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-2 virus 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 Virology Laboratory, Dr Soliman Fakeeh Hospital, Jeddah, Saudi Arabia. In addition, a cell culture isolate of the virus was obtained from the Health Protection Agency (HPA), London, UK.

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Vero cells were infected with a cell culture isolate (unpublished data) at two different MOIs (multiplicities of infection) of ca. 0.1 and ca. 10 TCID50 per cell) and harvested after 0, 12, 24, and 36 h post-infection. Cells were then harvested and the RNA was extracted from the samples as described earlier [2] by using a viral RNA mini kit (Qiagen). Sputum samples were pretreated with 2h sputum lysis buffer (10 g of N-acetyl-cysteine/ml, 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 sulphate), 1.4 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulphate solution (Invitrogen – not provided with the kit), 1 µl of non-acetylated bovine serum albumin (Sigma), 400 mM concentrations of primer upE-Fwd (GCACAGCCGGCAGATTGAGT) and primer upE-Rev (GGTCTCAGACGAGGACCATCA), as well as 200 mM of probe upE-prb (6-carboxy-fluorescein [FAM]-CTCTTCAATTATGGCCGAGCTCG-6-carboxy–6-carboxy-

N,N'-bismethylimidazoline (TMAPR)). 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.
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 x 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 for the ORF1b assay indicated that this assay had a lower sensitivity.

Figure 1. Replication of hCoV-EMC monitored by the upE and ORF1b RT-PCR assays, 2012

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).

Figure 2. Probit regression analysis to determine limit of detection for the upE assay., 2012

Table 1. Results of sensitivity and specificity tests for hCoV-EMC assays, 2012

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-titre 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.

Table 2. Known respiratory viruses in clinical samples used for testing the specificity of hCoV-EMC assays, 2012
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 our 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. Consequently, 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.

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