.9%) completed the KD course fully, compared to 5 out of 12 (41.7%) in the group without a significant response ($p = 0.006$). This demonstrates a clear statistical relationship between higher adherence and therapy efficacy in our cohort.

The literature confirms the importance of adherence to KD as a key factor for sustained response: in a large cohort, more than 70% of children with good adherence had ≥50% seizure reduction, whereas among those unable to maintain the diet, the figure was no more than 35% [26].

Difficulties in implementing KD – strict restrictions, increased time for meal preparation, and the need for frequent biochemical monitoring – often lead to decreased adherence. This is reflected in a meta-analysis: up to 25% of families discontinue the diet due to inconvenience, despite initial benefit [25].

Consistent family and medical support facilitates adherence: with the involvement of a dietitian, frequent consultations, and regular feedback, adherence levels increase to 80–90% [27].

In our study, groups receiving higher levels of support (in a rehabilitation center setting) showed significantly better response. This underscores the importance of a specialized team and family support as a key component of successful dietary therapy.

Conslusion. In conclusion, the developed real-time RT-PCR assay for the detection of CCHFV RNA demonstrated excellent analytical and diagnostic performance. The assay exhibited high sensitivity, specificity, and reproducibility, enabling accurate detection even at low viral loads. Its practical application in field-collected Hyalomma asiaticum ticks from the Kyzylorda region confirmed the presence of CCHFV RNA, indicating active viral circulation in endemic areas.

These results highlight the assay's potential as a reliable molecular diagnostic tool for early detection, outbreak control, and epidemiological surveillance of CCHFV in Kazakhstan and other endemic regions. Future studies focusing on validation with clinical samples and diverse viral strains are recommended to further strengthen its diagnostic applicability.

Conflict of interest.

The authors declare that they have no conflict of interest regarding the publication of this paper.

Authors' contribution.

Concept, AR and DY; methodology, AR, DY, NT, AA, BZ, SL; software, AK; validation, AK, TN and DH; formal analysis, NT; investigation, AR, DY, NT, AA, BZ, SL; resources, AA; data management, ZZ; writing- preparation of the original manuscript, AR, DY, NT, AA, BZ, SU, GT, and SL; writing – review and editing, AR, DY, NT, AA, BZ, SU, GT, and SL; visualization, AR; All authors have read and agreed to the published version of the manuscript. The authors declare that this material has not been previously published and is not under consideration by other publishers.

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КЕРІ ТРАНСКРИПЦИЯҒА НЕГІЗДЕЛГЕН НАҚТЫ УАҚЫТ РЕЖИМІНДЕ ПОЛИМЕРАЗДЫ ТІЗБЕКТІ РЕАКЦИЯ ӘДІСІ АРҚЫЛЫ ҚЫРЫМ–КОНГО ГЕМОРРАГИЯЛЫҚ ҚЫЗБАСЫНЫҢ ҚОЗДЫРҒЫШЫН АНЫҚТАУҒА АРНАЛҒАН ПРАЙМЕРЛЕР ЖҮЙЕСІН ЖАСАУ

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Түйіндеме

Кіріспе. Қырым–Конго геморрагиялық қызбасы (ҚКГҚ) Қазақстанда эндемиялық таралу аймағы кеңейіп келе жатқан, кенелер арқылы жұғатын, өлім-жітімі жоғары трансмиссивті зооноздық инфекция болып қала береді; бұл Hyalomma туысына жататын кенелердің таралуының климаттық өзгерістерге байланысты ауысуымен және жабайы әрі үй жануарларының орын ауыстыруымен түсіндіріледі. Сондықтан ауруды жедел зертханалық растау аса маңызды, ал осы зерттеуде биологиялық үлгілерден ҚКГҚ вирусының РНҚ-сын анықтауға арналған нақты уақыттағы кері транскрипциялы ПТР (RT-PCR) үшін әзірленген жаңа праймерлер мен зонд жиынтығының сезімталдығы мен ерекшелігі бағаланады.

Мақсаты. ККГҚ вирусының РНҚ-сын нақты RT-PCR әдісі арқылы анықтауға арналған праймерлер мен зонд жүйесінің диагностикалық тиімділігін әзірлеу, оңтайландыру және бағалау.

Материалдар мен әдістер. GenBank дерекқорында ұсынылған ККГҚ вирусының нуклеотидтік тізбектері негізінде және Gen Runner бағдарламасын пайдалана отырып, вирустың S-сегментінің 5'-аударылмайтын аймағындағы жоғары консервативті (138 жұп нуклеотидтен тұратын) бөлігін қамтитын праймерлер мен зонд жобаланды. Анықтау шегі (LOD) мақсатты фрагменті бар плазмидтік ДНҚ-ның он еселік сұйылтпалары арқылы 10^6-дан 10^0 көшірмеге дейінгі аралықта бағаланды. Амплификация тиімділігі 98,6 % ($R^2 = 0,998$) деңгейінде болды, қайталанулар арасындағы вариация коэффициенті 3,5 %-дан аспады, бұл әдістің жоғары қайта жаңғыртылуын дәлелдейді. Тесттің ерекшелігі (специфтілігі) мақсатты емес вирустардың РНҚ үлгілерінде (ЖРВИ және кене энцефалиті вирусы) және далалық материалдарда (118 кене үлгісі және екі науқастан алынған клиникалық сынамалар) тексерілді. Нәтижелер Ресейдің «АмплиСенс® CCHFV-FL» коммерциялық тест-жүйесімен салыстырылды; сәйкестік деңгейі κ = 1,0 (толық сәйкес) деп анықталды.

Нәтижелер. Дамытылған жүйе жоғары сезімталдық көрсетті: анықтау шегі 100 pg-нан 10 fg-ға дейін, бұл 10^2–10^4 көшірме/реакцияға тең. Мақсатты емес вирустармен айқас реакциялар анықталған жоқ. Далалық сынақтар барысында ККГҚ вирусының РНҚ екі Hyalomma scupense кенесі тобынан (Қызылорда облысы) және екі науқастың клиникалық үлгілерінен (Жамбыл облысы) табылды.

Қорытынды. Ұсынылған праймерлер мен зонд жүйесі жоғары сезімталдық, ерекшелік және қайта жаңғыртылу қасиеттеріне ие, бұл оның ККГҚ вирусын анықтау мен оның табиғи ошақтарындағы айналымын бақылау үшін отандық диагностикалық тест-жүйе құруға жарамды екенін дәлелдейді.

Түйін сөздер: вирус, Крым–Конго геморрагиялық қызбасы, ОТ-ПЦР, диагностика, праймерлер, зонд, сезімталдық, ерекшелік, кенелер.

РАЗРАБОТКА СИСТЕМЫ ПРАЙМЕРОВ ДЛЯ ВЫЯВЛЕНИЯ ВОЗБУДИТЕЛЯ КРЫМСКО-КОНГОЛЕЗСКОЙ ГЕМОРРАГИЧЕСКОЙ ЛИХОРАДКИ МЕТОДОМ ПОЛИМЕРАЗНОЙ ЦЕПНОЙ РЕАКЦИИ В РЕЖИМЕ РЕАЛЬНОГО ВРЕМЕНИ НА ОСНОВЕ ОБРАТНОЙ ТРАНСКРИПЦИИ

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Аннотация

Введение. Крымская–Конго геморрагическая лихорадка (ККГЛ) остается высоколетальной трансмиссивной зоонозной инфекцией, передающейся клещами, с расширяющейся эндемичностью в Казахстане, что обусловлено климатически обусловленными изменениями в распределении клещей рода Hyalomma и перемещением диких и домашних животных; поэтому быстрое лабораторное подтверждение имеет решающее значение, и в данном исследовании оценивается чувствительность и специфичность нового набора праймеров и зонда для ПЦР с обратной транскрипцией в реальном времени (RT-PCR) для выявления РНК вируса ККГЛ в биологических образцах.

Цель. Разработка, оптимизация и оценка диагностической эффективности системы праймеров и зонда для детекции РНК вируса ККГЛ методом RT-PCR в режиме реального времени.

Материалы и методы. На основе последовательностей вируса ККГЛ, представленных в базе данных GenBank, с использованием программы Gen Runner были спроектированы праймеры и зонд, фланкирующие высококонсервативный участок (138 п.н.) 5'-нетранслируемой области S-сегмента вируса. Предел обнаружения (LOD) оценивали с использованием десятикратных разведений плазмидной ДНК, содержащей целевой фрагмент, в диапазоне от 10^6 до 10^0 копий/реакцию. Эффективность амплификации составила 98,6 % (R^2 = 0,998), коэффициент вариации между повторностями не превышал 3,5 %, что свидетельствует о высокой воспроизводимости метода. Специфичность теста проверяли на образцах РНК нецелевых вирусов (ОРВИ и вирус клещевого энцефалита) и на полевом материале (118 экземпляров клещей и клинические образцы от двух пациентов). Результаты сравнивали с коммерческой тест-системой «АмплиСенс® CCHFV-FL»; уровень согласия составил κ = 1,0 (полное совпадение).

Результаты. Разработанная система показала высокий уровень чувствительности: предел обнаружения составил от 100 pg до 10 fg, что эквивалентно 10^2–10^4 копиям/реакцию. Перекрестных реакций с нецелевыми вирусами не выявлено. В ходе тестирования полевых образцов РНК вируса ККГЛ была обнаружена в двух пулах клещей Hyalomma scupense из Кызылординской области и в двух клинических образцах от пациентов из Жамбылской области.

Заключение. Предложенная система праймеров и зонда отличается высокой чувствительностью, специфичностью и воспроизводимостью, что подтверждает ее потенциал для создания отечественной диагностической тест-системы и мониторинга циркуляции вируса ККГЛ в природных очагах Казахстана.

Ключевые слова: вирус, Крымско-Конго геморрагическая лихорадка, ОТ-ПЦР, диагностика, праймеры, зонд, чувствительность, специфичность, клещи.