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Sequence Optimized Real-Time Reverse Transcription Polymerase Chain Reaction Assay for Detection of Crimean-Congo Hemorrhagic Fever Virus

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Abstract. Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* within the family Bunyaviridae. Infection can result in general myalgia, fever, and headache with some patients developing hemorrhagic fever with mortality rates ranging from 5% to 30%. CCHFV has a wide geographic range that includes Africa, Asia, the Middle East, and Europe with nucleotide sequence variation approaching 20% across the three negative-sense RNA genome segments. While phylogenetic clustering generally aligns with geographic origin of individual strains, distribution can be wide due to tick/CCHFV dispersion via migrating birds. This sequence diversity negatively impacts existing molecular diagnostic assays, leading to false negative diagnostic results. Here, we updated a previously developed CCHFV real-time reverse transcription polymerase chain reaction (RT-PCR) assay to include strains not detected using that original assay. Deep sequencing of eight different CCHFV strains, including three that were not detectable using the original assay, identified sequence variants within this assay target region. New primers and probe based on the sequencing results and newly deposited sequences in GenBank greatly improved assay sensitivity and inclusivity with the exception of the genetically diverse strain AP92. For example, we observed a four log improvement in IbAr10200 detection with a new limit of detection of 256 PFU/mL. Subsequent comparison of this assay to another commonly used CCHFV real-time RT-PCR assay targeting a different region of the viral genome showed improved detection, and both assays could be used to mitigate CCHFV diversity for diagnostics. Overall, this work demonstrated the importance of continued viral sequencing efforts for robust diagnostic assay development.

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV; family Bunyaviridae, genus *Nairovirus*) infection of humans can result in a disease spectrum ranging from a nonspecific febrile illness to hemorrhagic fever manifestations with a mortality rate of 5%–30%.[1] The relatively low rate of disease in seropositive populations has spurred research into potential host susceptibility factors,[2–5] although the availability of appropriate supportive care may provide a more direct correlation with clinical outcomes. CCHFV is predominantly transmitted by ixodid ticks of the genus *Hyalomma*, and CCHFV has a wide geographic distribution with endemic foci in Eastern Europe, sub-Saharan and southern Africa, the Middle East, and Asia.[6,7] Handling of tick-infested livestock and proximity to vegetated areas with high tick burdens are significant risk factors for CCHFV infection. In addition, nosocomial exposure to CCHFV-infected individuals in low resource facilities can result in severe disease among healthcare workers.[1,7]

CCHFV is an enveloped virus with a trisegmented, negative-sense RNA genome that encodes an RNA-dependent RNA polymerase (L), two major structural glycoproteins (G_N and G_C), and a nucleoprotein (N) on the L, M, and S genome segments, respectively. CCHFV has the largest genome of any bunyavirus at 19.1 kb total with 12.1, 5.4, and 1.6 kb in the three genome segments, respectively. To date, the National Institute of Allergy and Infectious Diseases (NIAID) Virus Pathogen Database and Analysis Resource (ViPR) lists complete genome sequences available for 54 (L), 75 (M), and 102 (S) genome segments of CCHFV strains.[8] Pairwise alignments of these sequences indicate that their mean sequence identities are 89.4% (L), 80.0% (M), and 88.1% (S).

Currently, there are no CCHFV vaccines or therapeutics approved for human use by the United States Food and Drug Administration, although immunoglobulin therapy and ribavirin have been used abroad with mixed results.[9] In the absence of approved countermeasures, effective diagnostics remain an invaluable means to identify and control CCHFV outbreaks. A variety of assay platforms for CCHFV can detect viral nucleic acids to include low density macroarrays,[10] high density resequencing arrays,[11] padlock probes with colorimetric readout,[12] loop-mediated isothermal amplification (LAMP),[13] and polymerase chain reaction.[14–18] Real-time reverse-transcription polymerase chain reaction (RT-PCR) remains the gold standard for quantitative, sensitive, and specific detection of CCHFV; however, these assays have sensitivity issues due to the genetic diversity of different CCHFV strains.[19]

Previously, Garrison et al.[20] developed a TaqMan MGB real-time RT-PCR assay (Garrison assay) capable of detecting eighteen strains of CCHFV. Subsequent testing of this assay identified several additional strains which were undetectable by this assay.[15] We suspected whether the inherent diversity of CCHFV genome contributed to inefficient primer/probe hybridization. To improve the assay performance, we sequenced these isolates and designed a set of degenerate primers and probes to take into account CCHFV diversity in the assay target region. This optimization increased assay sensitivity compared with the original Garrison assay and to a commonly used assay developed by Atkinson et al.[15,21–26]

MATERIALS AND METHODS

Viruses. Multiple CCHFV strains including IbAr10200 (UCC# R4401), DAK8194 (UCC# R4416), SPU 128/81 (UCC# R4417), SPU 115/87 (UCC# R4448), UG 3010 (UCC# R4432),

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JD-206 (UCC# R4413), HY-13 (UCC# R4459), and Drosdov (UCC# R4405) were acquired from the Unified Culture Collection (UCC) maintained at the US Army Medical Research Institute of Infectious Diseases (USAMRIID). Total RNA was extracted from 200 µL of cell culture supernatant using TRIzol LS (Thermo Fisher Scientific, Waltham, MA), the EZ1 Advanced XL (Qiagen, Valencia, CA), and the EZ1 Virus Mini Kit V 2.0 (Qiagen) according the manufacturers' recommendations. Total nucleic acid was eluted in 90 µL of elution buffer and stored at –80°C until use. Previously extracted RNA from additional CCHFV strains maintained at the USAMRIID, including I-40, 2219 KKK28, I-248, SPU 97/85, SPU 134/ 87, SPU 415/85, and SPU 41/84, were used for assay inclusivity testing.

S segment analysis. For assay primer/probe design optimization, sequences from the UCC virus strains used in this study were sequenced and analyzed.[27] While S segments for some of these viruses have previously been sequenced[28] and there have been additional GenBank submissions by Lofts, Hodgson, and Smith, the specific viruses used in this study were sequenced to 1) characterize the virus stocks being used, 2) characterize strains not previously sequenced, and 3) meet the FDA-ARGOS reference genome standards (BioProject #PRJNA231221). Consensus sequences used include GenBank (IbAr10200 [KY484036], DAK8194 [KY484027], SPU 128/81 [KY484044], SPU 115/87 [KY484040], UG3010 [KY484048], JD-206 [KY484037], HY-13 [KY484031], and Drosdov [KY484028]).[27]

The S segments from these sequenced viruses were aligned, and the assay target region was isolated for variant analysis and assay redesign. In addition, existing CCHFV S segment sequences from GenBank that covered the assay target region were aligned with the CLC Genomics Workbench (Supplemental Figure 1).

Real-time RT-PCR assays. The Garrison assay[20] was run as previously described with modifications described below. For the new assay described here (CCHF-S2), primers and probe were designed within the same assay target region based on the data from the new sequencing data. See Table 1 for the primer and probe sequences and concentrations. Both assays (Garrison assay and CCHF-S2) were run on a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) using the SuperScript One-Step RT-PCR Kit (Thermo Fisher Scientific), 5 µL of purified nucleic acid, and a final concentration of 3 mM $MgSO_4$. Cycling conditions were 50°C for 15 minutes, 95°C for 5 minutes, and then 45 cycles of 94°C for 1 second, 55°C for 20 seconds, and 68°C for 5 seconds. For the comparison with the Atkinson assay, primers, probe, and reaction conditions were the same as previously published.[15] The fluorescence was measured at the end of each 68°C extension step, and a positive call required a quantification cycle (Cq) value of less than 40 cycles. All negative calls were given a Cq value of 40. The modified assay (CCHF-S2) was optimized for primer and probe concentrations using CCHFV IbAr10200 RNA. This process involved testing multiple primer concentrations ranging from 0.5 to 1.0 mM with 0.2 mM probe. The optimal primer concentration was selected based on the lowest Cq value and the highest endpoint fluorescence (data not shown).

A preliminary limit of detection (LOD) determination was conducted for both assays by serially diluting viral RNA either 10-fold or 5-fold in two different series, and samples were run by real-time RT-PCR in triplicate. The preliminary LOD was the lowest RNA dilution where all replicates were positive. The LOD was confirmed by running 60 replicates at the LOD, requiring at least 58 of 60 replicates to be positive. Inclusivity for both assays was determined using the 15 different strains of CCHFV maintained at USAMRIID and the UCC described previously. Synthetic RNAs (Bio-Synthesis, Lewisville, TX) comprising the Atkinson and the Garrison assay target regions of CCHFV AP92 were used for additional inclusivity testing.

Exclusivity testing was conducted using a viral RNA reference panel maintained at USAMRIID and acquired from the UCC. These viruses included Rift Valley fever virus (MP12), Hantaan virus (76118), yellow fever virus (17D), dengue virus serotype 1 (WestPac, UCC# R4423), dengue virus serotype 2 (S16803, UCC# R4424), dengue virus serotype 3 (CH53489, UCC# R4425), dengue virus serotype 4 (341750, UCC# R4426), West Nile virus (EG101 [UCC# R4310T] and NY99 [UCC# R4272T]), Chikungunya virus (B8636 and 38635), Lassa fever virus Josiah (UCC# R4086T), and Ebola virus variant Mayinga (UCC# R3828S).

Statistics. Statistical analyses were performed using GraphPrism 6 (GraphPad Software, San Diego, CA). Assay linearity based on the preliminary LOD was determined based on the linear range of the curve using a nonlinear regression analysis. A two-way analysis of variance with Sidak's multiple comparisons test was conducted to determine differences between the Garrison assay and CCHF-S2 assay using multiple CCHFV strain RNAs.

RESULTS

CCHFV sequence analysis. Since the development of the Garrison assay,[20] we (Table 2) and others[15] identified decreased assay performance including nondetection of several CCHFV strains (JD-206, Drosdov, and DAK8194). To address this problem, we conducted deep sequencing of multiple

TABLE 1
Crimean-Congo hemorrhagic fever virus (CCHFV) real-time primers and probe

Assay	Primers/probe	Sequence (5′-3′)	Conc. (µM)	Amplicon	Reference
CCHFV-S2	CCHF-SF2	GGAVTGGTGVAGGGARTTTG	1.0	57	here
	CCHF-SR2	CADGGTGGRTTGAARGC	1.0		
	CCHF-N2	6FAM-CAARGGCAARTACATMAT-MGBNGQ	0.2		
Garrison assay	CCHF forward	GGAGTGGTGCAGGGAATTTG	1.25	57	Garrison et al.[20]
	CCHF reverse	CAGGGCGGGTTGAAAGC	1.25		
	CCHF-N	6FAM-CAAGGCAAGTACATCAT-MGBNGQ	0.1		

TABLE 2
Crimean-Congo hemorrhagic fever virus (CCHFV) assay detection

Virus	Detection (average Cq ± STDEV)		
	Atkinson assay	CCHFV-S2	Garrison assay*
AP92†	34.57 ± 0.49	Nd	nd
I-40	27.62 ± 0.3	20.66 ± 0.380	17.13 ± 0.242
2219 KKK28	35.01 ± 0.16	25.12 ± 0.04	22.34 ± 0.290
I-248	31.23 ± 0.27	22.95 ± 0.04	23.56 ± 0.174
JD-206	31.69 ± 0.3	34.29 ± 0.511	nd
Drosdov	39.37 ± 0.64	29.00 ± 0.091	nd
HY13	24.94 ± 0.21	19.66 ± 0.07	17.85 ± 0.11
SPU 97/85	38.94 ± 0.51	21.69 ± 0.133	34.10 ± 1.308
SPU 134/87	31.61 ± 0.13	25.48 ± 0.182	30.30 ± 2.081
SPU 115/87	31.96 ± 0.26	24.28 ± 0.489	34.35 ± 0.474
SPU 415/85	26.81 ± 0.22	18.97 ± 0.025	32.18 ± 0.219
SPU 41//84	25.68 ± 0.2	25.68 ± 0.083	32.86 ± 0.321
SPU 128/81	28.97 ± 0.04	23.16 ± 0.216	37.37 ± 0.525
UG3010	29.84 ± 0.14	24.20 ± 0.059	24.01 ± 0.050
IbAr10200	30.55 ± 0.52	24.28 ± 0.201	39.47 ± 0.924
DAK8194	33.46 ± 0.54	28.89/29.28‡	nd

STDEV = standard deviation.
* nd is not detected.
† Synthetic RNA for each assay target was used for AP92. AP92 was not detected using the CCHFV-S and CCHFV-S2 assays while the Atkinson assay had positive detection down to ~1×10^5 copies/mL.
‡ Two of three replicates were positive.

CCHFV S segments, including the three nondetectable CCHFV strains, from the UCC.[27] The S segment consensus sequences for these viruses were aligned to identify mismatches within the assay target region (Figure 1).

Multiple nucleotide variants identified in the primer and probe region for each strain sequenced (Figure 1) could have negatively impacted primer/probe binding. Of note, a deletion in the 5′ end of the published probe sequence, along with additional 3′ probe variants for JD-206 and DAK8194, likely resulted in nondetection of those two virus strains. Multiple variants in the reverse primer of Drosdov likely contributed to that strain's nondetection.

Assay evaluation. New primers and probe (assay CCHF-S2, Table 1) were redesigned to incorporate as much sequence diversity at the assay target location as possible. Comparison of the CCHFV-S2 assay primer/probe sequence to all CCHFV S segment sequences available in GenBank at the time of the assay redesign (N = 149, Supplemental Figure 1) showed that the forward, reverse, and probe sequences had no greater than one mismatch (and an exact match within the last two bases of the 3′ end) for 94.0%, 98.7%, and 99.3% of these S segment sequences, respectively.

Analytical characteristics of both the Garrison assay and CCHF-S2 assays using a well-characterized stock of IbAr10200 showed differences in performance metrics for these two assays (Figure 2, Table 3). The preliminary LOD, the highest dilution of virus where three of three replicates were all positive, was 1.28 PFU/reaction or 256 PFU/mL for the CCHF-S2 assay (Figure 2). Considering the linear segment of the dilution series, the R^2 value was 0.980, and the y-intercept was 43.64. This LOD was confirmed by running 60 replicates at this LOD, resulting in 58 of 60 positive replicates (Figure 2). The Garrison assay, using the same IbAr10200 RNA, identified the preliminary LOD, confirmed by 59 of 60 replicates being positive, to be 1.28×10^4 PFU/rxn or 2.56×10^6 PFU/mL (Figure 2). The assay linearity over the linear part of the dilution series was 0.845, and the y-intercept was 53.41.

We next compared the new CCHF-S2 assay with the Garrison assay and another commonly used CCHFV assay, the Atkinson assay,[15] that targets a different region of CCHFV. Using the same RNA as a template and the reaction conditions described previously,[15] we identified a greater assay sensitivity compared with the Garrison assay but decreased sensitivity compared with the CCHF-S2 assay (Figure 2, Tables 2 and 3). The Atkinson assay LOD with IbAr10200 RNA was 2.56×10^4 PFU/mL with 60/60 replicates being positive. We noted several nucleotide variants for the Atkinson assay within the assay target region of the CCHFV strains previously sequenced; however, incorporating sequence-optimized reverse primer and the probe into the Atkinson assay did not change assay sensitivity (data not shown).

Screening of an inclusivity panel of 15 different CCHFV strains showed different levels of detection for each of the three assays (Table 3). The CCHF-S2 assay and the Atkinson assay detected all of the CCHFV strains including the three that the Garrison assay did not detect. Sensitivity, reflected in the Cq values, was generally better for the CCHF-S2 assay (Table 3). On comparing the Garrison and the CCHF-S2 assays, almost all of these viruses had large improvements in the Cq values. For example, SPU 115/87 had ~10 Cq (~3 log) improvement in sensitivity, and IbAr10200 had ~15 Cq (> 4 log) improvement (Table 3). Because the genetically divergent CCHFV AP92 strain was not available for inclusivity testing, synthetic RNA encompassing each assay target was tested using each assay. While the Atkinson assay readily detected this synthetic RNA target, the CCHF-S2 assay did not detect this virus. Exclusivity testing for multiple viruses (see Materials

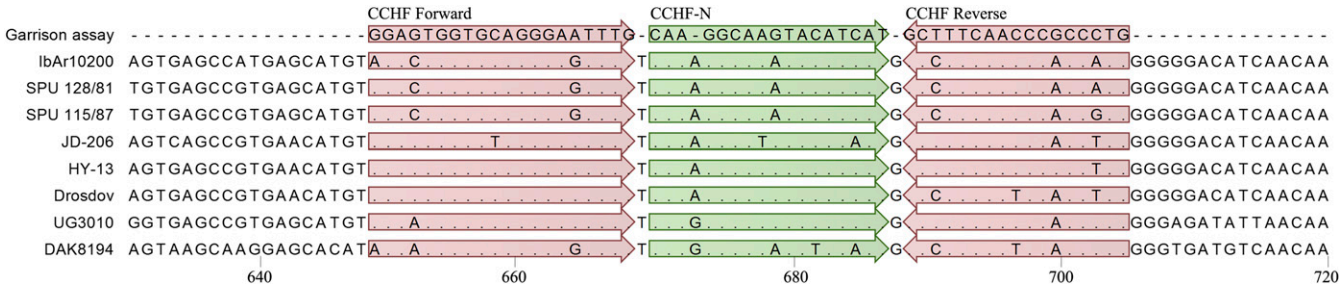

FIGURE 1. Crimean-Congo hemorrhagic fever virus (CCHFV) strain sequence analysis. Consensus sequences from eight CCHFV strains and the Garrison assay primer/probe sequences,[20] indicated with red and green arrows, respectively, were aligned. Nucleotides identical to the primer and probe sequence are shown as dots, and nucleotide numbers are relative to IbAr10200. Degenerate primers and probe for the Garrison assay (see Table 1) were designed based off of these aligned sequences. This figure appears in color at www.ajtmh.org.

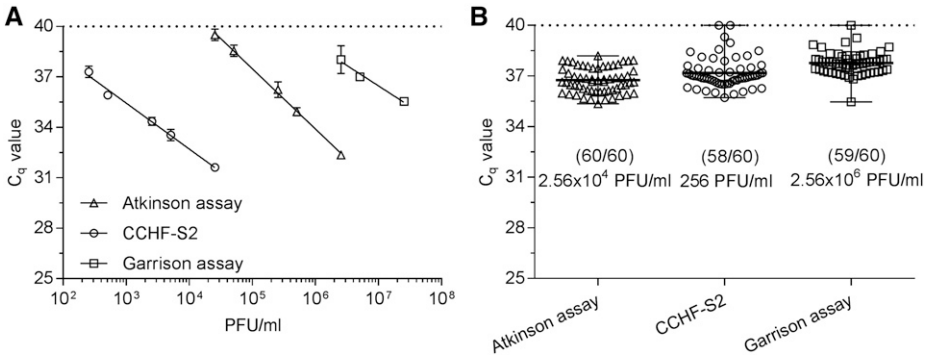

FIGURE 2. Crimean-Congo hemorrhagic fever virus (CCHFV) assay characterization. (**A**) CCHFV IbAr10200 RNA was serially diluted and assayed with the CCHFV assays. Shown is a nonlinear fit of the linear range where all three of the replicates were positive. (**B**) The preliminary limit of detection (LOD) was confirmed by running 60 replicates at the preliminary LOD. The dashed line in each figure indicates the Cq positive/negative cutoff (40 cycles).

and Methods) with the CCHF-S2 assay resulted in negative detection for each virus tested.

DISCUSSION

Because of the low fidelity of the viral RNA-dependent RNA-polymerase (RdRp), RNA viruses generally rapidly evolve under selective pressure, resulting in significant phylogenetic heterogeneity. For CCHFV, a broad geographic range, tick vector and host diversity, and the low fidelity RdRp likely contribute to the high level of diversity seen among CCHFV strains.[28] This diversity can be problematic for diagnostic assays and therapeutics, requiring assay modification as additional sequence information becomes available. Indeed, two recently published studies investigating the genomic diversity of the Ebola virus circulating in West Africa[29,30] identified multiple nucleotide variants among several commonly used Ebola virus real-time RT-PCR assays and therapeutics in development. These studies suggested such variants could negatively impact efficacy of diagnostic assays and therapeutics. To mitigate the diagnostic risk of CCHFV diversity, we redesigned our currently fielded CCHFV assay by incorporating sequencing data from several CCHFV strains that were previously undetectable with the original assay.

Deep sequencing of the CCHFV S segments of these and other CCHFV strains identified multiple nucleotide variants within the Garrison assay target region, likely leading to the decreased assay performance we observed in the Garrison assay. Differences in the Garrison assay performance previously[20] compared with the current effort (ex. DAK8194 detection) are likely the result of differences in how stock virus was generated. Previously, virus was harvested when cytopathic effect was observed at about 4 days post-infection; virus is currently harvested 2 days post-infection as virus viability is negatively impacted significantly at 4 days post-infection. Harvesting at this later time point resulted in a higher RNA to infectious virus ratio and likely led to the discordant results.

Variant analysis within the Garrison assay region did not identify intra-viral nucleotide differences (data not shown), suggesting some signature stability within each strain and supporting continued targeting of this genomic region as a diagnostic signature. Based on these sequencing data and the CCHFV genomic data deposited into GenBank since the original assay design, a new assay (CCHF-S2) incorporated degenerate primers and probe taking into account the assay target sequence diversity. These primers greatly improved CCHFV detection, reflected in lower Cq values and detection of the three strains not identified by the Garrison assay.

For highly diverse viruses like CCHFV, it is advantageous to have several diagnostic assays that target different regions of the viral genome to further minimize the diagnostic risk of a false negative call due to primer/probe mismatches. We conducted a comparison with another commonly used CCHFV assay developed by Atkinson and colleagues that targets the 5′ untranslated region of the CCHFV S segment.[15] While both the CCHF-S2 assay and the Atkinson assay positively detected all of the CCHFV strains tested here, the CCHF-S2 assay had improved sensitivity for most of the tested strains. However, evaluation of the CCHF-S2 assay using synthetic assay target RNA for the genetically diverse AP92 strain resulted in nondetection while the Atkinson assay resulted in positive detection using synthetic RNA. Since both of these assays target different regions of the CCHFV genome, both assays could be used for increased confidence (inclusivity and sensitivity) in diagnostic and biosurveillance efforts to mitigate the risk of nondetection due to CCHFV's diversity.

In summary, we redesigned a CCHFV real-time RT-PCR assay that was initially developed when limited sequence information was available and did not perform optimally with

TABLE 3
Analytical assay characteristics with IbAr10200

Assay	Linearity (R^2)	Slope	*y*-intercept	LOD, PFU/mL (positives/60 replicates)	Cq ± STDEV	Coefficient of variance (%)
Atkinson assay	0.987	–3.581	55.4	2.56×10^4 (60/60)	36.75 ± 0.74	2.02
CCHF-S2	0.980	–2.730	43.64	256 (58/60)	37.18 ± 0.91	2.95
Garrison assay	0.845	–2.419	53.41	2.56×10^6 (59/60)	37.77 ± 0.68	1.79

LOD = limit of detection; STDEV = standard deviation.

newly acquired CCHFV strains. This new assay contains degenerate primers and probe that accounts for a significant amount of the diversity within CCHFV, resulting in dramatically improved strain detection and assay sensitivity. These data increase the confidence in the new assay detecting true positives, and this approach can be used to improve assay sensitivity of existing nucleotide-based assays.

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