Dolgova *et al. BMC Infectious Diseases* (2026) 26:376
https://doi.org/10.1186/s12879-026-12641-y

BMC Infectious Diseases

RESEARCH Open Access

# Development and evaluation of a real-time RT-PCR assay for *Mammarenavirus guanaritoense* virus detection

Anna S. Dolgova[1*], Anna S. Gladkikh[1], Dmitrii E. Polev[1], Pierina D'Angelo[2], Victor Alarcón[2], Norca García[2], Yurianni Arias[2], Iraima Monsalve[2], Lieska Rodriguez[2], Anna V. Shabalina[1], Vera A. Chayeb[1], Igor S. Sukhikh[1], Esperanza Briceño[2] and Vladimir G. Dedkov[1,3]

**Abstract**

*Mammarenavirus guanaritoense* (formerly Guanarito virus, GTOV) is an important etiological agent of an emergent disease with a high mortality rate, namely Venezuelan hemorrhagic fever (VHF). Traditionally, VHF is an important public health problem and is a mandatory reportable disease. Moreover, global interconnection has increased the probability of worldwide spread of GTOV. Therefore, national healthcare organizations need tools for the management of GTOV-associated risks. These tools should definitely include diagnostic kits based on real-time reverse transcription PCR (RT-PCR) because it is the most suitable method for the diagnosis of viral hemorrhagic fever diseases. Here, we describe a real-time RT-PCR assay for the detection of GTOV. This assay was developed and evaluated using armored positive control particles (ARC). The LOD of the assay is in the range 1–2.5 × $10^2$ RNA copies/mL on the CFX96 PCR plate machine and is in the range 1–2.0 × $10^3$ RNA copies/mL on the Rotor-Gene Q PCR rotary machine. Our assay was evaluated using positive GTOV samples from the collection of the Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela (INHRR). The assay provides a fast and sensitive tool for GTOV detection. The high specificity and sensitivity of the assay make it useful for clinical and epidemiological investigations in the field of VHF and their etiological agents.

**Keywords** *Mammarenavirus guanaritoense*, Guanarito virus, Venezuela, RT-PCR, Venezuelan hemorrhagic fever

## Background

Mammarenavirus *guanaritoense* (formerly Guanarito virus, GTOV) belongs to the genus *Mammarenavirus* within the *Arenaviridae* family [1]. GTOV is known as the etiological agent of Venezuelan hemorrhagic fever (VHF) [2]. The genome of GTOV is represented by two segments (large 'L', small 'S') of single-stranded RNA with negative polarity. Like other arenaviruses, GTOV has an ambisense genetic organization [3]. The L segment (7,141 bp) of GTOV encodes viral RNA-dependent RNA polymerase (RdRp) and a zinc finger motif protein (Z protein), while the S segment (3,942 bp) encodes the

*Correspondence:
Anna S. Dolgova
annadolgova@inbox.ru
[1]St. Petersburg Pasteur Institute, Federal Service for the Oversight of Consumer Protection and Welfare, St. Petersburg, Russia
[2]Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela
[3]Martsinovsky Institute of Medical Parasitology, Tropical and Vector Borne Diseases, Sechenov First Moscow State Medical University, Moscow, Russia

© The Author(s) 2026. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

nucleocapsid protein (NP) and a glycoprotein precursor (GPC) [4, 5].

From a geographic, phylogenetic and serological point of view, GTOV belongs to clade B of New Word arenaviruses, also known as the Tacaribe complex [3, 6]. Besides GTOV, clade B also includes four human-pathogenic New World arenaviruses. These are: *Mammarenavirus juninense* (formerly Junin virus, JUNV), the etiological agent of Argentine hemorrhagic fever; *Mammarenavirus machupoense* (formerly Machupo virus, MACV), the agent of Bolivian hemorrhagic fever; *Mammarenavirus brazilense* (formerly Sabia virus, SABV), the agent of Brazilian hemorrhagic fever; and *Mammarenavirus chapareense* (formerly Chapare virus, CHAPV), the agent of Chapare hemorrhagic fever. Clade B also contains three New World arenaviruses non-pathogenic for humans: *Mammarenavirus tacaribeense* (formerly Tacaribe virus, TCRV); *Mammarenavirus amapariense* (formerly Amapari virus, AMAV); and *Mammarenavirus cupixiense* (formerly Cupixi virus, CPXV). Based on partial NP gene sequences, it is customary to distinguish nine phylogenetically distinct GTOV genotypes that differ by 4–17% in nucleotide sequence or up to 9% in amino acid sequence [6].

The first outbreak of VHF was described in the municipality of Guanarito (Portuguesa State, Venezuela) in September, 1989, and the lethality rate of confirmed cases was 60% (9/15) [7]. From September 1989 through December 2006 in the state of Portuguesa there were recorded 618 VHF cases, with a case-fatality rate of 23.1% [8]. The latest report (dated 2021) describes 36 confirmed cases of VHF [9].

The short-tailed cane mouse (*Zygodontomys brevicauda*) is considered the principal reservoir of GTOV [10]. However, it has also been isolated from hispid cotton rats (*Sigmodon hispidus*) and Alston's cotton rats (*Sigmodon alstoni*). Seropositivity has been detected in hamster species belonging to the genus *Oryzomys* as well as in black rats (*Rattus rattus*) [7, 11]. Infected rodents shed the virus in urine, and contamination of unprotected food and water is a likely mode of GTOV transmission.

The clinical manifestation of VHF is the same as other hemorrhagic fevers. After a 3 to 19 day incubation period, VHF usually begins as a mild, nonspecific febrile illness that progresses in severity over approximately six days. Patients report fever and progressive onset of symptoms, including melena, hematemesis, petechiae, epistaxis, and rectal bleeding as the main hemorrhagic signs [12].

Isolation of GTOV remains the gold standard method for laboratory diagnosis of VHF. Detection of neutralizing antibodies is another important diagnostic method. It should be noted, however, that neutralizing antibodies, while specific and informative, appear too late after infection to be ideal or useful in early diagnostics [13]. Both methods are time-consuming and require skilled, qualified staff, as well as BSL-4 biosafety level conditions. Frequently, these conditions are not available in endemic areas [13]. Detection of IgM by enzyme-linked immunosorbent assay (ELISA), or indirect immunofluorescence assay (IFA), are also possible. However, these methods are not specific due to cross-reactions between GTOV and other arenaviruses. Therefore, other methods for rapid diagnosis based on real-time RT-PCR are more convenient for use. These methods are fast, specific, sensitive and could be implemented in BSL-3 biosafety conditions.

Despite the obvious attractiveness and necessity of molecular assays for VHF diagnosis, only several assays based on real-time RT-PCR are currently described in the scientific literature [14–18]. Although all of the assays described have shown sufficient analytical sensitivity and high specificity, it has been noted that all of them were evaluated using only one prototype GTOV strain (INH-95551) originally isolated from a fatal VHF case [14], or synthetic RNA fragments of GTOV [15–18], without testing using real clinical samples. Moreover, these assays represent multiplex panels for differential diagnosis of a number of various viruses known to be causative agents of hemorrhagic fevers. Such assay designs are more suitable for diagnosis of imported hemorrhagic fever cases and not suitable for the diagnosis and surveillance of GTOV in endemic areas.

This study aimed to develop and evaluate a monoplex, real-time RT-PCR assay for the detection of GTOV, targeting the L gene, using clinical samples from patients with VHF. This assay may be suitable for detection of various GTOV genotypes circulating in Venezuela and in a broader perspective, it can be used as a diagnostic method both in other endemic regions and for monitoring imported cases in all parts of the world.

## Materials and methods

### Identification of conserved sites

All GTOV L gene sequences available in GenBank (NCBI), as of December 2022, were aligned to identify conserved sites. BioEdit 7.2.5 software package (Ibis Biosciences, USA) was used to perform the alignment. As a target for amplification it was selected a 157 nucleotide (nt) fragment of the L gene (Fig. 1) which corresponds to nt positions 6931–7087 in the GTOV reference sequence (strain VHF-1608, GenBank accession no. KU059748).

### Primer and probe design for RT-qPCR

Primers and probes were designed according to standard RT-qPCR guidelines [19, 20]. Primer melting point calculations were performed using the OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) [21]. The thermodynamic characteristics of the probes and the

**Fig. 1** Alignment of available GTOV sequences (NCBI GenBank), primers, and probe. *Mammarenavirus guanaritoense* sequences with the following GenBank acc. nos. were aligned: KU059748, KU746274, KU746279, KU746275 – Venezuela, 1995; KU746283, KU746282 – Venezuela, 1996; KU746278, KU746285, KU746288 – Venezuela, 1997; KR260725 – Australia, 1996; KR260719 – Australia, 2008; KR260723 – Australia, 2009; KR260729 – Australia, 2011; and NC_005082 (strain INH-95551)

**Table 1** Primer and probe sequences for *Mammarenavirus guanaritoense* virus (GTOV) RNA detection

| Primer/probe | Sequence (5′–3′) | Probe type | Product size |
|---|---|---|---|
| GTOV-fa | AAAAGCTTGAAGCCCTCTGTTAA | TaqMan | 156 bp |
| GTOV-fb | AGGAGCTTGAAGCCCTCTGTTA | | |
| GTOV-r | ATCCTAGGCGAAATTTGACAATG | | |
| GTOV-prb | R6G-CCTCCTTCTGATAAGACAGTTCAGG***ag*** -BHQ1 | | |
| IC-for | CCGGATTGCGTATCTCCGGACT | TaqMan | 122 bp |
| IC-rev | CACGGCGGCATCTCTATCACGA | | |
| IC-prb | FAM-CTAGCTGGGCGTCAGGAATCCCAGG-BHQ1 | | |

* Two nucleotides added to the 3′-end of the probe to form a hairpin marked in bold italics

**Table 2** Oligonucleotides used for de novo cDNA synthesis by two-step PCR

| Name | Sequence (5′-3′) |
|---|---|
| GUAV_1 | AAAAGCTTGAAGCCTCTGTTAAAACCATCGGTATTTCAACTTGTGAAAGC |
| GUAV_2 | CTGATATACCTGAACTGTCTTATCAGAAGGAGGCATTGCTTTCACAAGTTGAAATACCGA |
| GUAV_3 | CTGATAAGACAGTTCAGGTATATCAGGTACTTGCCTTCTGATGAAGTCCCTCAAAACTAC |
| GUAV_4 | ATCCTAGGCGAAATTTGACAATGGATGAAAAAGTTGTAGTTTTGAGGGACTTCATCAG |

probability of secondary structure formation were determined by MFOLD (http://unafold.rna.albany.edu/?q=mfold/download-mfold). Two nucleotides were added to the 3' end of the probe to form a hairpin. The donor dye rhodamine 6G (R6G) was covalently attached to the 5' end of the GTOV-specific probe and its 3' end was modified by adding the quencher BHQ1. Primer and probe sequences targeting IC have been described previously [22–24]. All primers and probes (Table 1) were synthesized by Genterra PLC (Moscow, Russia),

### RT-PCR control samples

The assay uses several types of control samples. The control sample set is similar to that already described [22–24] and includes internal extraction control (IC), negative extraction control (NEC), armored RNA control (ARC), and PCR controls (positive 'C+' and negative 'C-').

To produce positive DNA and RNA controls a 157 bp GTOV targeting fragment was synthesized de novo by the previously described method two-step PCR method [25] from the primers listed in Table 2.

### Reaction mixture and amplification conditions

The amplification was carried out in a final volume of 25 µL consisting of 10 µL of sample, 12.5 µL of 2X reaction buffer (Biolabmix, Russia), 1 µL of BioMaster Mix (Biolabmix, Russia), 10.5 pmol of each GTOV specific primer (GTOV-fa, GTOV-fa, GTOV-r), 7.5 pmol of GTOV-probe, 10.5 pmol of internal control primers and 7.5 pmol of internal control probe. Reactions were brought to 25 µL with $H_2O$ (milli Q, Merck Millipore, USA). The thermal cycling parameters were as follows: 15 min at 50 °C; 5 min at 95 °C; followed by 40 cycles (10 s at 95 °C and 20 s at 55 °C). Fluorescence data were collected during the 55 °C step in the HEX/yellow channel (for GTOV) and FAM/Green channel (for IC). Reactions were performed using the CFX96 C1000 Touch (Bio-Rad, USA) and Rotor-Gene Q (Qiagen, Germany) PCR machines.

### Limit of detection

The limit of detection (LOD) was determined in the way described previously [23, 24] as the ARC minimum dilution found in all replicates and additionally using Probit analysis.

### Analytical specificity

Analytical specificity was tested on a panel of viral samples (RNA/DNA solutions) of heterologous viral strains obtained from the collection of the Saint Petersburg Pasteur Institute. Viral loads in the samples were assessed using specific real-time RT-PCR (Table 3).

### GTOV positive samples

Serum and necroscopy samples from individuals with VHF ($n = 28$) were provided by the Instituto Nacional de Higiene "Rafael Rangel" in Caracas, Venezuela (INHRR). The samples were previously collected as a part of the INHRR clinical sample collection. They were obtained between days 1 and 22 of disease onset and stored.

The diagnosis of VHF in individuals was confirmed using *in house* RT-PCR with electrophoretic detection. Briefly, viral RNA was extracted from 140 µL of serum using the QIAamp® Viral RNA Extraction Mini Kit® (QIAGEN, Hilden, Germany) in accordance with manufacturer instructions or from 30 mg of necropsy material by QIAamp® RNeasy Mini Kit (QIAGEN, Hilden, Germany). A one-step RT-PCR assay targeting a 589 bp fragment of the S segment was used for GTOV detection with primers UGtov (5´-ACAGTTCAGGGAGGAGAAAC-3´) and LGtov (5´-TGTCCATCCAGATTGTGTC-3´) [30]. A protocol was designed for one-step reverse transcription and amplification using the Super Script III One Step RT-PCR kit with Platinum Taq polymerase (Invitrogen TM, USA). GTOV-specific primers were added at a concentration of 10 µM (up to 45 µL), followed by addition of 5 µL of the extracted RNA. Reactions were performed using the GeneAmp® PCR 9700 thermal cycler

**Table 3** Viral species used in this study

| Species | Acronym | Family | Genus | Type of nucleic acid | RT-PCR kit/RT-PCR assay | Ct value | GTOV RT-PCR assay |
|---|---|---|---|---|---|---|---|
| Zaire ebolavirus | EBOV | *Filoviridae* | Ebolavirus | RNA | AmpliSens® FiloA-screen-FRT PCR kit (Russia) [26] | 19.8 | negative |
| Sudan ebolavirus | SUDV | *Filoviridae* | Ebolavirus | RNA | | 16.7 | negative |
| Marburg virus | MARV | *Filoviridae* | Marburgvirus | RNA | | 21.5 | negative |
| Lassa virus | LASV | *Arenaviridae* | Arenavirus | RNA | LASV-FI assay [27] | 21.3 | negative |
| Crimean-Congo hemorrhagic fever virus | CCHFV | *Bunyaviridae* | Nairovirus | RNA | AmpliSens® CCHFV-FRT PCR kit (Russia) | 20.8 | negative |
| Influenza A/H1N3 | FLUAV(H1N3) | *Orthomyxoviridae* | Influenzavirus A | RNA | AmpliSens® Influenza virus A-type-FRT PCR kit (Russia) | 18.4 | negative |
| Influenza A/H3N2 | FLUAV(H3N2) | *Orthomyxoviridae* | Influenzavirus A | RNA | | 17.9 | negative |
| Influenza B | FLUBV | *Orthomyxoviridae* | Influenzavirus B | RNA | AmpliSens® Influenza virus A/B-FRT PCR kit (Russia) | 23.6 | negative |
| Yellow fever virus | YFV | *Flaviviridae* | Flavivirus | RNA | AmpliSens® Yellow fever virus-FRT PCR kit (Russia) | 16.8 | negative |
| West Nile virus | WNV | *Flaviviridae* | Flavivirus | RNA | AmpliSens® WNV-FRT PCR kit (Russia) | 22.7 | negative |
| Zika virus | ZIKV | *Flaviviridae* | Flavivirus | RNA | AmpliSens® Zika virus -FRT PCR kit (Russia) | 23.1 | negative |
| Denge virus | DENV | *Flaviviridae* | Flavivirus | RNA | AmpliSens® Dengue virus-FRT PCR kit (Russia) | 22.6 | negative |
| Tick borne encephalitis virus | TBEV | *Flaviviridae* | Flavivirus | RNA | AmpliSens® TBE-FRT PCR kit (Russia) | 22.5 | negative |
| Chikungunya virus | CHIKV | *Togaviridae* | Alphavirus | RNA | Chikungunya Detection kit (Real-Time Probe Based PCR) (HiMedia Laboratories Pvt Ltd., India) | 23.8 | negative |
| Kemerovo virus, strain 21/10 | KEMV-21/10 | *Reoviridae* | Orbivirus | RNA | KEMV-FI assay [28] | 25.7 | negative |
| Human Rotavirus A | RVA | *Reoviridae* | Rotavirus | RNA | AmpliSens® Rotavirus/ Norovirus/ Astrovirus-FRT PCR kit (Russia) | 19.9 | negative |
| Rabies virus | RABV | *Rhabdoviridae* | Lyssavirus | RNA | RABV-FI assay [29] | 15.2 | negative |
| Human Cytomegalovirus 5 | HCMV-5 | *Herpesviridae* | Cytomegalovirus | DNA | AmpliSens® CMV-FRT PCR kit (Russia) | 19.9 | negative |
| Human parvovirus B19 | B19 | *Parvoviridae* | Erythroparvovirus | DNA | AmpliSens® Parvovirus B19-FRT PCR kit (Russia) | 22.1 | negative |
| Severe acute respiratory syndrome-related coronavirus-2 | SARS-CoV2 | *Coronaviridae* | Betacoronavirus | RNA | COVID-19Amp (St. Petersburg Pasteur Institute, Russia) [22] | 17.3 | negative |

(Applied Biosystems, USA) as follows. After reverse transcription (55 °C, 60 min) and denaturation (94 °C, 2 min), 35 cycles of amplification were performed (denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 90 s), followed by a final extension (72 °C, 10 min). RNA extracted from GTOV-infected cell culture supernatant was used as a positive control for RT-PCR. Presence of virus in supernatant was confirmed using IFA with GTOV-specific monoclonal antibodies [31, 32]. Products obtained were processed using electrophoretic separation in a 2% agarose gel with 0.1 mg/mL ethidium bromide (Bio-Rad, USA). Bands corresponding to the amplified products were visualized in a PhotoDoc-ItTM65 Imaging System (Ultra-Violet Products Ltd., UK) ultraviolet light transilluminator. Samples were re-tested (using the approach described above) directly before GTOV-assay evaluation (Table 5).

### Target fragment sequencing

RNA samples were obtained by extraction and purification using the QIAamp® Viral RNA Extraction Kit® or QIAamp® RNeasy Mini Kit (QIAGEN, Hilden, Germany). One step RT-RCR was conducted with SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (ThermoFisher, USA). The 157 bp fragments were purified with AMPure XP beads (Beckman Coulter, USA). Sequencing of 14 of 28 fragments was performed using the ONT MinION. Libraries were prepared using the ONT SQK-NBD114.96 kit. Amplicon (130 ng) was repaired using the NEBNext Ultra II End Repair/dA Tailing Module (NEB, USA). Barcodes were ligated with NEB Blunt/TA Ligase Master Mix, and adapter ligation was performed with the NEBNext Quick Ligation Module (NEB, USA). Samples were purified with 1× Ampure XP beads (Beckman Coulter, USA), washed with ONT Long Short Buffer, and eluted with 15 µL ONT Elution Buffer. MinION R10.4 (FLO-MIN114) flow cells were prepared and run according to ONT instructions: 20 fmol of prepared library was loaded per flow cell, and the run time was 24 h. Base-calling was performed using Dorado (v.0.6.2), wherein duplex BAM files were generated from the raw data and subsequently demultiplexed for downstream.fastq file generation. Obtained GTOV fragment sequences were deposited to GenBank with accession numbers PV471245-PV471258.

## Results

The alignment of the GTOV sequences available in GenBank (NCBI) was carried out and highly conserved regions required for the design of the GTOV-specific primers and probe (Fig. 1; Table 1) were identified. After that primers and probes were designed and synthesized. Developed assay for GTOV detection includes all components required for real-time RT-PCR.

It allows the verification of all steps of the analysis (extraction, reverse transcription, PCR). The risk of false-positive results because of cross-contamination is minimized is minimized by using NEC and C – controls. Limit of detection (LOD) was assessed using flatbed (Bio-Rad CFX96 C1000 Touch, USA) and rotary (Rotor-Gene Q, Qiagen, Germany) devices. The LOD of the assay on the CFX96 measured as the minimum dilution found in all replicates, was $10^2$ RNA copies per mL, while the LOD* measured using Probit analysis was $2.5 \times 10^2$ RNA copies per mL. For the Rotor-Gene Q it was above $10^3$ and $2 \times 10^3$ RNA copies per mL, respectively (Table 4, Figure S1, Supplementary Materials).

Analytical specificity was verified on 20 viral species belonging to 11 viral families with no cross-reactivity. Thus, the evaluated analytical specificity was 100%.

In addition, a total of 28 GTOV-positive samples examined previously by the INHRR were re-tested using *in house* RT-PCR with electrophoretic detection, as described above, and then tested using the GTOV RT-PCR assay (Table 5). Fourteen among 'previously tested as positive' samples (50%) were assessed as positive again using both *in house* RT-PCR and the developed real-time RT-PCR assay. The $C_t$ values of these samples ranged from 24 to 37 cycles. Eleven among 'previously tested as positive' samples (39%) were assessed as a negative using both *in house* RT-PCR and the developed real-time RT-PCR assay. Discordance for 3 samples was also observed. Three among 'previously tested as positive' samples (11%) were assessed as negative using *in house* RT-PCR.

**Table 4** Threshold cycles (Ct) of the HEX/yellow channel by ARC Dilution

| ARC concentration, copies/mL | CFX96 C1000 Touch (Bio-Rad, USA) | | | | Rotor-Gene Q (Qiagen, Germany) | | | |
|---|---|---|---|---|---|---|---|---|
| | Replicate, $C_t$ value | | | | Replicate, $C_t$ value | | | |
| | 1 | 2 | 3 | median | 1 | 2 | 3 | median |
| $10^4$ | 34.17 | 34.35 | 34.36 | **34.3** | 34.21 | 33.84 | 33.44 | **33.8** |
| $10^3$ | 36.96 | 36.57 | 37.48 | **37.0** | 38.68 | 37.42 | 38.17 | **38.0** |
| $10^2$ | 39.14 | 39.95 | 39.30 | **39.5** | negative | 38.57 | negative | negative |
| $10^1$ | negative | negative | negative | negative | negative | negative | negative | negative |
| LOD, copies/mL | **$10^2$** | | | | **$10^3$** | | | |
| LOD*, copies/mL | **$2 \times 10^2$** | | | | **$2.5 \times 10^3$** | | | |

LOD*- limit of detection measured using Probit analysis

**Table 5** GTOV-positive samples used in this study

| | Sample ID | Collection region | Collection year | Days since onset of VHF disease | Sample type | Type of tissue | Assessment using conventional RT-PCR assay (initial) | Assessment using conventional RT-PCR assay (this study) | GTOV-RT PCR assay, Ct value (this study) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | GTOV-1 | Barinas | 2012 | 6 | serum | | **positive** | **negative** | **negative** |
| 2 | GTOV-2 | Barinas | 2016 | 3 | serum | | positive | positive | 32 |
| 3 | GTOV-3 | Barinas | 2017 | 15 | serum | | positive | positive | 28 |
| 4 | GTOV-4 | Barinas | 2016 | 5 | necropsy | liver | positive | positive | 36 |
| 5 | GTOV-5 | Barinas | 2017 | 22 | necropsy | liver | positive | positive | 36 |
| 6 | GTOV-6 | Barinas | 2017 | 22 | necropsy | spleen | **positive** | **negative** | **37** |
| 7 | GTOV-7 | Barinas | 2017 | 6 | necropsy | liver | positive | positive | 31 |
| 8 | GTOV-8 | Barinas | 2017 | 9 | necropsy | spleen | positive | positive | 31 |
| 9 | GTOV-9 | Barinas | 2016 | 4 | serum | | **positive** | **negative** | **negative** |
| 10 | GTOV-10 | Portuguesa | 2012 | 1 | serum | | positive | positive | 32 |
| 11 | GTOV-11 | Barinas | 2017 | 7 | serum | | positive | positive | 35 |
| 12 | GTOV-12 | Barinas | 2017 | 7 | necropsy | liver | **positive** | **negative** | **31** |
| 13 | GTOV-13 | Portuguesa | 2019 | 21 | serum | | **positive** | **negative** | **negative** |
| 14 | GTOV-14 | Barinas | 2024 | 8 | serum | | positive | positive | 36 |
| 15 | GTOV-15 | Barinas | 2017 | 6 | serum | | **positive** | **negative** | **negative** |
| 16 | GTOV-16 | Barinas | 2017 | 6 | necropsy | liver | positive | positive | 37 |
| 17 | GTOV-17 | Portuguesa | 2013 | 7 | serum | | positive | positive | 34 |
| 18 | GTOV-18 | Portuguesa | 2013 | 6 | serum | | **positive** | **negative** | **negative** |
| 19 | GTOV-19 | Barinas | 2017 | 7 | necropsy | spleen | **positive** | **negative** | **negative** |
| 20 | GTOV-20 | Barinas | 2017 | 6 | necropsy | spleen | **positive** | **negative** | **negative** |
| 21 | GTOV-21 | Barinas | 2017 | 7 | necropsy | liver | **positive** | **negative** | **negative** |
| 22 | GTOV-22 | Barinas | 2017 | 7 | necropsy | liver | **positive** | **negative** | **negative** |
| 23 | GTOV-23 | Barinas | 2017 | 6 | necropsy | spleen | **positive** | **negative** | **negative** |
| 24 | GTOV-24 | Barinas | 2016 | 5 | necropsy | spleen | **positive** | **negative** | **negative** |
| 25 | GTOV-25 | Barinas | 2017 | 9 | serum | | positive | positive | 30 |
| 26 | GTOV-26 | Barinas | 2017 | 15 | necropsy | spleen | positive | positive | 24 |
| 27 | GTOV-27 | Barinas | 2019 | 2 | serum | | **positive** | **negative** | 37 |
| 28 | GTOV-28 | Barinas | 2019 | 11 | serum | | positive | positive | 34 |

However, these samples tested positive using the real-time RT-PCR assay ($C_t$ values ranging from 31 to 37 cycles). The differences in the testing results using the *in-house* RT-PCR earlier and during this study are probably due to degradation of viral RNA during long-term storage. In addition, 'GTOV RT-PCR assay positive' samples were partially sequenced successfully in the target region of the assay studied.

In silico analysis of primer and probe sequences (target regions) within viral sequences was performed to identify if significant substitutions exist which might cause interference. Significant substitutions were not noted for GTOV isolates available in GenBank (Fig. 1) or clinical GTOV isolates used in this study (Fig. 2). Thus, the GTOV RT-PCR assay contributed to INHRR workflow, including analysis and interpretation of results obtained.

## Discussion

Hemorrhagic fever diseases (HFD) remain a major global health threat, primarily through their emergent status and high mortality. It is a mistake to assume that HFDs are a public health problem only in endemic regions. Globalization and climate change increase the risk of imported HFD cases in non-endemic countries, and these factors could lead to the introduction of hemorrhagic fever viruses into previously non-endemic regions.

An example is the largest-ever recorded epidemic of Ebola virus disease (EVD) that occurred in West Africa from 2013 to 2016, featuring 28,616 total cases and 11,310 deaths [33]. The epidemic spread throughout three West African countries: Guinea, Liberia, and Sierra Leone. Sporadic cases of EVD were also reported in Mali, Nigeria, and Senegal [34]. Human-to-human transmission has begun in urban areas. Moreover, visitors from the UK, Spain, Italy, and the US were infected, which created a pandemic threat [35].

In most cases, emergent pathogens of viral hemorrhagic fevers are endemic in countries with a tropical climate. And, as a rule, these are countries with limited health system capabilities. These countries need to be supported by the global health system, not only in the diagnosis of HFD cases that have arisen, but also in

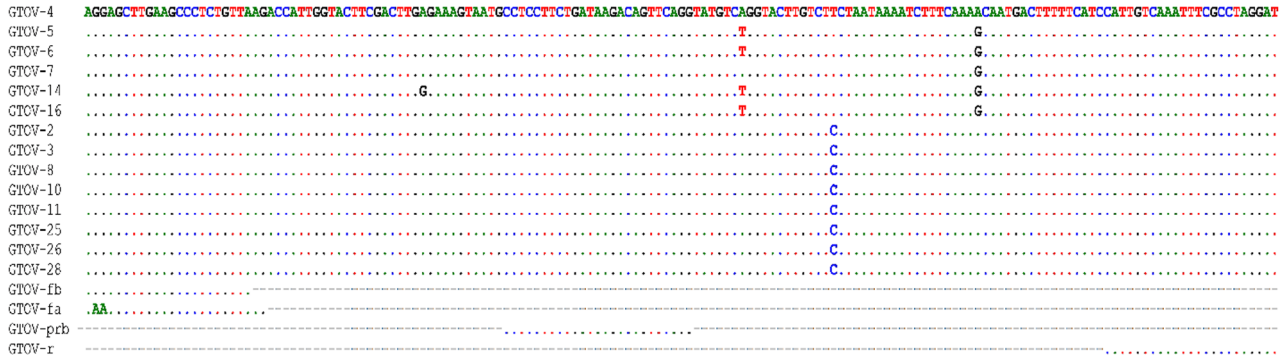


**Fig. 2** Alignment of GTOV isolate sequences used in the study, primers, and probe. *Mammarenavirus guanaritoense* isolate *sequences* with the following sample IDs were aligned: GTOV-4 (PV471245), GTOV-5 (PV471246), GTOV-6 (PV471247), GTOV-7 (PV471248), GTOV-14 (PV471249), GTOV-16 (PV471250), GTOV-2 (PV471251), GTOV-3 (PV471252), GTOV-8 (PV471253), GTOV-10 (PV471254), GTOV-11 (PV471255), GTOV-25 (PV471256), GTOV-26 (PV471257), and GTOV-28 (PV471258) (Table 5)

performing surveillance within the framework of the One Health conceptual approach. In this regard, specific methods for detection of hemorrhagic fever viruses that are rapid and sensitive are needed. They are valuable not only for HFD diagnosis, but also for surveillance and identification of viral sources in the environment.

Real-time RT-PCR assay for GTOV detection is a sensitive, specific, and rapid diagnostic tool, valuable both for outbreak control in endemic regions and for excluding the possibility of imported cases in non-endemic areas. Its combination of speed, accuracy, and quantitative capability positions it as a strong candidate for the gold standard for laboratory confirmation of VHF.

In endemic areas, real-time RT-PCR can be applied for early detection and differentiation from other febrile illnesses such as dengue, chikungunya, and others. This assay demonstrates high analytical sensitivity, supported by both bioinformatic predictions and experimental data (Table 3). Validation studies using samples collected over multiple years from endemic regions of Venezuela have shown that real-time RT-PCR offers greater sensitivity than conventional PCR, even with long-term sample storage, enabling its use in retrospective diagnostics.

The assay's adaptability to high-throughput formats (96-well plates for PCR and automated nucleic acid extraction systems) supports large-scale surveillance in both rodent populations and human samples during outbreak investigations. For suspected imported VHF cases, the real-time RT-PCR approach enables rapid exclusion or confirmation without the need for BSL-4 laboratory facilities.

Thus, the developed diagnostic method can be applied to a wide range of GTOV-related tasks. Future work will involve this approach to analyze field material collected in Venezuela and potentially in neighboring countries. Its high-throughput capacity will allow the study not only of the short-tailed cane mouse, the primary reservoir host, but also of other rodent species within the virus's potential range. We anticipate that such studies—whether conducted by our group or others—will contribute valuable new information on the biology, distribution, and reservoir ecology of *Mammarenavirus guanaritoense*.

## Conclusions

This manuscript reports the development and evaluation of a real-time RT-PCR assay for GTOV detection. The assay contains all of the necessary components to perform the analysis, including internal extraction control (IC), positive control for reverse transcription armored RNA control (ARC), negative control of extraction (NEC), and PCR controls (positive 'C+' and negative 'C-'). The advantage of this assay is that it allows the verification of all steps of the analysis, including extraction, reverse transcription and PCR.

Our results revealed that the LOD of the assay is in the range 100 to 250 RNA copies/ml on the CFX96 PCR plate machine. On the Rotor-Gene Q PCR rotary machine, it is in the range 1000 to 2000 RNA copies/ml. Thus, our GTOV real-time RT-PCR assay provides a fast and sensitive tool for Guanarito virus detection. The high specificity and sensitivity of the assay make it useful for clinical and epidemiological investigations in the field of VHF.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-026-12641-y.

Supplementary Material 1

**Acknowledgements**

We thank Ms. Mayling Castillo, Instituto Nacional de Higiene "Rafael Rangel", for her technical assistance.

**Author contributions**

All authors read and approved the final manuscript. ASD genome alignment, writing—review and editing, organization of RT-PCR assay development, control samples design, visualization. ASG genome alignment, PCR analysis

of clinical samples, sequencing of clinical samples. DEP sequencing of clinical samples. PA coordination of the work, PCR analysis of clinical samples, data analysis. VA collection and storage of clinical samples. NG, YA, IM working with clinical samples, extraction and storage of nucleic acids, conventional RT-PCR assay. LR data curation, primary analysis of samples, conventional RT-PCR assay curation. AVS control samples development and production. VAC, PCR during RT-PCR assay development, writing—original draftISS RT-PCR assay development protocol tracking, PCR during RT-PCR assay development. EB project administration, writing—review and editing. VGD RT-PCR primers and probe design, writing—review and editing, supervision, project administration.

**Funding**
The study was supported by the Russian federal governmental program "Sanitary Shield".

**Data availability**
The datasets generated during the current study are available in the GenBank (NCBI) with accession numbers PV471245-PV471258. Other analysed datasets are available in the GenBank (NCBI) with accession numbers specified in the article.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

Received: 4 April 2025 / Accepted: 16 January 2026
Published online: 24 January 2026

**References**

1. https://ictv.global/taxonomy
2. Hallam SJ, Koma T, Maruyama J, Paessler S. Review of *Mammarenavirus* biology and replication. Front Microbiol. 2018;9:1751. https://doi.org/10.3389/fmicb.2018.01751.
3. Günther S, Lenz O. Lassa virus. Crit Rev Clin Lab Sci. 2004;41(4):339–90. https://doi.org/10.1080/10408360490497456.
4. Gonzalez JP, Sanchez A, Rico-Hesse R. Molecular phylogeny of Guanarito virus, an emerging arenavirus afecting humans. Am J Trop Med Hyg. 1995;53(1):1–6.
5. Cajimat MN, Fulhorst CF. Phylogeny of the Venezuelan arenaviruses. Virus Res. 2004;102(2):199–206. https://doi.org/10.1016/j.virusres.2004.01.032.
6. Silva-Ramos CR, Montoya-Ruíz C, Faccini-Martínez ÁA, Rodas JD. An updated review and current challenges of Guanarito virus infection, Venezuelan hemorrhagic fever. Arch Virol. 2022;167(9):1727–38. https://doi.org/10.1007/s00705-022-05453-3.
7. Salas R, de Manzione N, Tesh RB, Rico-Hesse R, Shope RE, Betancourt A, Godoy O, Bruzual R, Pacheco ME, Ramos B, et al. Venezuelan haemorrhagic fever. Lancet. 1991;338(8774):1033–6. https://doi.org/10.1016/0140-6736(91)91899-6.
8. Fulhorst CF, Cajimat MN, Milazzo ML, Paredes H, de Manzione NM, Salas RA, Rollin PE, Ksiazek TG. Genetic diversity between and within the arenavirus species Indigenous to Western Venezuela. Virology. 2008;378(2):205–13. https://doi.org/10.1016/j.virol.2008.05.014.
9. Rodríguez-Morales AJ, Bonilla-Aldana DK, Risquez A, Paniz-Mondolfi A, Suárez JA. Should we be concerned about Venezuelan hemorrhagic fever? - A reflection on its current situation in Venezuela and potential impact in Latin America amid the migration crisis. New Microbes New Infect. 2021;44:100945. https://doi.org/10.1016/j.nmni.2021.100945.
10. Milazzo ML, Cajimat MN, Duno G, Duno F, Utrera A, Fulhorst CF. Transmission of Guanarito and pirital viruses among wild Rodents, Venezuela. Emerg Infect Dis. 2011;17(12):2209–15. https://doi.org/10.3201/eid1712.110393.
11. Tesh RB, Wilson ML, Salas R, De Manzione NM, Tovar D, Ksiazek TG, Peters CJ. Field studies on the epidemiology of Venezuelan hemorrhagic fever: implication of the cotton rat Sigmodon alstoni as the probable rodent reservoir. Am J Trop Med Hyg. 1993;49(2):227–35. https://doi.org/10.4269/ajtmh.1993.49.227.
12. de Manzione N, Salas RA, Paredes H, Godoy O, Rojas L, Araoz F, Fulhorst CF, Ksiazek TG, Mills JN, Ellis BA, Peters CJ, Tesh RB. Venezuelan hemorrhagic fever: clinical and epidemiological studies of 165 cases. Clin Infect Dis. 1998;26(2):308–13. https://doi.org/10.1086/516299.
13. Zhirnov IV, Ryabinin VA, Sinyakov AN, Ternovoy VA, Shikov AN. A prototype of oligonucleotide microarray for detection of pathogens relating to arena- and Filoviridae families. Bioorg Khim. 2015;41(1):54–66. https://doi.org/10.1134/s1068162014050136.
14. Vieth S, Drosten C, Charrel R, Feldmann H, Günther S. Establishment of conventional and fluorescence resonance energy transfer-based real-time PCR assays for detection of pathogenic new world arenaviruses. J Clin Virol. 2005;32(3):229–35. https://doi.org/10.1016/j.jcv.2004.07.011.
15. Fajfr M, Neubauerová V, Pajer P, Kubíčková P, Růžek D. Detection panel for identification of twelve hemorrhagic viruses using real-time RT-PCR. Epidemiol Mikrobiol Imunol. 2014;63(3):238–44.
16. Trombley AR, Wachter L, Garrison J, Buckley-Beason VA, Jahrling J, Hensley LE, Schoepp RJ, Norwood DA, Goba A, Fair JN, Kulesh DA. Comprehensive panel of real-time TaqMan polymerase chain reaction assays for detection and absolute quantification of filoviruses, arenaviruses, and new world hantaviruses. Am J Trop Med Hyg. 2010;82(5):954–60. https://doi.org/10.4269/ajtmh.2010.09-0636.
17. Pang Z, Li A, Li J, Qu J, He C, Zhang S, Li C, Zhang Q, Liang M, Li D. Comprehensive multiplex one-step real-time TaqMan qRT-PCR assays for detection and quantification of hemorrhagic fever viruses. PLoS ONE. 2014;9(4):e95635. https://doi.org/10.1371/journal.pone.0095635.
18. Choi Y, Kim Y. Application of multiplex realtime PCR detection for hemorrhagic fever syndrome viruses. J Infect Public Health. 2023;16(12):1933–41. https://doi.org/10.1016/j.jiph.2023.10.012.
19. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 2019;37(7):761–74. https://doi.org/10.1016/j.tibtech.2018.12.002.
20. Van Pelt-Verkuil E, van Belkum A, Hays JP. Principles and technical aspects of PCR amplification. Springer; 2008.
21. Kibbe WA. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 2007;35(Suppl 2):W43–46.
22. Goncharova EA, Dedkov VG, Dolgova AS, Kassirov IS, Safonova MV, Voytsekhovskaya Y, Totolian AA. One-step quantitative RT-PCR assay with armored RNA controls for detection of SARS-CoV-2. J Med Virol. 2021;93(3):1694–701. https://doi.org/10.1002/jmv.26540.
23. Dolgova AS, Kanaeva OI, Antonov SA, Shabalina AV, Klyuchnikova EO, Sbarzaglia VA, Gladkikh AS, Ivanova OE, Kozlovskaya LI, Dedkov VG. Qualitative real-time RT-PCR assay for nOPV2 poliovirus detection. J Virol Methods. 2024;329:114984. https://doi.org/10.1016/j.jviromet.2024.114984.
24. Chayeb VA, Dolgova AS, Popova MR, Zheleznova NV, Shirobokova SA, Shabalina AV, Sharova AA, Gladkikh AS, Antipova AY, Kirichenko AD, et al. Development and evaluation of a new measles detection assay using Real-Time RT-PCR. Int J Mol Sci. 2025;26(5):1801. https://doi.org/10.3390/ijms26051801.
25. Dolgova AS, Stukolova OA. High-fidelity PCR enzyme with DNA-binding domain facilitates de Novo gene synthesis. 3 Biotech. 2017;7(2):128. https://doi.org/10.1007/s13205-017-0745-2.
26. Dedkov VG, Magassouba N, Safonova MV, Bodnev SA, Pyankov OV, Camara J, Sylla B, Agafonov AP, Maleev VV, Shipulin GA. Sensitive multiplex real-time RT-qPCR assay for the detection of filoviruses. Health Secur. 2018;16(1):14–21. https://doi.org/10.1089/hs.2017.0027.
27. Dedkov VG, Magassouba N, Safonova MV, Naydenova EV, Ayginin AA, Soropogui B, Kourouma F, Camara AB, Camara J, Kritzkiy AA, Tuchkov IV, Shchelkanov MY, Maleev VV. Development and evaluation of a One-Step quantitative RT-PCR assay for detection of Lassa virus. J Virol Methods. 2019;271:113674. https://doi.org/10.1016/j.jviromet.2019.113674.
28. Dedkov VG, Markelov ML, Gridneva KA, Bekova MV, Gmyl AP, Kozlovskaya LI, Karganova GG, Romanova LIu, Pogodina VV, Yakimenko VV, Shipulin GA. Prevalence of Kemerovo virus in Ixodid ticks from the Russian federation. Ticks Tick Borne Dis. 2014;5(6):651–5. https://doi.org/10.1016/j.ttbdis.2014.04.017.

29. Dedkov VG, Deviatkin AA, Poleshchuk EM, Safonova MV, Blinova EA, Shchelkanov MY, Sidorov GN, Simonova EG, Shipulin GA. Development and evaluation of a RT-qPCR assay for fast and sensitive rabies diagnosis. Diagn Microbiol Infect Dis. 2018;90(1):18–25. https://doi.org/10.1016/j.diagmicrobio.2017.09.009.
30. Ríos M, Velásquez RodríguezM, Villarroel F, Salas R, Revista. De La Sociedad Venez De Microbiología. 2017;37:82–6. (in Spanish).
31. Ministerio. para el Poder Popular para la Salud.Viceministerio de Redes de Salud Colectiva. Fiebre Hemorrágica Venezolana. 2012 Versión 2.0 (in Spanish).
32. Vásquez C, Salas RA, De Manzione N, Paredes H, Tesh R. Fiebres hemorrágicas por Arenavirus en Venezuela. Vitae Academia Biomédica Digital. 2005; 21 Disponible en: http://vitae.ucv.ve/pdfs/VITAE_2963.pdf. Acceso: 27 abril 2017 (in Spanish).
33. Gillespie AM, Obregon R, El Asawi R, Richey C, Manoncourt E, Joshi K, Naqvi S, Pouye A, Safi N, Chitnis K, Quereshi S. Social mobilization and community engagement central to the Ebola response in West africa: lessons for future public health emergencies. Glob Health Sci Pract. 2016;4(4):626–46. https://doi.org/10.9745/GHSP-D-16-00226.
34. Gire SK, Goba A, Andersen KG, Sealfon RS, Park DJ, Kanneh L, Jalloh S, Momoh M, Fullah M, Dudas G, Wohl S, Moses LM, Yozwiak NL, Winnicki S, Matranga CB, Malboeuf CM, Qu J, Gladden AD, Schaffner SF, Yang X, Jiang PP, Nekoui M, Colubri A, Coomber MR, Fonnie M, Moigboi A, Gbakie M, Kamara FK, Tucker V, Konuwa E, Saffa S, Sellu J, Jalloh AA, Kovoma A, Koninga J, Mustapha I, Kargbo K, Foday M, Yillah M, Kanneh F, Robert W, Massally JL, Chapman SB, Bochicchio J, Murphy C, Nusbaum C, Young S, Birren BW, Grant DS, Scheiffelin JS, Lander ES, Happi C, Gevao SM, Gnirke A, Rambaut A, Garry RF, Khan SH, Sabeti PC. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science. 2014;345(6202):1369–72. https://doi.org/10.1126/science.1259657.
35. Gupta S, Gupta N, Yadav P, Patil D. Ebola virus outbreak preparedness plan for developing nations: lessons learnt from affected countries. J Infect Public Health. 2021;14(3):293–305. https://doi.org/10.1016/j.jiph.2020.12.030.

## Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.