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Colorimetric Nucleic Acid Testing Assay for RNA Virus Detection Based on Circle-to-Circle Amplification of Padlock Probes▽

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We developed a molecular diagnostic method for detection of RNA virus based on padlock probes and colorimetric readout. The feasibility of our approach was demonstrated by using detection of Crimean-Congo hemorrhagic fever (CCHF) virus as a model. Compared with conventional PCR-based methods, our approach does not require advanced equipment, involves easier assay design, and has a sensitivity of 10^3 viral copies/ml. By using a cocktail of padlock probes, synthetic templates representing different viral strain variants could be detected. We analyzed 34 CCHF patient samples, and all patients were correctly diagnosed when the results were compared to those of the current real-time PCR method. This is the first time that highly specific padlock probes have been applied to detection of a highly variable target sequence typical of RNA viruses.

Outbreaks of viral diseases highlight the need for sensitive, rapid, reliable, and convenient diagnostic methods. Diagnostic methods based on nucleic acid testing, such as PCR, can fulfill the requirements of being sensitive and reliable. However, PCR-based methods are very unlikely to be convenient when the test needs to be carried out far away from a well-equipped laboratory. This problem is even more pronounced in low-income and developing countries. Therefore, new methods are required for this purpose. In this paper, we present an approach for RNA virus detection based on padlock probes (16) and colorimetric readout, which can potentially overcome the drawbacks of PCR-based nucleic testing methods. We used Crimean-Congo hemorrhagic fever virus (CCHFV) as a model for establishing the assay.

CCHFV is a member of the *Bunyaviridae* family (genus *Nairovirus*) and is known to cause severe disease and mortality (3 to 50%) in humans. The disease, for which the underlying molecular mechanisms are poorly understood, is manifested by symptoms like fever, prostration, and severe hemorrhage (6). CCHFV is widely distributed throughout large areas of sub-Saharan Africa, Bulgaria, the former Yugoslavia, northern Greece, European Russia, Pakistan, the Xinjiang Province of northwest China, the Arabian Peninsula, Iraq, and Iran. In addition, in areas of endemicity, viremia and the presence of antibodies have been documented in a long list of domestic and wild vertebrates, including horses, sheep, goats, pigs, camels, donkeys, mice, and dogs. Thus, CCHFV circulates unnoticed in nature in an enzootic tick-vertebrate-tick cycle. Enzootic foci of the virus mainly occur where one or several *Hyalomma* species are the predominant ticks feeding on domestic and wild animals. The virus is also mainly transmitted to humans through ixodid ticks of the genus *Hyalomma*, in particular, *Hyalomma marginatum marginatum*. Human infection also occurs through contact with blood or tissue material from infected animals or humans. In addition, person-to-person transmission can occur via bloody vomit, body fluids, or aerosols from patients in advanced stages of disease (20–22).

Current CCHFV diagnostic methods involve the detection of viral genomic RNA and/or viral antigen and detection of specific IgM and IgG antibodies (7, 10, 17). Conventional reverse transcription (RT)-PCR protocols take several hours for reverse transcription, amplification, and analysis, and in some cases, a second round of nested amplification is necessary to achieve the desired sensitivity. Designing primers and probes for RT-PCR for CCHFV is hampered by a remarkable genetic variability among virus strains. In the last couple of years, several RT-PCR protocols with a higher sensitivity and a shorter running time than conventional PCR analysis have

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been developed. However, the requirement for expensive RT-PCR equipment is still a problem. The disease is endemic in low-income and developing countries, and outbreaks mostly occur in villages far away from equipped laboratories. Many locations experience sudden power outages that can damage delicate equipment, which is then costly to repair or replace. This has created an urgent demand for a sensitive detection method that does not require expensive equipment.

In this study, a sensitive detection assay for CCHFV using padlock probes (16) and circle-to-circle amplification (C2CA) technology (4) combined with enzymatic readout has been developed (Fig. 1). A padlock probe is a linear oligonucleotide that contains half of a unique target recognition sequence at each end and a target-independent sequence in between. The target recognition sequences are designed to bind head to tail on a genomic target DNA. Upon the hybridization to target DNA, the probes become circularized by enzymatic ligation, while if there is no target DNA present, the padlock probes remain linear. Padlock probes have previously been used for genotyping (9, 13), gene copy number (23), expression analysis (14), target sequencing (5), and mRNA splicing (3), as well as for detection of bacteria and other infectious pathogens (1, 8, 15). Only the circularized padlock probes are then amplified by the exponential rolling-circle amplification (RCA)-based C2CA reaction (2, 4). The ligation reaction is highly specific and will not occur if there is a single mismatch between the target and the probe at the ligation site. This ensures high specificity but may be a problem when addressing highly variable target sites. The main aim of this study was to investigate whether it is feasible to detect highly variable target sequences by using cocktails of padlock probes covering the range of variation. In the current study, the amplification product is finally detected using a horseradish peroxidase (HRP)-catalyzed colorimetric readout to eliminate the use of sophisticated amplification and detection instruments.

We show that our method can detect CCHFV with high sensitivity. Using a combination of padlock probes, CCHFV strains with known sequences can be detected. The colorimetric readout makes it possible to read the results with the naked eye or semiquantitatively in a simple absorbance reader. Our approach is not based on PCR amplification and does not require sophisticated or expensive instrumentation. It is possible to use this method for field-based diagnosis and monitoring applications in developing countries.

MATERIALS AND METHODS

Probe sequences. All oligonucleotide sequences are shown in Table 1. The polarity of the padlock probe is referred to as positive (+), and that of its complement is referred to as negative (−). Padlock probes were designed using ProbeMaker software (18) to target the most conserved sequences in the L segment of the CCHFV genome. Due to the high genetic variability of the viral genome, 7 additional probes were designed to ensure detection of all strains (total of 8 probes).

Serum samples. Serum samples from a total of 20 patients were included in the study. Samples were taken from patients displaying symptoms of CCHFV infection, and where possible, both acute-phase samples (A) and convalescent-phase samples (B) were taken from the patients. All samples were also analyzed by conventional real-time RT-PCR as described by Wolfel et al. (24).

Virus propagation. Viral RNA for the concentration standard curve was prepared from the CCHFV Iranian strain. Confluent Vero cells (ATCC CCL-81) were inoculated with virus and grown for 2 to 3 days in minimal essential medium (MEM; Invitrogen, Life Technologies, Paisley, United Kingdom) supplemented with penicillin-streptomycin solution (Gibco), HEPES (Gibco), and 2% heat-inactivated fetal calf serum (FCS; Gibco) at 37°C. The supernatant was collected after clarification by centrifugation at 1,000 × *g* for 10 min and subsequently aliquoted and stored at −80°C prior to RNA extraction. All handling of live virus was carried out in biosafety level 4 (BSL4) facilities.

RNA extractions. The CCHFV supernatants and the serum samples were treated with TRIzol LS reagent (ratio, 1 + 3; Invitrogen) in the BSL4 laboratory for a minimum of 5 min, before decontamination of the tubes and transport to a BSL2 laboratory following the safety instructions. After phase separation by chloroform (Merck) treatment and centrifugation, viral RNA was purified from the aqueous phase using a QIAamp viral RNA minikit (Qiagen), according to the manufacturer's instructions.

RT-PCR. The extracted RNA was analyzed by CCHFV real-time RT-PCR, positive reactions were plotted against a standard curve, and the genome content/ml was calculated (24). The cycling reactions were performed in a Roche LightCycler 2.0 or 480 apparatus.

Synthesis of biotin-labeled cDNA. In a 21-μl reaction volume, a mixture of 1.5 μl (100 ng/μl) 5′ biotin-labeled random hexamers (Gene Link, Hawthorne, NY), 1 μl 10 mM deoxynucleoside triphosphate (dNTP) mix (Invitrogen), 2.5 μl distilled water, and 5 μl RNA was incubated at 65°C for 5 min and then immediately chilled on ice. A mixture of 2 μl 10× RT buffer, 4 μl $MgCl_2$ (25 mM), 2 μl dithiothreitol (DTT; 0.1 M), 1 μl (40 U/μl) RNaseOUT, and 1 μl SuperScriptIII reverse transcriptase (200 U/μl; Invitrogen) was added and the mixture was incubated at 25°C for 10 min, 50°C for 50 min, and 65°C for 5 min and then kept at 4°C. Finally, 1 μl RNase H (2 U/μl; Invitrogen) was added and the mixture was incubated at 37°C for 20 min. The biotinylated cDNA was stored at −20°C until use. The cycling reactions were performed in a thermocycler (Gene Amp 2700; ABI).

PCR analysis of cDNA. RNA and cDNA were analyzed simultaneously by the previously described RT-PCR, and the genome content/ml was calculated (24). The cycling reactions were performed in a Roche LightCycler 480 apparatus.

Padlock probe ligation and circle-to-circle amplification. All padlock probes were phosphorylated prior to use. Briefly, 100 μl of a phosphorylation mixture containing 10 μM padlock probes, 1× PNK buffer A (Fermentas), 1 mM ATP, and 0.1 U/μl T4 polynucleotide kinase was incubated at 37°C for 30 min and 60°C for 20 min. The phosporylated probes were then stored at −20°C until use. A schematic illustration of the following protocol can be found in Fig. 1. First, 10 μl biotinylated cDNA from either clinical samples or the cultivated Iranian reference strain was added to 10 μl ligation mix in a 96-well PCR plate (Thermo Electron). Final concentrations were 1× Ampligase buffer (Epicentre), 100 nM each padlock probe, and 5 U Ampligase (Epicentre). The ligation reaction was carried out by incubation at 55°C or 60°C for 5 min, forming DNA circles. Then, 10 μl 10-mg/ml streptavidin-coupled MyOne T1 magnetic beads (Invitrogen) suspended in 3× wash buffer (15 mM Tris-HCl [pH 7.5], 1.5 mM EDTA, 3 M NaCl, 0.1% Tween 20) was added to each sample, followed by gentle vortexing and incubation at room temperature for 5 min. Unbound material was then separated from the beads using a magnetic rack (Invitrogen). The beads were washed once with wash buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 M NaCl, and 0.1% Tween 20, and the wash buffer was discarded. The DNA circles captured by the beads were primed by adding 20 μl RCA mix containing 1× phi29 DNA polymerase buffer (33 mM Tris-acetate [pH 7.9 at 37°C], 10 mM magnesium acetate, 66 mM potassium acetate, 0.1% [vol/vol] Tween 20, 1 mM DTT; Fermentas), 100 μM dNTPs, 0.2 mg/ml bovine serum albumin (BSA), 50 nM primer, and 4 U phi29 DNA polymerase. The reaction mixture was incubated at 37°C for 20 min, followed by 1 min at 65°C to inactivate the phi29 DNA polymerase. The amplified single molecules were then monomerized by adding 5 μl restriction digestion mixture containing 1 U/μl AluI restriction enzyme (New England BioLabs), 1× phi29 DNA polymerase buffer, 400 nM replication oligonucleotides CCHF_RO+, and 0.2 mg/ml BSA. The reaction mixture was incubated at 37°C for 5 min, and AluI was inactivated at 65°C for 3 min. The monomers were then recircularized and amplified by addition of 10 μl ligation and RCA mix containing 1× phi29 DNA polymerase buffer, 0.2 mg/ml BSA, 3 mM ATP, 250 μM dNTP, 0.05 U/μl T4 DNA ligase (Fermentas), and 0.3 U/μl phi29 DNA polymerase. To initiate the second restriction digestion, 5 μl restriction digestion mix containing 1 U/μl AluI, 1× phi29 DNA polymerase buffer, 1.6 μM replication oligonucleotides CCHF_RO−, and 0.2 mg/ml BSA was added. Finally, the third RCAs were then carried out by adding the same ligation and RCA mix mentioned above.

HRP detection. Magnetic beads were preincubated in capture probe solution (50 nM capture probe in hybridization buffer containing 20 mM EDTA, 20 mM Tris-HCl, 1.4 M NaCl, and 0.1% Tween 20) at a 1:3 volume ratio. The mixture was then incubated for 5 min at room temperature and placed in a magnetic rack (Invitrogen) for 1 min, and the supernatant containing unbound capture probes

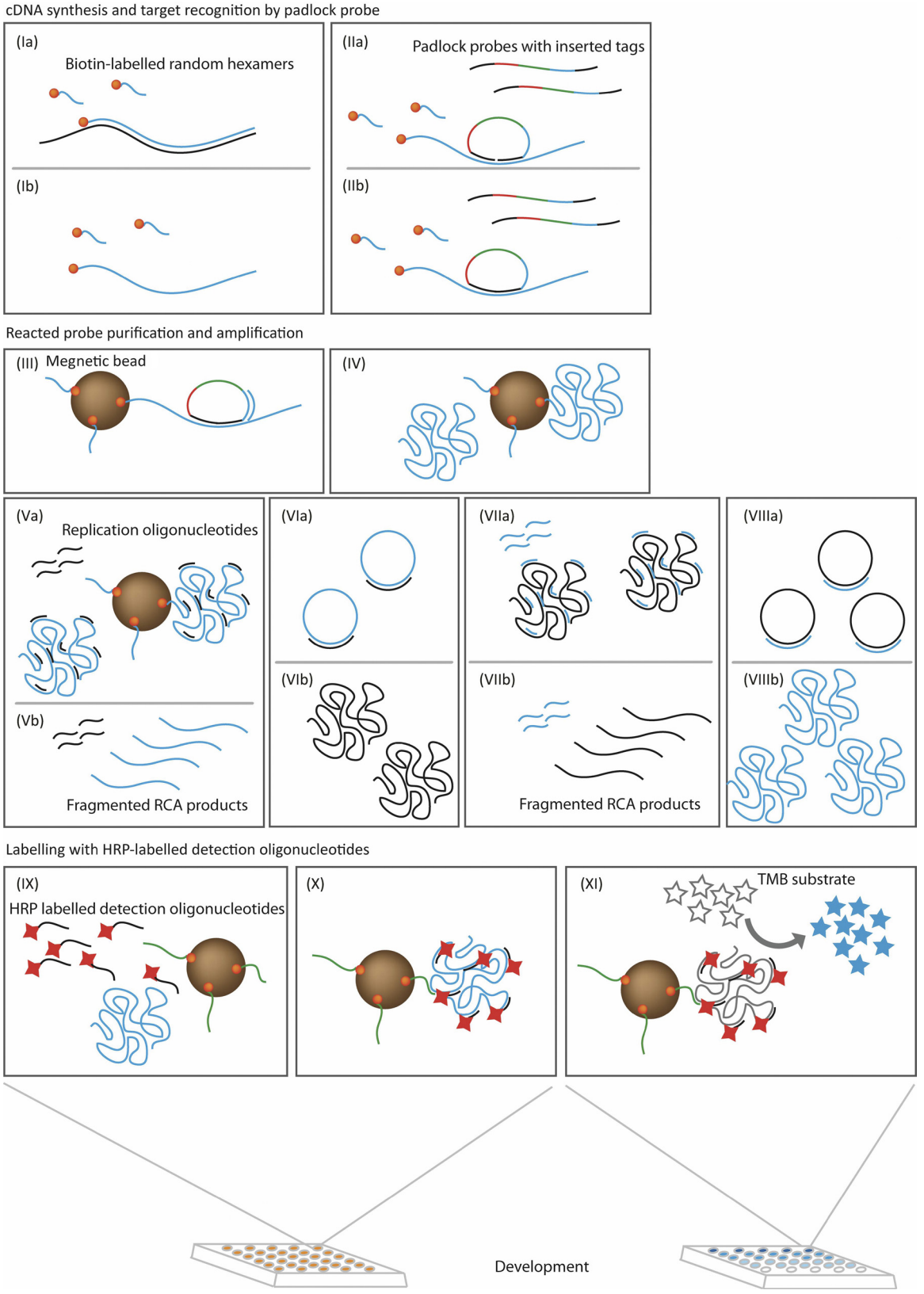

FIG. 1. Schematic illustration of CCHFV detection using padlock probes and enzymatic readout. (I) Synthesis of biotin-labeled cDNA from CCHF mRNA fragments. (II) Padlock probes are added to samples and specifically circularized by DNA ligase when hybridized to the correct template. (III) Ligated padlock probes are captured by streptavidin-coated magnetic beads, whereas unbound probes are removed by washing. (IV) Ligated padlock probes are amplified by RCA. (V) RCA products are digested by restriction enzyme to generate monomers. (VI) The monomers are recircularized and amplified by RCA to generate second-generation RCA products. (VII) Second digestion of RCA products. (VIII) Monomers are recircularized and again amplified by RCA to generate third-generation RCA products. (IX to X) Third-generation RCA products are hybridized to HRP-labeled detection probes and bound by capture probes immobilized on magnetic beads. (XI) Signal is developed by adding TMB substrates to the beads.

TABLE 1. Oligonucleotides for CCHFV detection

Name	Sequence[a]	5′ end modification	Function	GenBank accession no(s).[b]
T	*CTCTCTCTCTCTCT*AAACCAAGCCTGACCAT|GTTGATACAGACT	Biotin	Template	GQ337055.1, EU044832.1, DQ211623.1, DQ211618.1, DQ211617.1, DQ211614.1, AY389361.2, AY995166.2
T1	*CTCTCTCTCTCTCT*AAACCAAGCCTGAC**T**AT|GTTGATACAGACT	Biotin	Template	AY675240
T2	*CTCTCTCTCTCTCT*AAACC**T**AGCCTGACCAT|GTTGATACAGACT	Biotin	Template	AY720893
T3	*CTCTCTCTCTCTCT*AAACCAAGCCTGACCAT|GTT**A**ATACAGACT	Biotin	Template	DQ211621
T4	*CTCTCTCTCTCTCT*AAACCAAGCCTGACCAT|G**C**TGATACAGACT	Biotin	Template	DQ076417.1 DQ076414.1
T5	*CTCTCTCTCTCTCT*AAACCAAG**T**CTGACCAT|GTTGATACAGAC**A**	Biotin	Template	HM452307.1 AY947890.1
T6	*CTCTCTCTCTCTCT*AAACCAAGCCTGACCAT|GTTGATACA**A**ACT	Biotin	Template	DQ211613.1
T7	*CTCTCTCTCTCTCT*AAACCAAGCCTGACCAT|GTT**A**ATACAGAC**C**	Biotin	Template	DQ211622.1 DQ211620.1
T8	*CTCTCTCTCTCTCT*AAACCAAG**T**CTGACCAT|G**C**TGATACAGAC**G**	Biotin	Template	DQ211619.1
T9	*CTCTCTCTCTCTCT*AAACCAAG**TT**TGACCAT|GTTGATACA**A**AC**C**	Biotin	Template	DQ076412.1
T10	*CTCTCTCTCTCTCT*AAACCAAGCCT**A**ACCAT|GTT**A**ATACAGAC**C**	Biotin	Template	DQ211615.1
T11	*CTCTCTCTCTCTCT*AAACCAAG**TTTA**ACCAT|GTTGATACAGAC**A**	Biotin	Template	DQ211616.1
T12	*CTCTCTCTCTCTCT*AAACC**G**AG**T**CT**A**AC**A**AT|GTTGATACAGAC**C**	Biotin	Template	DQ211612.1
T13	*CTCTCTCTCTCTCT*AAACC**G**AG**TT**TGACCAT|G**C**TGATACA**A**AC**C**	Biotin	Template	DQ211624.1 DQ099335.2
P_CCHF	ATGGTCAGGCTTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCAAC		Padlock probe	
P_CCHF_1	ATGGTCAG**A**CTTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCA**G**C		Padlock probe	
P_CCHF_2	ATT**G**TT**A**GA**C**TTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCAAC		Padlock probe	
P_CCHF_3	ATGGTCAGGCTTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT AT**T**AAC		Padlock probe	
P_CCHF_4	ATGGTCA**A**A**C**TTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCAAC		Padlock probe	
P_CCHF_5	ATGGTCA**A**A**C**T**C**GGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACT**G**G**T**TGT ATCA**G**C		Padlock probe	
P_CCHF_6	ATGGTCAGGCTTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCA**G**C		Padlock probe	
P_CCHF_7	AT**A**GTCAGGCTTGGTTTGTGGATAGTGTCTTACACGAAGAGTG TACCGACCTCAGTAAGTCTCCTAGCTCGGTGAACTAGTCTGT ATCAAC		Padlock probe	
CCHF_RO+	AGTCTCCTAGCTCGGTGAAC		Replication probe	
CCHF_RO−	GTTCACCGAGCTAGGAGACT		Replication probe	
HRP_1° RCA	HRP-CTTGCGACGTCAGTGGATAGTGTCTTACACGATTT	HRP	Detection probe	
B2_CO	TTTTCTGGATCGTCAGGGAAAGAGTGTACCGACCTCAGTA	Biotin	Capture probe	

[a] Italics, inserted spacer sequences; |, ligation site; underline, target complementary sequences; bold letters, SNPs.
[b] GenBank accession number(s) for the representative virus strain(s) containing the sequence of synthetic templates.

was aspirated. The plate was removed from the rack, and beads were resuspended in 5 μl hybridization buffer per sample to be detected. Five microliters of this bead-capture probe complex was added to each sample well along with 50 μl detection probe (30 nM HRP-coupled probes in hybridization buffer), and the plate was incubated at 55°C for 15 min in a heating block. The beads were then washed two times with 150 and 250 μl washing buffer (phosphate-buffered saline plus 0.05% Tween 20) with the aid of the magnetic rack (Invitrogen). After the last washing, buffer was aspirated, 100 μl tetramethylbenzidine (TMB; Thermo Scientific) was added, and the plate was incubated at 37°C for 20 min. The result was quantified in an enzyme-linked immunosorbent assay reader (Multiscan FC; Thermo Scientific) at 650 nm but could also be examined visually.

RESULTS

Method design. Padlock probes targeting the most conserved sequence in the L segment of the CCHFV genome were designed. The padlock probes in the current study are equipped with two end sequences that recognize the target sequence and three sequence elements that are used for different purposes: a detection sequence for hybridization with an HRP-labeled detection oligonucleotide, a capture sequence for hybridization to capture oligonucleotide immobilized on magnetic beads, and finally, a replication sequence for C2CA (Fig. 1).

Viral CCHF cDNA was synthesized from viral mRNA by reverse transcription using 5′ biotin-labeled random hexamers. The cDNA was probed using a high concentration of padlock probes to promote hybridization and ligation kinetics. Excess unreacted padlock probes were eliminated by washing enabled by capture of the target sequence to streptavidin-coated mag-

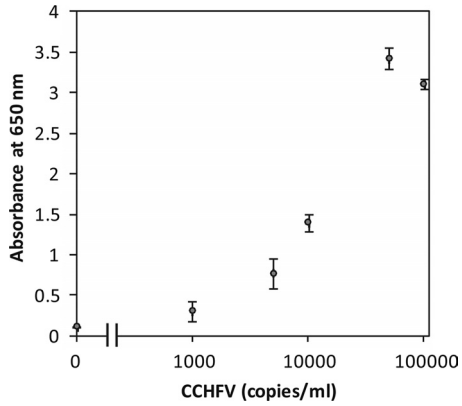

FIG. 2. Typical standard curve of CCHFV detection using diluted cDNA samples. Error bars, ±1 standard deviation; $n = 3$.

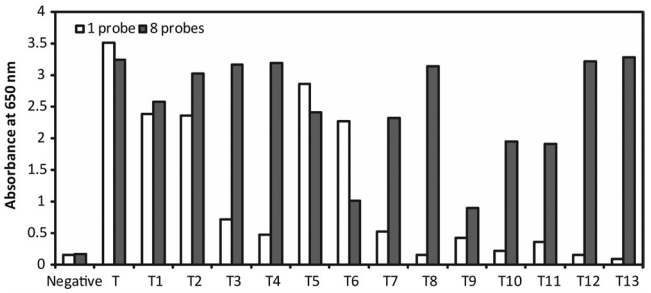

FIG. 4. Detection of different CCHFV variants by using either a single padlock probe or a set of eight padlock probes. T to T13 correspond to variants of the target sequence (listed in Table 1).

netic beads. Reacted padlock probes were then amplified by three cycles of RCA. The C2CA products were thereafter hybridized with HRP-labeled detection oligonucleotides, followed by separation of free detection oligonucleotides by capturing the labeled product in a sandwich hybridization reaction using capture oligonucleotides immobilized on magnetic beads. Finally, the signal was developed by adding HRP colorimetric substrate.

Two different probing approaches were taken. The first aimed to target as many viral strains as possible with a single padlock probe (P_CCHF). The second approach involved the addition of seven different padlock probes (P_CCHF_1 to P_CCHF_7) targeting the same site for detection of variants of other CCHFV strains (in total, 8 probes).

Sensitivity. The sensitivity of the approach was investigated by detection of serial dilutions of cDNA from cultivated CCHFV of an Iranian strain, whose concentration was validated by real-time RT-PCR. The limit of detection for the current method is 10^3 copies/ml using the single-padlock-probe approach (Fig. 2).

Patient samples. We applied our method for analysis of 34 samples from 20 patients, which were also analyzed by RT-PCR. By applying an absorbance value higher than 0.5 as a threshold, we scored eight of the samples positive and the rest negative, results which are in agreement with the RT-PCR results (Fig. 3).

Capability of the approach. We investigated the capability of our approach by detection of synthetic templates that represented different CCHFV strains found in different regions. We found that when we used a single padlock probe for detection, some strains gave false-negative results. To overcome this problem, we designed seven additional padlock probes with slightly different sequences (Table 1). The eight padlock probes were then pooled and applied for detection. Using this approach, all 14 synthetic templates representing the target sequences of the different strains were detected at a concentration of 6×10^4 copies per ml, which is equal to 600 copies per reaction (Fig. 4). The negative controls were either double-distilled H_2O or Rift Valley virus, which also belongs to the *Bunyaviridae* family (12), and neither gave a positive signal.

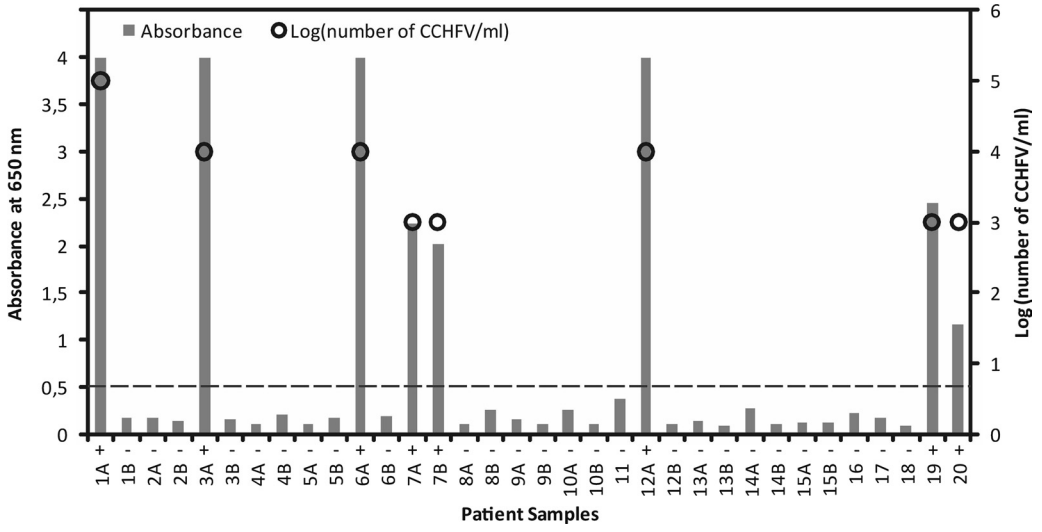

FIG. 3. Detection of CCHF patient samples. The results for all samples were confirmed by RT-PCR; circles, positive signals from RT-PCR. The cutoff value for oversaturated signals is set to 4. +, positive results; −, negative results.

DISCUSSION

We have established a non-PCR-based nucleic acid detection assay applying padlock probe and C2CA for the detection of CCHFV. The isothermal amplification strategy and simple colorimetric readout together make the assay independent of sophisticated instrumentation. Therefore, it is potentially suitable for field-based diagnosis and monitoring applications in developing countries. Even though our approach is PCR independent, the sensitivity is comparable to that of PCR-based methods. In a typical PCR-based detection method, a pair of primers, sometimes along with a TaqMan probe, is needed to amplify the target sequence. Hence, every target sequence needs two to three hybridization sequences to set up a detection assay. However, the CCHFV genome is highly variable due to high mutation rates. Thus, it may be difficult to find conserved sequences for all the required probe binding sites. For our padlock probe-based detection, only one hybridization sequence is required, which increases the chance of finding a suitable target sequence.

In our experience, even though the reagents need to be added into the reaction tubes several times during the protocol, cross contamination was not observed, which was in accordance with the findings of previous studies (4). This property is important, because for simple readout assays, open-tube manipulation of amplified products to generate a signal is often required. In contrast, PCR-based assays are more sensitive to open-tube manipulations. The reason why our method is less prone to cross contamination may be because the RCA products are macromolecules, which are not as easily spread into the air. In addition, the C2CA generates amplified products with opposite polarity between two successive generations. Even though the risk of cross contamination is low, it is still possible. Therefore, careful handling of samples and reagents is important when performing the experiment.

In previous studies that involve C2CA technology, RCA products were detected by radiation-labeled detection oligonucleotides, fluorescence-labeled detection oligonucleotides, or paramagnetic beads (4, 11, 19). However, these methods often require advanced and expensive instrumentation, which made them unsuitable for field-based diagnosis. The only equipment absolutely required in the field using our method would be heating blocks. In this study, we used HRP-labeled oligonucleotides for detection of amplified RCA products (Fig. 1). The result can be determined either by a regular spectrophotometer or by the naked eye, which makes our method more suitable for on-site detection in developing countries.

To address the problem of high variability in the target sequence of CCHFV (Table 1), we designed seven additional padlock probes for detection of the variants in different viral strains (eight probes in total). By using a combination of all eight padlock probes for the detection of different CCHF strains, all 14 strains investigated could be detected with a minimal loss of sensitivity. The result indicates that when analyzing a new variant of CCHFV, the sensitivity is altered. However, once the new variant strain is sequenced, if the existing pool of padlocks cannot already detect it, a new padlock probe can be designed and added to the pool for high-sensitivity detection.

The limitation of the current approach is the multiple steps for adding reagents in the protocol. However, even though the protocol has many steps and may seem complicated, it is made simpler by using premade mixes and can be learned quickly. Also, scaling up to analyze many samples at the same time does not add either much time or much effort to the protocol due to the 96-well format. In well-equipped laboratories, automated pipetting devices could be used, eliminating the problem completely.

To summarize, we have developed a PCR-free but highly sensitive and high-capability CCHFV detection assay. This is the first time that highly specific padlock probes have been applied to detection of a highly variable target sequence, typical of RNA viruses. By designing new probes, our method could easily be adapted for detection of other RNA or DNA viruses.

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M.N. holds shares in the company Olink Bioscience, which holds commercial rights to the technology.

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