Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction

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We present two real-time reverse-transcription polymerase chain reaction assays for a novel human coronavirus (CoV), targeting regions upstream of the E gene (upE) or within open reading frame (ORF)1b, respectively. Sensitivity for upE is 3.4 copies per reaction (95% confidence interval (CI): 2.5–6.9 copies) or 291 copies/mL of sample. No cross-reactivity was observed with coronaviruses OC43, NL63, 229E, SARS-CoV, nor with 92 clinical specimens containing common human respiratory viruses. We recommend using upE for screening and ORF1b for confirmation.

Introduction

Coronaviruses (CoV) are large positive-stranded RNA viruses causing mainly respiratory and enteric disease in a range of animals and in humans. Humans are known to maintain circulation of four different human coronaviruses (hCoV) at a global population level. These are part of the spectrum of agents that cause the common cold. The SARS-CoV constitutes a fifth hCoV, which was in circulation for a limited time during 2002 and 2003, when a novel virus appeared in humans and caused an outbreak affecting at least 8,000 people. Mortality was high, at ca. 10% [1]. Symptoms matched the clinical picture of acute primary viral pneumonia, termed severe acute respiratory syndrome (SARS).

During September 2012, health authorities were notified of two cases of severe hCoV infection caused by a novel virus type. Both patients had travelled, or resided, in Saudi Arabia. Laboratories dealing with each of these unlinked cases were situated in Jeddah, Rotterdam and London, respectively.

In a collaborative activity co-ordinated by major European and national epidemic response networks we have developed diagnostic real-time reverse-transcription polymerase chain reaction (RT-PCR) assays

suitable for qualitative and quantitative detection of the new agent. Here we summarise the technical evaluation and analytical performance of these assays.

Materials and methods

Template for design of assays

A provisional genome sequence as well as an isolate of the new virus were obtained from author RM Fouchier on 24 September 2012, after public notification of the second case case, who was in the United Kingdom (UK), to be most probably infected by the same virus as the first case, yet unrelated. The sequence (GenBank accession number: JX869059 for the Rotterdam virus isolate, termed hCoV-EMC) served as the template for assay design, and the virus was used for initial validation experiments.

Clinical samples

Respiratory swab, sputum, and endotracheal aspirate material was obtained during 2010–2012 from several hospital wards of the University of Bonn Medical Centre.

Cell culture

Vero cells were infected with a the cell culture isolate (unpublished data) at two different doses (multiplicities of infection (MOI) of ca. 0.1 and ca. 10 TCID50 per cell) and harvested after 0, 12, 24, and 36 hours for RT-PCR analysis.

RNA extraction

RNA was extracted from the samples as described earlier [2] by using a viral RNA mini kit (Qiagen). Sputum samples were pretreated with 2× sputum lysis buffer (10 g of N-acetylcysteine/litre, 0.9% sodium chloride) for 30 minutes in a shaking incubator. Swabs were immersed in lysis buffer.

Real-time reverse-transcription polymerase chain reaction screening assay upstream of E gene (upE assay)

A 25-µl reaction was set up containing 5 µl of RNA, 12.5 µl of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each dNTP and 3.2 mM Magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), 1 µg of non-acetylated bovine serum albumin (Sigma), 400 nM concentrations of primer upE-Fwd (GCAACGCGCGATTCAGTT) and primer upE-Rev (GCCTCTACACGGGACCCATA), as well as 200 nM of probe upE-Prb (6-carboxyfluorescein [FAM])-CTCTTCACATAATCGCCCCGAGCTCG-6-carboxy-N,N,N,N´-tetramethylrhodamine [TAMRA]). All oligonucleotides were synthesized and provided by Tib-Molbiol, Berlin. Thermal cycling involved 55°C for 20 min, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 30 s.

It should be mentioned that common one-step real-time RT-PCR kits formulated for application with probes should all provide satisfactory results with default reaction mix compositions as suggested by manufacturers. In the particular case of our formulation the bovine serum albumin can be omitted if using a PCR instrument with plastic tubes. The component only serves the purpose of enabling glass capillary-based PCR cycling.

Real-time reverse-transcription polymerase chain reaction confirmatory assay (open reading frame (ORF)1b gene)

The assay had the same conditions as for the upE RT-PCR, except primer and probe sequences were ORF1b-Fwd (TTCGATGTTGAGGGTGCTCAT), primer ORF1b-Rev (TCACACCAGTTGAAAATCCTAATTG), and probe ORF1b-Prb (6-carboxyfluorescein [FAM])-CCCGTAATGCATGTGGCACCAATGT-6-carboxy-N,N,N,N´-tetramethylrhodamine [TAMRA]). This target gene did not overlap with those of known pan-CoV assays [3-5].

In-vitro transcribed RNA controls

PCR fragments covering the target regions of both assays, and some additional flanking nucleotides ('peri-amplicon fragments'), were generated using primers CTTCTCATGGTATGGTCCCTGT and AAGCCATACACACCAAGAGTGT for the upE assay, and CGAGTGATGAGCTTTGCGTGA and CCTTATGCATAAGAGGCACGAG for the ORF1b assay. Products were ligated into pCR 4 plasmid vectors and cloned in *Escherichia coli* by means of a pCR 4-TOPO TA

FIGURE 1

Replication of hCoV-EMC monitored by the upE and ORF1b RT-PCR assays, 2012
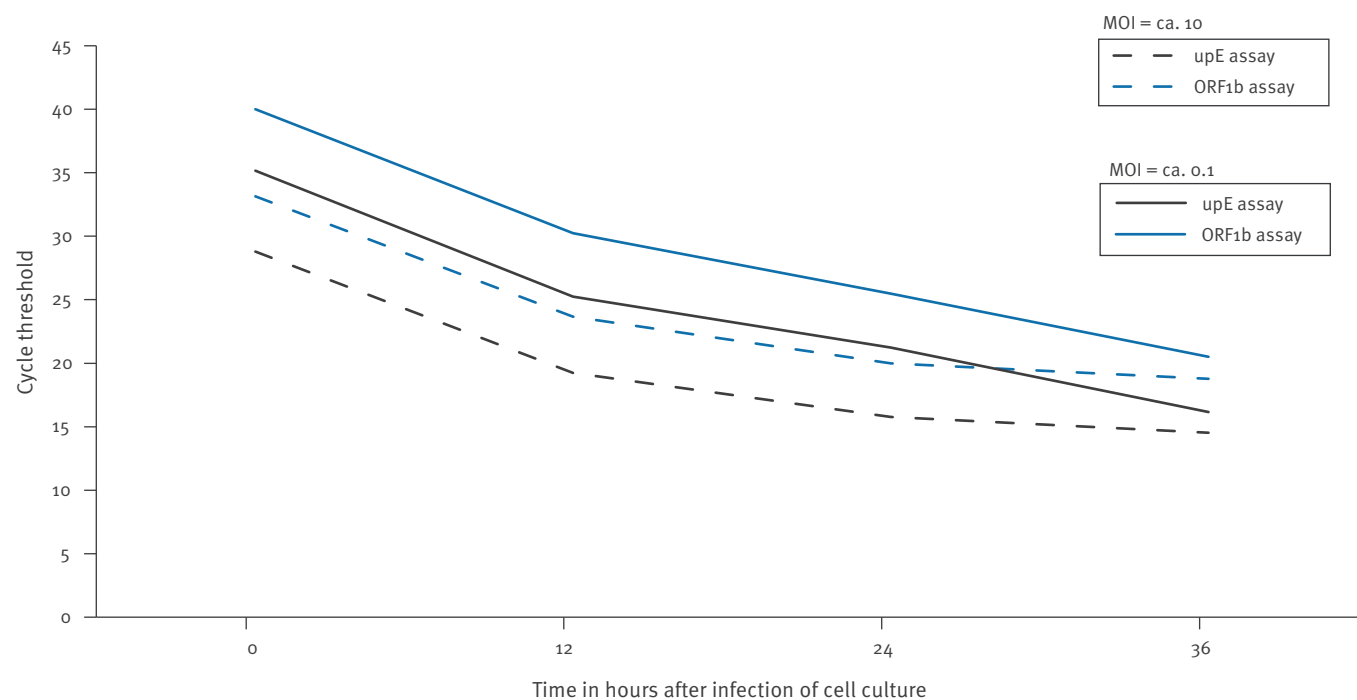
MOI : mulitplicity of infection (TCID50 per cell); RT-PCR: real-time reverse transcription-polymerase chain reaction; upE: upstream of the E gene; ORF1b: open reading frame 1b gene.

Vero cells were infected with hCoV-EMC at two different doses (MOI: ca. 10 and MOI: ca. 0.1) and standardised samples taken at different time points (after 0, 12, 24, and 36 hours) were tested by the upE and ORF1b RT-PCR assays.

cloning reagent set (Invitrogen). Plasmids were examined for correct orientation of inserts by PCR, purified, and re-amplified with plasmid-specific primers from the reagent set to reduce the plasmid background in subsequent in vitro transcription. Products were transcribed into RNA with the MegaScript T7 in vitro transcription reagent set (Ambion). After DNase I digestion, RNA transcripts were purified with Qiagen RNeasy columns and quantified photometrically. All transcript dilutions were carried out in nuclease-free water containing 10 µg/mL carrier RNA (Qiagen).

Determination of analytical sensitivities of real-time reverse-transcription polymerase chain reaction methods

Series of eight parallel reactions per concentration step were prepared and tested by the respective RT-PCR to determine concentration-dependent hit rates. Hit rates were subjected to probit regression analysis in StatgraphicsPlus software (version 5.0; Statistical Graphics Corp.).

Specificity of the assays

Assay specificity was determined using high-titred virus stock solutions, as well as clinical samples known to contain respiratory viruses. All material stemmed from the in-house strain and sample collection of University of Bonn, Institute of Virology. Identities and virus RNA concentrations were re-confirmed by specific real-time RT-PCRs for each virus before the experiment. Measured RNA concentrations are listed below along with the recorded stock virus titres.
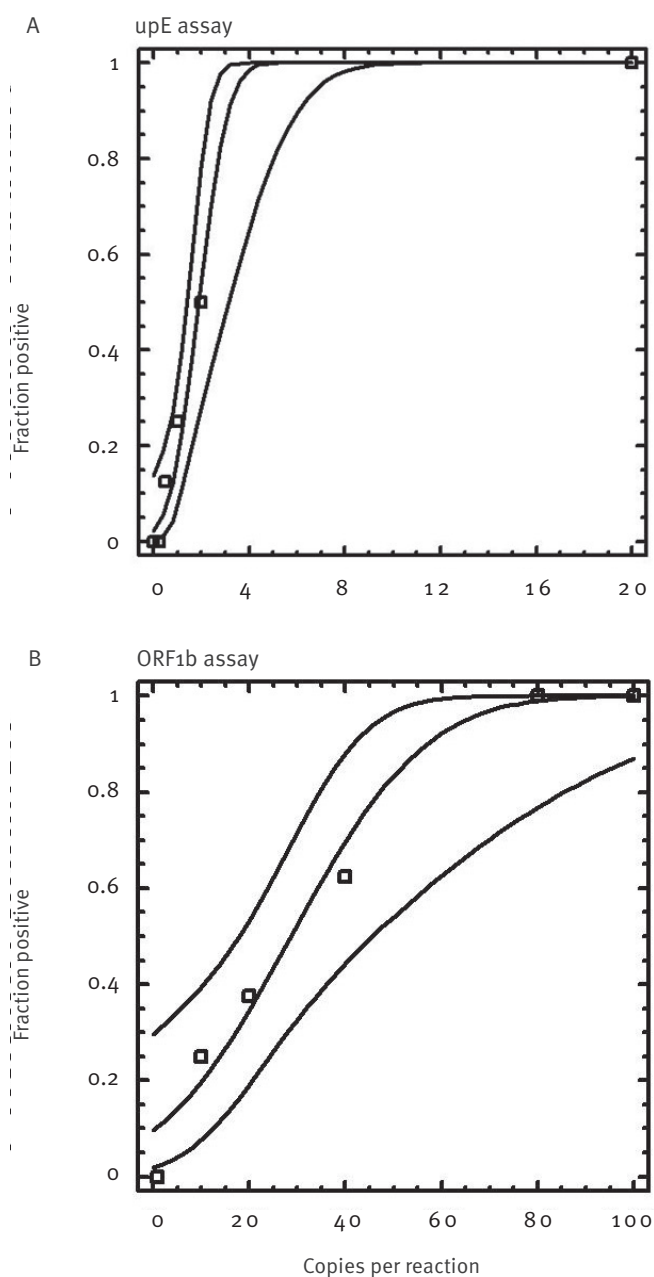
Results

Upon scanning of a provisional genome assembly, a region upstream of the putative E gene was identified as a particularly suitable target region for a real-time RT-PCR assay. The assay designed for this region is hereafter referred to as the upE-assay. A confirmatory test was designed in the open reading frame 1b (termed the ORF1b assay). This target gene did not overlap with those of known pan-CoV assays [3-5].

In order to obtain an estimate of the end point sensitivity of the assays, they were applied to cell culture-derived virus stock. The virus had a titre of 1.26×10^7 median tissue culture infective dose (TCID50)/mL. In limiting dilution experiments, the upE and ORF1b assays detected down to 0.01 and 0.1 TCID50 per reaction, respectively. The discrepancy between assays might be due to release of subgenomic RNA after onset of cytopathogenic effect (CPE) in cell culture, including the upE target fragment. As shown in Figure 1, PCRs on these samples indicated no divergence between the assays after onset of CPE (observed at 24h onwards). However, both assays deviated from each other by constant numbers of Ct values over the full duration of incubation, including time 0 (T0) when the cells were just infected and when no subgenomic RNA could have been present. It was concluded that the higher Ct values at each time point, and the lower dilution end point

A   upE assay

B   ORF1b assay

Copies per reaction

ORF: open reading frame of the frame; upE: upstream of the E gene.

The y-axis shows fractional hit-rates (positive reactions per reactions performed), the x-axis shows input RNA copies per reaction. Squares are experimental datum points resulting from replicate testing of given concentrations in parallels assays. The middle regression line is a probit curve (dose-response rule). The outer lines are 95% confidence intervals.

Experiment	upE assay	ORF1b assay
Detection end point for cell culture-derived virus	0.01 TCID50/reaction	0.1 TCID50/reaction
Technical LOD	3.4 RNA copies/reaction (95% CI: 2.5–6.9 copies/reaction)	64 RNA copies/reaction (95% CI: 47–126 copies/reaction)
Cross-reactivity with hCoV-229E	No reactivity with virus containing 10^5 PFU/mL (3×10^9 RNA copies/mL)	
Cross-reactivity with hCoV-NL63	No reactivity with virus containing 10^6 PFU/mL (4×10^9 copies/mL)	
Cross-reactivity with hCoV-OC43	No reactivity with virus containing 5×10^5 PFU/mL (3×10^{10} copies/mL)	
Cross-reactivity with SARS-CoV	No reactivity with virus containing 3×10^6 PFU/mL (5×10^{10} copies/mL)	

CI: confidence interval CoV: corona virus; LOD: limit of detection; ORF: open reading frame; PFU: plaque forming units; TCID50: median tissue culture infective dose; upE: upstream of the E gene.

for the ORF1b assay indicated that this assay had a lower sensitivity.

A more detailed assessment of technical sensitivity can be achieved using quantified, in-vitro transcribed RNA derived from the peri-amplicon region of each assay. These transcripts were generated and tested in serial ten-fold dilution experiments. Detection end points were two copies per reaction for the upE assay, and 10 copies per reaction for the confirmatory, ORF1b gene, assay. To obtain a statistically robust assessment of Limit Of Detection (LOD), transcripts were also tested in multiple parallel reactions in smaller dilution intervals above and below the end-point PCR limits. The results in terms of the fraction of positive reactions at each concentration were subjected to probit regression analysis and plotted as shown in Figure 2, where panel A shows the upE assay and panel B the ORF1b assay. The resulting LODs are summarised in Table 1. Based on the upE assay with a detection limit of 3.4 copies per reaction, and a cell-culture endpoint equivalent to 0.01 TCID50 per reaction, it was calculated that the RNA/infectious unit ratio of the virus stock must have been ca. 29 (100/3.4).

To exclude non-specific reactivity of oligonucleotides among each other, all formulations were tested 40 times in parallel with assays containing water and no other nucleic acids except the provided oligonucleotides. In none of these reactions was any positive signal seen. Cross-reactivity with known, heterospecific human CoVs was excluded by testing high-titred cell culture materials as summarised in Table 1. It should be noted that the unculturable hCoV-HKU1 was not included in these experiments.

To obtain a more clinically relevant figure on assay specificity, the assays were applied on 92 original clinical samples in which other respiratory viruses had already been detected during routine respiratory screening at Bonn University Medical Centre. These samples were prepared using the Qiagen Viral RNA kit, a formulation widely used to extract RNA in clinical laboratories. Of note, the tested panel included four samples containing hCoV-HKU1, which was not available as cultured virus stock. In total, none of the 92 original clinical samples as presented in Table 2, containing a wide range of respiratory viruses, gave any detection signal with either assay while positive controls were readily detected. It was concluded that the assay could be reliably applied to clinical samples.

Preliminary testing was also done on a patient hospitalised with acute infection during preparation of this report (Authors R Gopal and M Zambon, own unpublished observations). Both assays provided very clear amplification signal on various clinical samples. The upE assay again appeared more sensitive than the ORF1b assay.

Discussion

Here we provide the technical background data for RT-PCR assays developed in rapid response to the emergence of a novel human CoV (GenBank accession number: JX869059 for the Rotterdam virus isolate, termed hCoV-EMC).

Cell culture-derived virus is a useful source of reference material for the evaluation of molecular detection assays. However, detection end points determined on cell culture-derived virus are difficult to correlate to virus titre. Reasons include the discrepancy between

infectious viral particles and the number of copies of viral RNA, as well as the imbalance between viral genomic and subgenomic transcripts in the particular case of CoVs. This is important for laboratories using cell-cultured virus as reference, but also in the clinical setting. For example, SARS-CoV assays targeting structural protein genes tend to be slightly more sensitive than ORF1b-based assays when applied to clinical samples [6]. For the novel virus the ratio of RNA copies per infectious unit was ca. 29, while little imbalance seems to exist between genomic and subgenomic RNA in Vero cells up to 36 h post infection.

While we are not addressing the issue of quantitative PCR in this report, it should be mentioned that the availability of synthetic RNA standards enables immediate implementation of quantitative virus detection that is essential for case management and public health. Quantitative virus data can help assess the height and duration of virus excretion, and can also be useful as an early and robust parameter for the success of treatment [2,7,8]. Here we have used synthetic RNA to determine technical limits of detection in the style of standards applied by industry, taking inter-assay variation into account and providing statistically robust detection end points based on physically quantified target genes, which is impossible to achieve on cell-cultured virus. It is important to note that the detection limits we describe here are expressed as copies per reaction. We have chosen not to translate these numbers into other terms such as 'copies per ml of sputum', 'copies per swab sample', or 'copies per gram of faeces'. Such transformations vary greatly between different RNA extraction methods and clinical materials. However, we can project that the level of sensitivity, particularly for the upE assay, is very similar to those levels achieved with most advanced RT-PCR assays developed for the SARS-CoV [6,8]. For example, the Qiagen Viral RNA kit with an input volume of 140 µl of sample and an elution volume of 60 µl as recommended by the manufacturer involves a conversion factor of 85.7 between copies per reaction and copies per mL of sample. The upE assay should thus detect as little as ca. 291 copies per mL of sputum with 95% certainty. For solid samples such as swabs, which can be dipped into the lysis buffer, the resulting conversion factor is 12, resulting in a projected capability of the assay to detect as little as ca. 41 copies per swab with 95% certainty.

In this regard it is highly important to remember practical experiences made with SARS-CoV detection. Even with the highest levels of RT-PCR sensitivity it turned out that not all patients retrospectively shown to seroconvert could be diagnosed by RT-PCR in the acute phase of disease [6,8,9]. This has been ascribed to the fact the SARS-CoV replication occurs predominantly in the lower respiratory tract due to the anatomical localisation of its entry receptor, Angiotensin-converting enzyme 2 (ACE2). Should the novel virus use the same receptor, we might see a similar distribution of virus,

Virus	Number of samples tested
Parainfluenza virus	
Parainfluenza 1 virus	5
Parainfluenza 2 virus	5
Parainfluenza 3 virus	8
Parainfluenza 4 virus	1
Respiratory syncytial virus	7
Human metapneumovirus	8
Coronavirus	
hCoV-NL63	6
hCoV-OC43	4
hCoV-229E	2
hCoV-HKU1	4
Rhinovirus	8
Enterovirus	9
Adenovirus	8
Human Parechovirus	
Type 1	5
Type 3	3
Influenza A (H1N1, H3N2)	9
Influenza B	2
Total	**92**

and similar challenges in clinical application of molecular diagnostics. Studies of virus concentration in clinical samples are underway to address these highly critical issues.

Specificity is a very important issue in rare, highly critical virus infections for which a broad number of differential diagnoses exist. The risk associated with false positive PCR results posed a challenge in development of the assays described here. First, real-time PCR can yield artificial signals due to technical interference of oligonucleotides involved in the assay (resembling primer dimers in which probe sequences participate). These may be observed at infrequent intervals due to the statistical nature of nonspecific random molecular interactions. We have taken care to exclude the occurrence of those signals by testing large series of water-containing assays. Second, any virus detection assay might cross-react with related viruses, and there is worldwide circulation of four different human CoVs. Viral stock solutions were tested in order to exclude cross-reactivity even on high-titred materials. In spite of the favourable outcome of this experiment, it should

be mentioned that of the two assays investigated, the target gene of the ORF1b-based assay was most conserved between CoV. The genetic range of known CoV from animals is larger than those human viruses tested here. Theoretical comparisons between genomes of these viruses and our ORF1b assay suggested no risk of significant cross-reactivity (not shown). However, in absence of further investigation we tend to recommend using the upE assay for case management. This is also due to the lower sensitivity of the ORF1b assay.

The final proof of assay specificity was provided in a set of clinical samples that was assembled to realistically reflect the composition of patient groups presenting with Acute respiratory infections (ARI). Of note, also the four 'common-cold coronaviruses' hCoV-NL63, -229E, -OC43, and -HKU1 were included in this panel. Consequentially, we can say from these data that typical human CoV will not cross-react with the assay, even under adverse conditions such as those created by the additional presence of patient-derived nucleic acid and other components typical of clinical samples that may all interfere with the performance of PCR.

The open availability of proven diagnostic assays early in an epidemic is useful in order to equip and prepare public health laboratories efficiently [10,11]. However, there is a number of caveats associated with the wide and largely uncontrolled provision of such technology during the very early phase of an epidemic. In this phase public health authorities around the world have to monitor the development of case statistics in order to make projections and attain epidemic risk assessment. The notification of false positive laboratory results can be highly detrimental during this phase of the epidemic.

The authors of this paper will provide in-vitro transcribed RNA controls to health professionals (refer to Acknowledgements section) but will not be able to provide intense technical advice. Authors will follow the policy of providing only one control, namely that for the upE assay, in order to minimise opportunities for accidental laboratory contamination. If laboratories find patient samples positive by the upE assay and control, they can conduct confirmatory testing using the ORF1b assay. A positive result in this test would most likely not be due to contamination. Of note, the target gene of our ORF1b assay does not overlap with that of other, so-called 'pan-CoV' assays [3-5], excluding the possibility of contaminating our assay with high-titred controls or PCR products from these assays.

In this light we should mention that we have been working on an N gene-based assay as well, but our experience with testing clinical material strongly suggests N-gene assays should not be used for diagnostic application for the time being, i.e., as long as no direct sequence information of the N gene is available from clinical samples.

*Erratum:

Table 1 was corrected and replaced on 28 September 2012.

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