Short Communication

Performance and clinical validation of the RealStar® MERS-CoV Kit for detection of Middle East respiratory syndrome coronavirus RNA

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ABSTRACT

Background: A highly pathogenic human coronavirus causing respiratory disease emerged in the Middle East region in 2012. In-house molecular diagnostic methods for this virus termed Middle East respiratory syndrome coronavirus (MERS-CoV) allowed sensitive MERS-CoV RNA detection in patient samples. Fast diagnosis is important to manage human cases and trace possible contacts.
Objectives: The aim of this study was to improve the availability of existing nucleic acid amplification-based diagnostic methods for MERS-CoV infections by providing a real-time RT-PCR kit, including an internal control and two target regions recommended by the World Health Organization (WHO). And to validate this kit (RealStar® MERS-CoV RT-PCR kit 1.0, Altona Diagnostics GmbH, Hamburg, Germany) using clinical samples of one MERS-CoV case from Munich and respiratory samples of patients with other respiratory diseases.
Study design: An internal amplification control was included into the RT-PCR assays targeting the genomic region upstream of the Envelope gene (upE) and within open reading frame (ORF) 1A. Based on these assays, a ready-to-use real-time RT-PCR kit featuring both the upE and ORF1A assays was developed, validated and compared to the established in-house versions.
Results: The performance of both RT-PCR assays included in the kit is comparable to the in-house assays. They show high analytical sensitivity (upE: 5.3 copies/reaction; ORF1A: 9.3 copies/reaction), no cross-reactivity with other respiratory pathogens and detected MERS-CoV RNA in patient samples in almost the same manner as the in-house versions.
Conclusion: The kit is a valuable tool for assisting in the rapid diagnosis, patient management and epidemiology of suspected MERS-CoV cases.

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1. Background

The *Middle East respiratory syndrome coronavirus* (MERS-CoV) was first detected in a single patient with severe respiratory tract infection in Saudi Arabia in 2012. Evidence of spreading was provided by virus detection in a second patient from Qatar treated for similar symptoms in the United Kingdom [1,2]. Since then an increasing number of cases, including more than 80 fatal cases have been notified to the World Health Organization (WHO) [3]. All human cases were linked to the Middle East region but imported cases were also detected in Europe and Africa [4,5]. Virus detection in the majority of cases was done using specific real-time reverse-transcription PCR (rRT-PCR) assays in samples from the lower respiratory tract. Samples from the upper respiratory tract have been used for the investigation of mild cases and contacts, despite lower virus concentrations in these materials [6–9]. Two rRT-PCR assays previously published by our group [10,11] are recommended by the WHO for MERS-CoV molecular diagnostics [12]. These assays target genomic regions upstream of the *Envelope* gene (upE) and in the viral open reading frame 1A (ORF1A). Both assays proved to be highly sensitive and were successfully used for the identification of the majority of the diagnosed MERS cases. Based
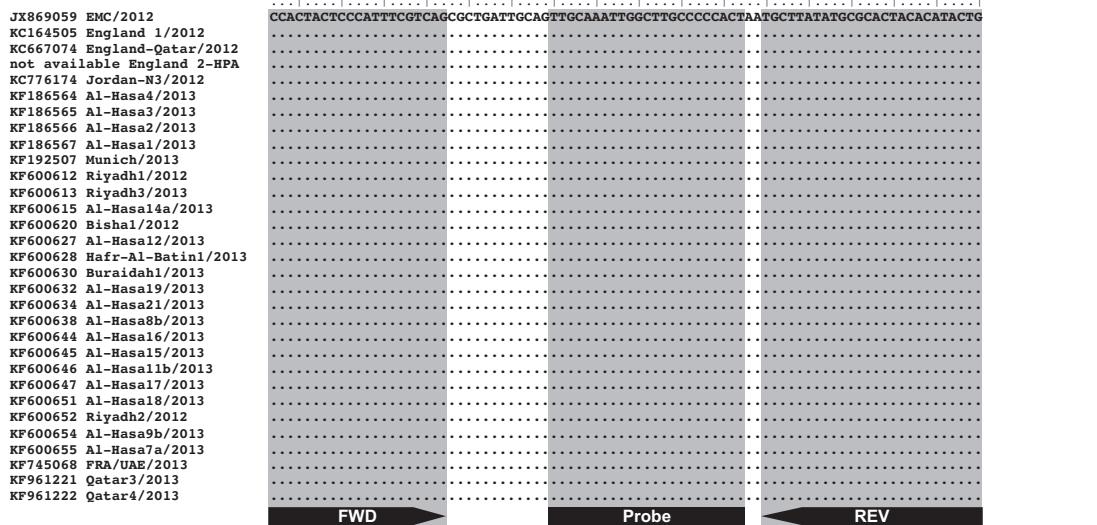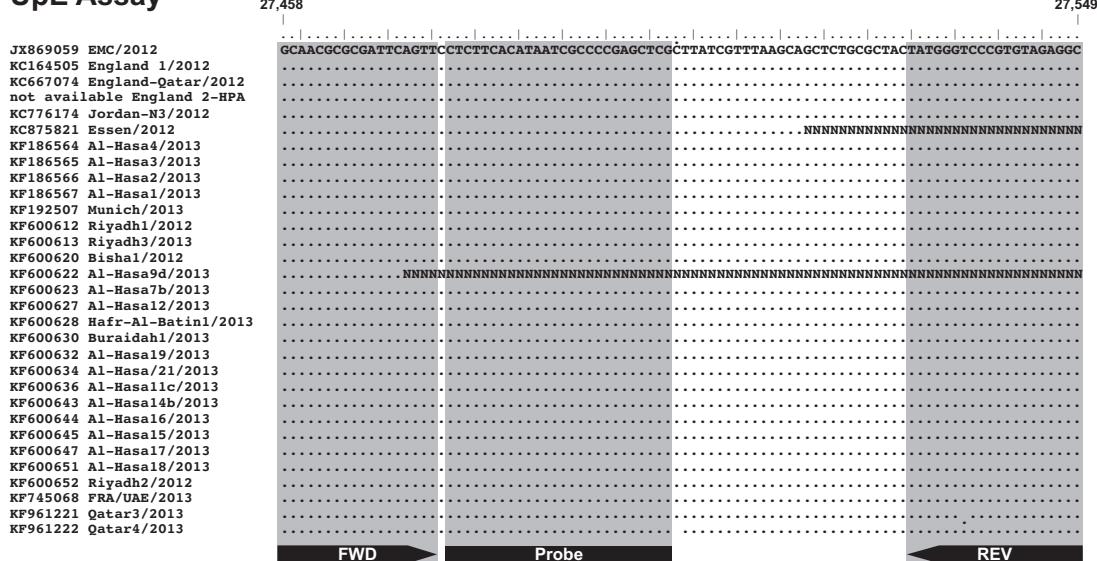
1A Assay

Fig. 1. Oligonucleotide binding sites for ORF1A and upE rRT-PCR assays. No target mismatches with any known MERS-CoV sequence (as of January 27th, 2014) was found. Dots represent identical nucleotides, "N"s represent sequence information missing. Numbers above alignments indicate nucleotide positions in the MERS-CoV EMC/2012 reference sequence. The first eight characters in the name correspond to the GenBank accession numbers if available.
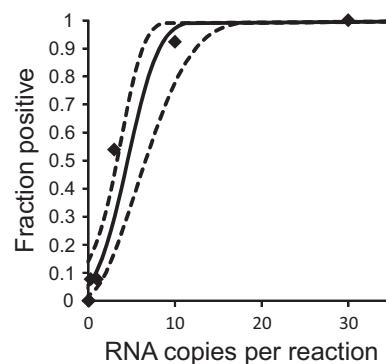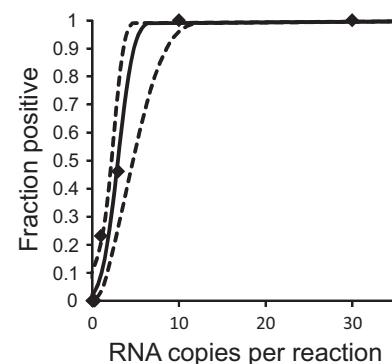
Fig. 2. Probit regression analyses of the two real-time reverse-transcription polymerase chain reaction assays included in the RealStar® MERS-CoV Kit as a results of 13 parallel replicates of both assays using 100 (not shown in the graphs) to 0.03 RNA copies per reaction The *y*-axis shows fractional hit-rates (positive reactions per reactions performed), the *x*-axis shows input RNA copies per reaction. Rhombs are experimental data points; solid lines represent the corresponding probit curve, the dashed lines the 95% confidence intervals.

Table 1
Patient samples tested with in-house MERS-CoV specific RT-PCR and the RealStar® MERS-CoV Kit.

Sample	Day after onset	In-house upE assay[a]	RealStar® MERS-CoV upE assay[a]	RealStar® MERS-CoV ORF1A assay[a]
Aspiration tube, flushed with PBS	16	40.00	40.00	37.74
Aspiration tube, flushed with PBS	16	35.80	35.00	36.17
Bronchoalveolar fluid	12	34.06	31.82	32.44
Bronchoalveolar fluid	12	34.96	32.67	33.16
Bronchoalveolar fluid	14	35.99	34.60	34.82
Bronchoalveolar fluid	13	–	–	–
Exudate, mouth	16	–	36.60	–
Exudate, mouth	16	–	–	–
Exudate, mouth	16	–	–	–
Exudate, nose	16	38.38	–	–
Exudate, nose	16	–	40.00	40.00
Exudate, mouth	16	–	–	–
Stool	12	38.98	31.69	31.78
Stool	12	39.37	40.00	–
Stool	16	40.00	40.00	–
Urine	12	–	40.00	37.86
Urine (catheter)	12	–	–	–
Urine (catheter)	13	–	–	–
Central venous catheter, flushed with PBS	12	–	–	–

[a] Numbers in columns indicate the respective Ct values of positive real-time RT-PCR runs.

on the upE and ORF1A assays, real-time RT-PCR assays using the same oligonucleotides were developed into ready-to-use reagent formulations available as a test kit.

2. Objectives

The aim of this study was to compare in-house versions of the upE and ORF1A rRT-PCRs with the analogous assays included in the kit and targeting the same loci in the MERS-CoV genome. The kit assays have a simple workflow to increase reliability of the PCR results and contained an additional heterologous internal amplification system to control for general PCR-inhibition [13]. The aim was to determine the limit of detection, to validate the kit using clinical samples from a MERS-CoV case from Munich, Germany [6], and to test possible cross-reactivity against other respiratory pathogens.

3. Study design

The protocols for the in-house assays (upE and ORF1A assays) were published previously [10,11]. Briefly, 12.5 μl of 2× reaction buffer (SuperScript® III One-Step RT-PCR System, Invitrogen, Karlsruhe, Germany), 1 μl of enzyme mixture, additional 0.8 mM MgSO$_4$, 0.4 μM of primer ORF1A-Fwd (CCACTACTCCCATTTCGTCAG) and 0.4 μM of primer ORF1A-Rev (CAGTATGTGTAGTGCGCATATAAGCA) and 0.2 μM of probe ORF1A-P (FAM-TTGCAAATTGGCTTGCCCCCACT-TAMRA) were mixed. Five μl of RNA and nuclease free-water were added up to a final reaction volume of 25 μl. Thermal cycling was performed at 55 °C for 20 min for reverse transcription, followed by 95 °C for 3 min and 45 cycles of 95 °C for 15 s, 58 °C for 30 s. Reaction conditions were the same for the upE RT-PCR but using the following primer and probe sequences: upE-Fwd (GCAACGCGCGATTCAGTT), upE-Rev (GCCTCTACACGGGACCCATA), upE-P (FAM-CTCTTCACATAATCGCCCCGAGCTCG-TAMRA). The test kit versions of the upE and ORF1A rRT-PCR assays are based on the same oligonucleotides and are both included in the RealStar® MERS-CoV RT-PCR kit 1.0 (altona Diagnostics GmbH, Hamburg, Germany). They have a final reaction volume of 25 μl with 10 μl of sample RNA input. During the RNA-extraction, the target for the internal amplification control (IC) is added to check for PCR inhibition or loss of RNA during the extraction procedure. Alternatively the IC could also be used as a RT-PCR inhibition control only, by adding the IC into the PCR reaction. The IC is an in vitro transcribed

RNA with an artificial sequence, targeted by additional primers and an additional probe included in the PCR mix. Two different real-time PCR instruments were used, the LC480II (Roche, Mannheim, Germany) and the Rotor-Gene Q (QIAGEN, Hilden, Germany). RNA-extraction was performed using the Viral RNA Mini Kit (QIAGEN). If not specified, 140 μl of sample were used and the RNA was eluted in 60 μl of AVE buffer (QIAGEN).

4. Results

All MERS-CoV sequence information data publicly available as of 27th January 2014 was analysed for variation within oligonucleotide binding sites which could negatively impact PCR-based viral detection. Fig. 1 shows the alignment for the upE and ORF1A target regions. No variation was found in 31 available sequences within binding regions of primers or probes.

The limits of detection for the RealStar® MERS-CoV RT-PCR upE and ORF1A assay were determined using in vitro transcribed RNA (IVT) quantified by spectrophotometry. The IVT was diluted in half-logarithmic steps (from 100 to 0.03 copies/reaction) in nuclease-free water containing 10 μg/mL carrier RNA (QIAGEN, Hilden, Germany) and tested in replicates ($n = 13$) for positive amplification. Probit regression analysis in SPSS (IBM, Ehningen, Germany) was used to determine the 95% cut-off value (Fig. 2). The upE-assay has a 95% cut-off value of 5.3 copies/reaction (95% confidence interval (CI): 4.0–9.7 copies/reaction); the ORF1A assay detects 9.3 copies/reaction with 95% probability (95% CI: 7.0–14.1 copies/reaction).

To determine the sensitivity in detecting the whole virus genome, RNA was extracted (50 μl input, 50 μl elution volume) from cell culture supernatant of MERS-CoV strain EMC/2012 grown on VeroB4 cells, serially diluted and tested in triplicates (equivalents of 2.8×10^2 to 2.8×10^{-6} plaque forming units (PFU)/reaction). Both assays showed positive triplicate results down to 2.8×10^{-3} PFU/reaction. The homogenous assays were as sensitive as the in-house assays.

The determine the analytical specificity of the homogenous assays we performed experiments to exclude non-specific signals caused by human nucleic acids or nucleic acids of other respiratory pathogens in patient samples. Clinical specimens positive for 15 other respiratory viruses were tested with the kit assays. The clinical samples contained Enterovirus ($n = 3$); Rhinovirus ($n = 5$); human Parainfluenzavirus 1 ($n = 2$), 2 ($n = 3$), 3 ($n = 2$) and 4 ($n = 2$); Respiratory Syncytial Virus ($n = 5$); Human

Metapneumovirus ($n = 2$); human CoVs HCoV-NL63 ($n = 2$), HCoV-OC43 ($n = 3$), HCoV-229E ($n = 2$) and HCoV-HKU1 ($n = 1$), Influenza A(H1N1)pdm09 ($n = 2$), Influenza A(H3N2) ($n = 2$) and Influenza B ($n = 2$). No false-positive results were obtained with any of the MERS-CoV assays. The internal amplification control was detected in all reactions showing that none of the PCR reactions was inhibited.

Nineteen samples from a patient were tested with the in-house upE assay and compared to the RealStar® MERS-CoV RT-PCR kit 1.0 (Table 1). Among the samples, eight were tested positive and seven negative with both assays. Four samples showed discrepant results, with 3 samples testing positive only with the kit assay and 1 testing positive only by the in-house assay formulation. All discrepant results were seen with samples with very low virus concentrations indicated by a high Ct-value (>36).

5. Conclusions

Here we have shown by testing laboratory-defined as well as original clinical materials that the RealStar® MERS-CoV RT-PCR kit 1.0 is comparable to recommended in-house formulations in terms of sensitivity and specificity. The availability of a homogenous assay format which can be provided as a test kit provides an important benefit to general diagnostic laboratories which have to deal with requests to investigate cases of suspected MERS-CoV infection. Handling of the test kit is more straightforward as it requires mixing of only two instead of seven or eight different reagents, which reduces the risk of human errors during preparation [13].

The vast majority of diagnostic investigations of suspected MERS-CoV cases will be done with the intention to rule out the infection, which is why the RealStar® assays include a second heterologous amplification system to control for PCR inhibition or general reaction failure. Conversely, the availability of two different detection targets included in the kit is of great utility in positive cases, whereby a first step into confirmatory testing is achieved through results from the second target gene [12]. As long as MERS-CoV infection is a rare clinical entity [14–16], however, diagnostic confirmation should never rely on results from one test alone, and the possibility of general errors on laboratory level should be considered [12,17]. For the time being, clinical samples from cases with positive initial results should be forwarded to reference laboratories in order to achieve confirmation and to provide reference materials for a severe but rare infection.

Note added in proof

During the review of this paper further MERS-CoV sequences including the target genes of our assays have become available [18,19]. An alignment of 26 new sequences targeted by the 1A assay showed no mismatch within primer- and probe binding sites. Among the 24 new sequences for the upE target region, two sequences showed single nucleotide mismatches, including one at the second position of the upE forward-primer in the Wadi-Ad-Dawasir_1_2013 sequence (GenBank no. KJ156881) and another at the second position of the upE-Probe in the Riyadh_13b_2013 sequence (GenBank no. KJ156873). We have generated an in vitro-transcribed RNA containing both mismatches and encountered no loss of sensitivity using both the in-house as well as the kit-based formulation of the upE assay.

Conflict of interest

Authors S.O. and M.H. are employees of Altona Diagnostics GmbH. The authors declare no other conflicts of interest.

Ethical approval

Not required.

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