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DEVELOPMENT OF A REAL-TIME RT-PCR ASSAY FOR DETECTION OF HENDRA AND NIPAH VIRUSES

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Abstract. The article is devoted to the development of a method for detection of viral RNA of two highly pathogenic zoonotic viruses from the genus *Henipavirus* — Hendra and Nipah using real-time reverse transcription polymerase chain reaction. In the natural environment, these viruses are carried by flying foxes in the family *Pteropodidae*. Horses and pigs, respectively, are susceptible to infection. The diseases are also transmitted to humans through contact with sick animals, their biological excreta and from person to person. In infected humans and animals, clinical signs of infection may be asymptomatic, or may present with flu-like symptoms at the onset of the disease and progress to neurologic disease and acute respiratory infection, followed by death. In Australia, the subunit vaccine HeV-sG is used against Hendra virus in horses. There is no treatment or vaccine for Hendra or Nipah viruses for humans. The need to develop new detection methods and search for new viral targets remains an urgent task due to the large area of distribution of the described viruses, high contagiousness and mortality of animals and humans. The study describes the original designed primers and probes for conserved regions of the genomes of two viruses: the gene encoding the nucleocapsid protein of Hendra virus and the gene encoding the glycoprotein of Nipah virus. Synthetic controls for the extraction and reverse transcription PCR stages have been created, confirming the quality of the developed method. Biological samples from healthy people (blood plasma, swabs from oral and nasopharyngeal mucous membranes, cerebrospinal fluid) with the addition of artificial controls passed the stages of sample extraction and real-time reverse transcription PCR, thus confirming the quality of control samples. The detection limit of the described viral RNA identification methods was determined as 100 copies/mL for Hendra virus and 1000 copies/mL for Nipah virus. The amplification transit time is less than 90 minutes. The developed method will help in epidemiologic control of the spread of these infections, can be used in the diagnosis of Hendra and Nipah viruses and for solving research tasks to study the properties of these pathogens.

Key words: *RT-qPCR, TaqMan probes, Hendra virus, Nipah virus, Henipavirus, diagnostics.*

РАЗРАБОТКА МЕТОДА ОТ-ПЦР В РЕЖИМЕ РЕАЛЬНОГО ВРЕМЕНИ ДЛЯ ОБНАРУЖЕНИЯ ВИРУСОВ ХЕНДРА И НИПАХ

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Резюме. Статья посвящена разработке способа обнаружения вирусной РНК двух высокопатогенных зоонозных вирусов из рода *Henipavirus* — Хендра и Нипах с помощью полимеразной цепной реакции с обрат-

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ной транскрипцией в реальном времени. В естественной среде эти вирусы переносятся летучими лисицами из семейства *Pteropodidae*. Заражению подвержены лошади и свиньи соответственно. Заболевания также передаются человеку через контакт с больными животными, их биологическими выделениями и от человека к человеку. У инфицированных людей и животных клинические признаки инфекции могут протекать бессимптомно, либо проявляющимися гриппоподобными симптомами на начальном этапе болезни и переходящие в неврологические заболевания и острую респираторную инфекцию с последующим летальным исходом. На сегодняшний день не разработано лечение против этих инфекций. Изученную субъединичную вакцину HeV-sG (Equivac®HeV, Zoetis Australia Pty Ltd.) используют в Австралии для лошадей против инфекции Хендра. Однако эта вакцина не используется для людей, и в настоящее время нет коммерчески доступных вакцин против вируса Нипах ни для человека, ни для животных. Необходимость разработки новых методов детекции и поиск новых вирусных мишеней по-прежнему остаются актуальным задачами в связи с большим ареалом распространения описанных вирусов, высокой контагиозностью и смертностью животных и людей. В исследовании описываются оригинальные разработанные праймеры и зонды на консервативные регионы геномов двух вирусов: гена, кодирующего нуклеокапсидный белок вируса Хендра и гена, кодирующего гликопротеин вируса Нипах. Созданы синтетические контроли прохождения этапов экстракции проб и постановки ПЦР с обратной транскрипцией в реальном времени, подтверждающие качество разработанного метода. Биологические образцы от здоровых людей (плазма крови, мазки со слизистых рото- и носоглотки, спинномозговая жидкость) с добавлением искусственных контролей проходили этапы выделения и постановку ПЦР с обратной транскрипцией в реальном времени, тем самым подтверждая качество контрольных образцов. Предел обнаружения описанных способов идентификации вирусной РНК определен как 100 копий/мл для вируса Хендра и 1000 копий/мл для вируса Нипах. Время прохождения амплификации составляет менее 90 минут. Разработанный способ поможет в эпидемиологическом контроле по распространениям данных инфекций, может применяться в диагностике вирусов Хендра и Нипах и для решения научно-исследовательских задач по изучению свойств данных патогенов.

Ключевые слова: *RT-qPCR, TaqMan probes, Hendra virus, Nipah virus, Henipavirus, diagnostics.*

Introduction

The *Henipavirus hendraense* (HeV) and *Henipavirus nipahense* (NiV) viruses belong to the family *Paramyxoviridae*, subfamily *Paramyxovirinae* [27]. The genus *Henipavirus* includes three species non-pathogenic to humans *(H. cedarense, H. ghanaense, H. angavokelyense)* and two highly pathogenic species *(H. hendraense, H. nipahense)* [16]. HeV and NiV are enveloped single-stranded, negative-sense RNA viruses. The genomes are represented by six genes encoding the main structural proteins, located in the following order from the 3' end: nucleocapsid protein (N); phosphoprotein (P); matrix protein (M); fusion protein (F); glycoprotein (G); and large protein (RNA polymerase (L)) [12]. The Hendra virus genome is 18 243 nucleotides in length [11], with several genotypes: HeV-g1 (discovered in 1994) and HeV-g2 (described in 2013) [2, 36]. There are several Nipah strains: the Malaysia strain (described in 1999) and the Bangladesh strain (identified in 2001) [19]. The genome sizes of these Nipah strains vary and are approximately 18 246 and 18 252 nucleotides, respectively [7, 29].

The natural hosts of the Hendra and Nipah viruses in nature are fruit bats belonging to the genus *Pteropus*, family *Pteropodidae*. The distribution area of these animals is wide: the east coast of Africa, the Indian subcontinent, Southeast Asia, north to Okinawa and south to Australia [11]. The viruses are transmitted to humans, in most cases, through intermediate hosts. For NiV, the intermediate host is pigs. For HeV, it is horses. NiV can be transmitted from bats to humans and from humans to humans. HeV is transmitted from bats to horses and from horses to humans through direct contact with the excreta of infected horses. Human-to-human transmission of HeV has not yet been recorded [12].

Outbreaks of Hendra virus have been recorded in northeastern Australia since 1994. To date, 105 cases of equine infection with Hendra virus are known, 88 of which have been fatal. Another 20 fatal cases remain with an unconfirmed diagnosis, but with symptoms similar to HeV infection [6]. Among humans during the same period of viral circulation, seven people were infected. Four died, and three recovered, albeit with various complications [6, 21, 23, 40]. The case fatality rates in horses and humans are approximately 80% and 60%, respectively [6, 40].

The main Nipah virus outbreak regions are Malaysia (43%), Bangladesh (42%), and India (15%) [31]. Since 2001, outbreaks have been registered in Bangladesh and nearby regions almost annually. According to the WHO, the last epidemiological cases in Bangladesh were recorded on January 30 and February 7, 2024 [4, 22, 25]. Nipah virus is apparently transmitted through direct contact with contaminated tissues/body fluids of infected pigs.

The incubation period of Nipah encephalitis ranges from 4 to 45 days [34, 38], while Hendra encephalitis averages 3 to 16 days, both in horses and

humans [40]. The main target organs of these viruses are the brain, lungs, heart, kidneys, and spleen [31]. Initial flu-like symptoms, fever, headache, and drowsiness very quickly develop into an encephalitis syndrome, including neurological symptoms [33].

As of 2022, the most studied vaccine against Hendra infection is the HeV-sG subunit vaccine (Equivac®HeV, Zoetis Australia Pty Ltd.) based on soluble recombinant glycoprotein HeV [15, 28]. However, this vaccine is not used for humans, and there are currently no commercially available vaccines against Nipah virus.

The development of effective and affordable methods for diagnosing infections caused by Hendra and Nipah viruses is relevant and important for several reasons. These include: poor knowledge of their viral pathogenesis; their highly contagious nature; and the large area of distribution involved (hosts, vectors, cases, etc.). Existing isothermal detection methods, such as LAMP, RPA, and NASBA, are inferior in sensitivity to classic RT-PCR methods [17, 18, 24, 32]. Variants of the latter aimed at detecting HeV and NiV have only been described in a few articles [8, 14, 20, 30, 37]. This study describes the development and evaluation of one-step real-time RT-PCR assays with original primers and probes for Hendra and Nipah virus detection.

Materials and methods

Identification of conserved genomic regions. Complete viral genomic sequences (19 Hendra, 83 Nipah) were aligned to identify conserved sites. Alignment was performed using the MEGA v.11 program (Oxford University, Great Britain). Figure 1 shows alignments of selected genomic segments of all Hendra virus isolates and 20 Nipah virus isolates, collected from different locations and in different years. A conserved region of the gene (RNA sequence) encoding nucleocapsid protein N was chosen as the target for oligonucleotide primers and a probe for Hendra virus. The gene encoding glycoprotein G was chosen for Nipah virus.

PCR control samples. The control sample set is similar to that already described [9, 13]. It includes internal extraction control (IC), armored RNA control (ARC), negative control of extraction (NEC), and PCR controls (C+, C–). To create DNA and RNA controls, fragments corresponding to target regions in the respective viral genomes (160 bp HeV, 174 bp NiV) were synthesized *de novo* by the previously described two-step PCR method [3] from the primers listed in Table 1.

Primer and probe design. Primer and probe sequences were by synthesized by Genterra (Moscow, Russia) as listed in Table 2. Primer melting temperature was estimated using the Integrated DNA Technologies OligoAnalyzer software (https://www.idtdna.com/calc/analyzer). Virus-specific probes were covalently modified with adducts: the fluorescent reporter dye rhodamine 6G (R6G) at the 5' end; and black hole quencher (BHQ1) at the 3' end. FAM was used as a fluorophore at the 5' end of the internal control, and BHQ1 was used as a quencher at the 3' end.

Reaction mixture and amplification conditions. Reactions were carried out in a volume of 25 µL including: 1 µl of BioMaster Mix (Biolabmix, Russia), 12.5 µl 2X reaction buffer (Biolabmix, Russia), and 1.5 µl specific primer and probe mixture. Forward and reverse primer concentrations were 10.5 pmol/µl for all three amplicon types (HeV, NiV, IC). Probe concentrations were 7.5 pmol/µl. Ten microliters of sample were used, and reactions were brought to 25 µL with H_2O (Milli-Q, Merck Millipore, USA). Reactions were performed using the "CFX96 C1000 Touch" (Bio-Rad, USA). The amplification conditions are listed in Tables 3 and 4.

Limit of detection. Limit of detection (LOD) values were determined using a series of 10-fold dilutions of armored RNA particles (same as armored ARC) at known concentrations. Concentrations were measured by droplet digital PCR (ddPCR). The concentrations used to determine the detection limit were 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies of armored RNA particles per mL (20 000–0.2 copies/reaction, respectively). Samples from each dilution (100 µL) were extracted in triplicate using the RIBO-prep kit (AmpliSens®, Russia) according to manufacturer instructions (elution volume 60 µL), followed by testing in the developed HeV and NiV Amp RT-PCR methods.

Analytical specificity. Analytical specificity was tested on a panel of samples (viral RNA/DNA) representing heterologous viral strains, as obtained from the St. Petersburg Pasteur Institute collection (Table 5)

Samples with added armored RNA control. Due to a lack of available clinical samples containing HeV and NiV viruses, three types of biological samples with the addition of armored genetic constructs (ARC) were used in the study. These were blood plasma samples, mucosal swabs (nasopharyngeal or oropharyngeal), and cerebrospinal fluid (CSF) samples obtained from healthy individuals. The same biologcal samples were also used as negative controls without the addition of artificial sequences. There were 25 samples in each biological group with three replicates. The average value of three replicates was used for analysis. The results are presented in Table 6.

Results

New primers and probes for identification of HeV and NiV viruses were developed based on the analyzed sequences of viral isolates from the GenBank database. In addition to definition of amplification

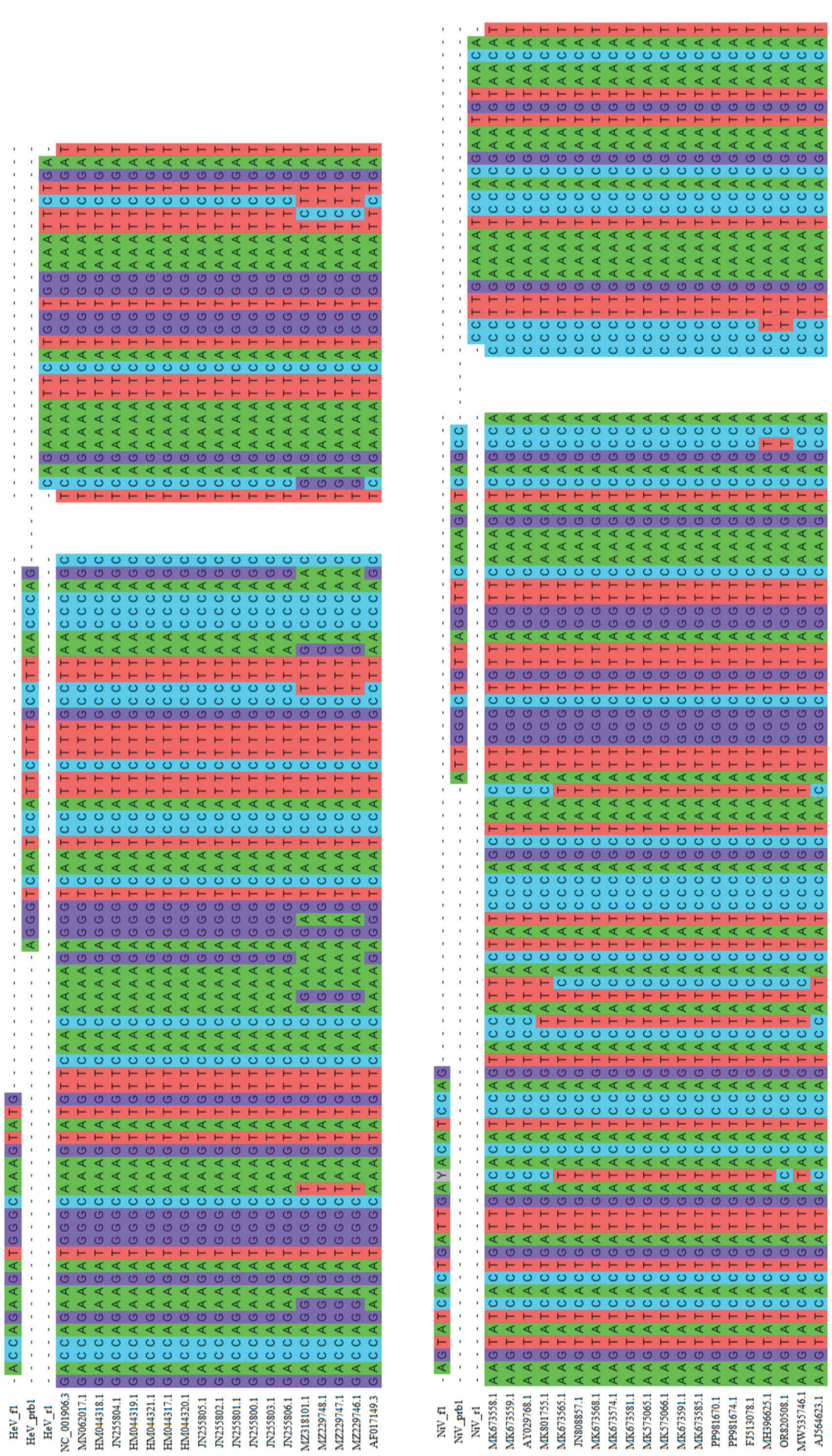

Figure 1. Alignment of target region nucleotide sequences

Note. A. Alignment of the Henipavirus hendraense N gene segment with isolates of this virus with GenBank accession numbers: NC_001906.3 — Hendra virus reference genome (no information about collection date); MN062017.1 — Australia, 1994; HM044318.1 — Australia, 2006; JN255804.1; HM044319.1; HM044321.1 — Australia, 2007; HM044317.1; HM044320.1; JN255805.1 — Australia, 2008; JN255802.1; JN255801.1; JN255800.1; JN255803.1; JN255806.1 — Australia, 2009; MZ318101.1 — Australia, 2015; MZ229748.1; MZ229747.1; MZ229746.1 — Australia, 2020; AF017149.3 (no information about place/date of sample collection). Primers and probe: HeV_f1, HeV_r1, HeV_prb1. B. Alignment of the Henipavirus nipahense G gene segment with isolates of this virus with GenBank accession numbers: MK673558.1; MK673559.1 — Malaysia (no information about collection date); MK801755.1 — Cambodia, 2003; MK673565.1 — Bangladesh, 2004; JN808857.1 — Bangladesh, 2008; MK673568.1; MK673574.1 — Bangladesh, 2011; MK673581.1 — Bangladesh, 2012; MK575065.1; MK575066.1 — Bangladesh, 2013; MK673591.1 — Bangladesh, 2014; MK673585.1 — Bangladesh, 2015; PP981670.1 — Bangladesh, 2020; PP981674.1 — Bangladesh, 2022; FJ513078.1 — India, 2007; MH396625.1 — India, 2018; OR820508.1 — India, 2023; MW535746.1 — Thailand, 2017; AJ564623.1 (no information about place/date of sample collection). Primers and probe: NiV_f1, NiV_r1, NiV_prb1

Table 1. Oligonucleotide sequences for *de novo* cDNA synthesis by two-step PCR

Name	Sequence (5'→3')	Fragment size, bp
HeV_1	AGGAAAGTGAGACCAGAAGATGGGCAAAGTATGTTCAACAAAAGAGGGTC	160
HeV_2	TGTCAGCCATTGCTGGGTTAAGGCAAAGAATGGATTGACCCTCTTTTGTTGAACATACTT	
HeV_3	ACCCAGCAATGGCTGACAGAGATGAGGAATCTCCTCTCACAAAGTCTCTCAGTCAGAAAA	
HeV_4	TTCTTTACCTCCATCAGAATTTCCACCATGAATTTTCTGACTGAGAGACTTTGTG	
NiV_1	AGGGCCCAAAGTATCACTGATTGACACATCCAGTACCATTACTATCCCAGCTAACAT	174
NiV_2	TGCAGTCGACTGGCTGATCTTTGAACCTAACAGCCCAATGTTAGCTGGGATAGTAATGGT	
NiV_3	TCAGCCAGTCGACTGCAAGTATAAATGAGAATGTGAATGAAAAATGCAAATTCACACTGC	
NiV_4	GGACAAGAAATGTTACATTCGTGGATTTTCAAGGGAGGCAGTGTGAATTTGCATTTTTCA	

Table 2. Primers and probes used in the study

Primer/ probe	Sequence 5'→3'	Modifications 5'→3'	Gene target	Probe type	Length	Amplified fragment size, bp
NiV_f1	AGT ATC ACT GAT TGA YAC ATC CAG	–	Attachment G glycoprotein gene	–	24	155
NiV_r1	TGT TAC ATT CGT GGA TTT TCA AGG	–		–	24	
NiV_prb1	ATT GGG CTG TTA GGT TCA AAG ATC AGC C	R6g — BHQ1		TaqMan	28	
HeV_f1	ACC AGA AGA TGG GCA AAG TAT G	–	Nucleocapsid (N) protein gene	–	22	133
HeV_r1	TCA GAA TTT CCA CCA TGA ATT TTC TG	–		–	26	
HeV_prb1	AGG GTC AAT CCA TTC TTT GCC TTA ACC CAG	R6g — BHQ1		TaqMan	30	
IC_f	CCG GAT TGC GTA TCT CCG GAC T	–	Artificial target	–	22	122
IC_r	CAC GGC GGC ATC TCT ATC ACG A	–		–	22	
IC_prb	TAG CTG GGC GTC AGG AAT CCC AGG	FAM — BHQ1		TaqMan	24	

Table 3. Hendra virus RNA amplification conditions

Step	Temperature, °C	Time	The number of cycles
Reverse transcription	50	15 min	1
Pre-denaturation	95	5 min	1
Denaturation	95	10 sec	40
Primer elongation and annealing + detection	55	30 sec	

Table 4. Nipah virus RNA amplification conditions

Step	Temperature, °C	Time	The number of cycles
Reverse transcription	50	15 min	1
Pre-denaturation	95	5 min	1
Denaturation	95	10 sec	40
Primer elongation and annealing + detection	60	20 sec	

Table 5. Viruses used in the assay to evaluate the analytical specificity of the method

Virus	Family	Genus	Nucleic acid type
Human parainfluenza virus type 3	*Paramyxoviridae*	*Respirovirus*	RNA
Influenza A virus (A/Puerto Rico/8/34 (H1N1))	*Orthomyxoviridae*	*Alphainfluenzavirus*	RNA
Influenza B virus (B/Florida/04/06)	*Orthomyxoviridae*	*Betainfluenzavirus*	RNA
Human adenovirus type 5	*Adenoviridae*	*Mastadenovirus*	DNA
Human rhinovirus type 1	*Picornaviridae*	*Enterovirus*	RNA
Tick-borne encephalitis virus	*Flaviviridae*	*Flavivirus*	RNA
Rabies virus	*Rhabdoviridae*	*Lyssavirus*	RNA
Measles virus	*Paramyxoviridae*	*Morbillivirus*	RNA
Human respiratory syncytial virus	*Pneumoviridae*	*Orthopneumovirus*	RNA
SARS-CoV-2	*Coronaviridae*	*Betacoronavirus*	RNA
Human coronavirus OC43	*Coronaviridae*	*Betacoronavirus*	RNA

Table 6. Average Ct values (three replicates) for three biological sample types with addition of HeV ARC and NiV ARC

	HeV	NiV		HeV	NiV		HeV	NiV
Sample name	**Ct indicator**		**Sample name**	**Ct indicator**		**Sample name**	**Ct indicator**	
blood 1	33.6	30.1	**swab 1**	31.7	29.2	**CSF 1**	32.9	29.1
blood 2	33.8	30	**swab 2**	31.6	29.9	**CSF 2**	34	30.1
blood 3	32.9	30.1	**swab 3**	31.7	31.1	**CSF 3**	33.1	31.4
blood 4	33.9	29.4	**swab 4**	31.7	29.2	**CSF 4**	34.3	29.6
blood 5	33.8	30	**swab 5**	31.7	29.2	**CSF 5**	33.5	31.1
blood 6	33.2	30	**swab 6**	31.6	29.2	**CSF 6**	33.5	31.2
blood 7	33.4	30.1	**swab 7**	31.7	31.3	**CSF 7**	33.6	31.3
blood 8	33.8	30	**swab 8**	31.8	29.7	**CSF 8**	34	29.1
blood 9	32.8	29.9	**swab 9**	31.5	29.2	**CSF 9**	33.6	29.5
blood 10	32.4	30.1	**swab 10**	32	29.3	**CSF 10**	33.5	30.8
blood 11	32.4	30	**swab 11**	31.8	29.2	**CSF 11**	32.9	29.1
blood 12	33.3	30	**swab 12**	32.2	29	**CSF 12**	32.5	29.2
blood 13	33.9	30	**swab 13**	31.7	29.1	**CSF 13**	33.7	29.8
blood 14	32.7	29.5	**swab 14**	30.9	29.2	**CSF 14**	32.3	30.1
blood 15	33.6	30.1	**swab 15**	31.8	29	**CSF 15**	34.1	29.4
blood 16	34	30.1	**swab 16**	31.2	30.1	**CSF 16**	33.2	30.2
blood 17	34	29.5	**swab 17**	31.1	29.3	**CSF 17**	33.4	29.5
blood 18	32.4	30.2	**swab 18**	30.6	29.3	**CSF 18**	33.7	29.3
blood 19	32.9	29.9	**swab 19**	31.3	29.7	**CSF 19**	34.1	29.8
blood 20	32.5	29.5	**swab 20**	32.1	29.2	**CSF 20**	33.9	29.2
blood 21	32.5	29.8	**swab 21**	31.1	29.2	**CSF 21**	33	29.3
blood 22	32.6	30.7	**swab 22**	31.5	31	**CSF 22**	33.9	29.9
blood 23	32.6	29.9	**swab 23**	31.6	29.2	**CSF 23**	32.1	29.9
blood 24	33.3	30.3	**swab 24**	31.8	29.2	**CSF 24**	33.4	30
blood 25	33.4	29.4	**swab 25**	31.1	31	**CSF 25**	32.4	29.6

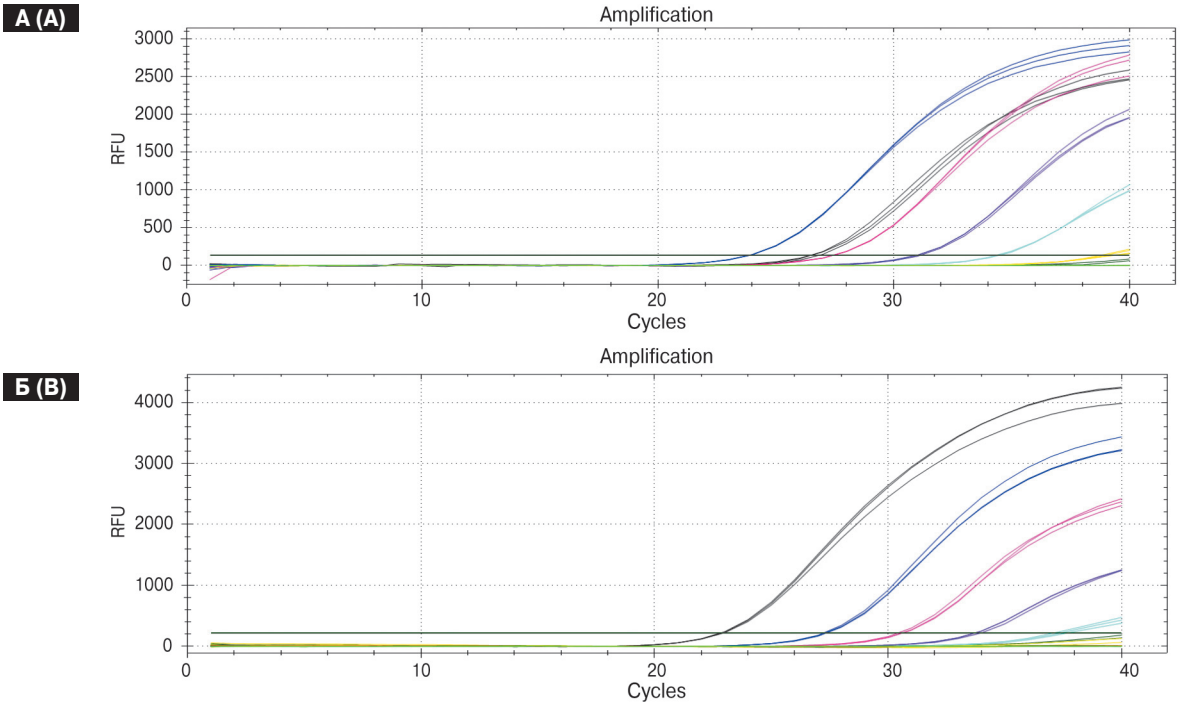

Figure 2. HEX fluorescence curves of Hendra (A) and Nipah (B) virus control samples

Note. Samples: black — C+, dark blue — ARC 10^6 copies/mL, pink — ARC 10^5 copies/mL, violet — ARC 10^4 copies/mL, blue — ARC 10^3 copies/mL, yellow — ARC 10^2 copies/mL. The ARC 10 copies/mL, negative control, and C — samples are negative. A plate type instrument, CFX96 C1000 Touch (Bio-Rad, USA), was used.

protocols, positive (ARC, C+) and negative (NEC, C–) controls were used at each stage of the study. These confirm the reliability of the obtained data.

Limit of detection (LOD) assessed using ARC dilutions was measured as the minimum dilution found in all replicates. Values were 1×10^2 RNA copies/mL and 1×10^3 RNA copies/mL for Hendra and Nipah virus, respectively (Table 7, Fig. 2).

Threshold (Ct) values for biological samples with the addition of ARC are presented in Table 6. The obtained results are within the LOD ranges for the corresponding virus. These data indicate: good reproducibility of the method; efficient ARC recovery during co-extraction and amplification with biological samples; and suitable sensitivity of the developed approach for the detection of viral RNA. The potential for cross-reactivity was assessed using RNA/DNA from 11 viral species (Table 5). None of them showed a positive reaction with the HeV or NiV real-time RT-PCR assays. The evaluated analytical specificity was 100% (data not shown).

Table 7. Threshold cycle values (Ct) of the HEX/yellow channel (ARC dilution) for the HeV and NiV virus protocols calculated from three replicates

Concentration, copies/ml	HeV replicates	NiV replicates
	Ct	Ct
10^6	24.3	27.8
10^5	28.3	31.1
10^4	31.5	34.3
10^3	35.1	37.7
10^2	39.3	N/A
10	N/A	N/A

Discussion

Hendra and Nipah viruses are zoonotic pathogens characterized by high pathogenicity and mortality, both in animals and humans [39]. Both viruses are classified as containment level 4 (CL4) pathogens. Moreover, NiV infection is currently included in the World Health Organization (WHO) list of priority diseases [1]. Episodic outbreaks of disease caused by HeV and NiV require highly sensitive diagnostic tools to effectively identify infected individuals and prevent further viral spread.

Proper selection of the target for molecular detection is an important factor in diagnostic tool development. Both sensitivity and specificity of the system depend on it. Viral genomes feature high mutation rates, which lead to the emergence of different genetic variants. In our research, we primarily selected conserved regions covering the entire known spectrum of genetic variants. Accordingly, we selected specific regions of the following genes for the annealing of primers and probes. For HeV, the gene encoding nucleocapsid protein N was chosen. For NiV, the gene encoding protein G was chosen. The N protein gene is a frequently used target for diagnostic methods based on Henipavirus nucleic acid amplification. For Hendra and Nipah virus, there are several such studies [8, 14, 20, 26]. Other genomic regions are used less frequently, for example genes encoding proteins L [35], C, M [8, 30], or G [5].

In the previously cited studies, the average amplification reaction time for Hendra and Nipah viruses is from 2 to 3 hours [14, 20, 30, 35, 37], with the exception of isothermal amplification in the work of Pollak [26]. It should be noted, however, that although isothermal amplification provides a faster result, it is inferior in sensitivity to real-time PCR, and sensitivity may be as low as 10^6 copies/mL. In the methods developed and described here, the overall processing times (reverse transcription and amplification) are 86 minutes for the HeV virus and 76 minutes for the NiV virus. Analytical sensitivity was assessed to be high for both assays as well: 1×10^2 copies/mL for HeV and 1×10^3 copies/mL for NiV.

Due to a lack of available clinical samples containing HeV and NiV viruses, the system was tested on three types of biological samples from healthy individuals with addition of specific genetic constructs (armored RNA controls, ARC) simulating real viruses. These were blood plasma, mucosal swabs (nasopharyngeal or oropharyngeal), and cerebrospinal fluid. In all cases, isolation and further RT-PCR detection were successful.

Conclusion

This manuscript reports the development and evaluation of real-time RT-PCR assays for Henipavirus hendraense (HeV) and Henipavirus nipahense (NiV) detection. For detection, the HeV and NiV assays target fragments of the N and G genes, respectively. The distinctive feature of our system is the presence of multiple controls, high sensitivity, and short reaction times. Both assays contain all of the necessary components to perform the analysis, including internal extraction control (IC), positive control for reverse transcription (ARC), negative control of extraction (NEC), and PCR controls (C+, C–). The advantage of this assay is that it allows the internal verification of all steps of the analysis, including extraction, reverse transcription, and PCR. Our results revealed that the LOD values for the assays are: 1×10^2 copies/mL for HeV viral RNA and 1×10^3 copies/mL for NiV viral RNA. In addition, both assays feature reaction times (one-step RT-PCR) less than 90 minutes.

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