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Evaluation of a Real-time Polymerase-chain Reaction for Severe Acute Respiratory Syndrome (SARS) Associated Coronavirus in Patients with Hospitalised Exacerbation of COPD*

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Abstract: One year after the first outbreak infections with SARS associated coronavirus were again reported and the clinical picture varied. Because health care facilities will have to initiate immediate preventive action in cases of probable SARS we tested the potential of PCR to exclude SARS associated coronavirus in patients hospitalized with respiratory symptoms. Based on primers published recently a real time Taqman® PCR was established and evaluated. Lower respiratory tract specimens of patients with acute exacerbation of COPD were investigated. 141 patients with mild to moderate COPD were included. The assay was specific, sensitive, precise and reproducible and allowed absolute quantification without cross-reactivity to other respiratory viruses. None of the samples were positive for SARS associated coronavirus. Our RT-PCR for SARS associated coronavirus is a valid and practicable method to further exclude SARS in X-ray negative patients with respiratory symptoms, even in the presence of other respiratory RNA viruses.

Key words: Severe Acute Respiratory Syndrome (SARS); Coronavirus; COPD

Introduction

The recent global outbreak of severe acute respiratory syndrome (SARS) has promoted the concern about the occurrence of respiratory diseases with symptoms similar to those seen in SARS. Although the epidemic nature of the outbreak has ceased, the recurrence of SARS during the influenza season 2004/05 cannot be ruled out. Health authorities and physicians are concerned that cases of influenza and other respiratory diseases, particularly when they occur as clusters in health care facilities, could raise suspicions of SARS, resulting in disruption of health services as well as costly precautionary measures and investigations (Schlagenhauf, 2003).

The number of hospitalisations with acute exacerbations of chronic obstructive pulmonary disease (AE-COPD) is seasonal and will pick up again during autumn and winter season. Although the clinical presentation of AE-COPD is in detail different from the case definition of SARS (lack of pulmonary infiltrate), it will be frequently mistaken for SARS. Moreover the clinical picture of SARS may vary(Chow, Lee et al., 2004; Fisher, Lim et al., 2003). Public health management guidelines for cases of SARS require complete isolation of the patient and health care personnel (World Health Organization, 2004). Emergency plans foresee those measurements also in cases with suspicion of SARS and may therefore lead to paralysis of ambulances in Europe through false positive suspicions of a SARS infection. A laboratory test for ruling out the disease in such patients would thus be desirable, but the specificity of PCR in this setting has not yet been characterized. Although cross-reactivity for some human and animal coronaviruses has been ruled out earlier(Drosten, Gunther et al., 2003).

We have investigated the potential of RT-PCR to exclude the presence of SARS-coronavirus in lower respiratory tract specimens in a cohort of patients with AE-COPD presenting to the hospital. In addition, we wanted to exclude cross-reactivity of SARS-coronavirus RT-PCR with other relevant RNA-viruses in human respiratory specimens.

Patients and Methods

Data and specimens were collected from October 1999 to July 2003 at a 600-bed university hospital in Bochum, Germany, for a prospective case-control study comprising patients with COPD (one group with AE-COPD and a control group with stable disease). The goal of this study was to identify viral infections in patients with AE-COPD; initial results for other respiratory viruses have been published recently(Rohde, Wiethege et al., 2003). For this investigation only specimens of patients with signs and symptoms of exacerbation of COPD were included in the analyses. The study was approved by the ethical committee

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of the Ruhr University Bochum Germany and all patients gave written informed consent for the study.

Clinical Evaluation

All patients underwent careful medical history and clinical examination. Spirometry was performed using a JAEGER Flowscreen device (E. Jaeger Würzburg, Germany). For spirometry the best out of three trials was selected and data were compared to reference values(Quanjer, Tammeling et al., 1993). Forced expiratory volume in one second (FEV1), forced and inspiratory vital capacity (FVC, IVC) were assessed. A routine posterior-anterior chest radiograph was evaluated on admission by expert radiologists for all COPD subjects.

Methods of sputum induction, processing and RNA extraction are described in detail elsewhere(Rohde, Wiethege et al., 2003).

Control of RNA Integrity in Sputum Samples

In order to exclude RNA degradation in case of negative SARS-Coronavirus RT-PCR results, samples with former positive virus results for other relevant respiratory RNA-viruses (Rhinovirus, Respiratory syncytial virus, Influenza A virus) have been tested again by real-time PCR. These control PCR experiments have been carried out as two-step TaqMan® real-time PCR as previously described for RSV-A detection(Borg, Rohde et al., 2003).

SARS-Coronavirus Real-time RT-PCR

A quantitative TaqMan® real-time RT-PCR was carried out using the Superscript II platinum Taq polymerase one-step RT-PCR kit (Invitrogen, Karlsruhe, Germany). Primers BNIoutS2 and BNIoutAs (TIB MOLBIOL, Berlin, Germany) designed by Drosten et al. (Drosten, Gunther et al., 2003) for conventional PCR were used in combination with a 6-carboxyfluorescein (FAM) -labeled TaqMan® probe BNITM-SARP (TIB MOLBIOL, Berlin, Germany). RT-PCR reactions were carried out with 5 µl RNA in a volume of 25 µl. Reactions contained 3.6 mM MgSO4 , 200 mM of each primer, 240mM of TaqMan® probe and 0.6 µl of the enzyme mixture. Positive and negative controls were included for all RT-PCR amplifications. A cloned and in vitro transcribed PCR fragment constructed by Drosten et al. was used as SARS-Coronavirus positive control standard RNA. Serial 10-fold dilutions of known quantities, ranging from 10^5 copies/µl to 10^{-2} copies/µl, were used. For negative controls water was used instead of RNA. The following TaqMan® RT-PCR profile was applied for all RT-PCR runs: 45 °C for 15 minutes; 95 °C for 3 minutes; 95°C for 10 seconds and 58 °C for 30 seconds (40 cycles). Amplification of target molecules and detection of RT-PCR products were performed with a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Amplified RT-PCR products were analysed on an ethidium bromide-stained 2% agarose gel under UV illumination.

Exclusion of Cross reactivity

Sputum samples positive for other RNA-viruses relevant for respiratory diseases as Rhinovirus, Influenza A virus and Respiratory syncytial virus were chosen to study SARS-Coronavirus RT-PCR specificity.

Sensitivity of SARS-Coronavirus RT-PCR

Exclusion of PCR-inhibiting components in sputum samples was tested by analysing nucleic acid isolates of respiratory tract specimens, which had been spiked with different concentrations of SARS-Coronavirus standard RNA.

Absolute Quantification and Statistical Analysis

The Applied Biosystems - ABI PRISMTM Sequence Detection System was used to monitor the increase of amplified FAM-labelled PCR products and the entire process of calculating Ct-values, preparing a standard curve and determining the starting copy numbers for unknown samples was performed by the software. Data were presented as amplification plot, showing the fluorescent values plotted versus the cycle number and as standard curve, displaying the threshold cycle versus the logarithm of defined quantities (copy numbers) of the positive standard samples. Copy numbers of unknown samples can be automatically inferred from the regression line. To evaluate reproducibility, intra-assay and inter-assay standard deviations (SDs) and coefficients of variation (CVs) were calculated for each standard concentration within and between individual PCR-runs.

Results

A total of 211 subjects with COPD were initially screened and 70 patients had stable disease (no exacerbation within the last 30 days prior to hospital admission and no changes in therapy within the last 14 days, including inhaled and oral medication). Thus, 141 patients were hospitalized for AE-COPD, characterized by worsening in dyspnoea, cough and expectoration in the presence of chronic airflow obstruction(Pauwels, Buist et al., 2001). None of the patients had a recent travelling history and patients were only included in the absence of pulmonary infiltrates suggestive of an infectious aetiology.

Patients demographic data are summarised in Table 1. All COPD patients had mild to moderate disease according to standardised criteria(Pauwels, Buist et al., 2001). The most frequent comorbidities were hypertension (37/141, 26%), coronary artery disease (28/141, 20%), diabetes (28/141, 20%) and hyperlipoproteinemia (24/141, 17%).

RNA from respiratory tract specimens had been stored at –70 °C for several months. Its integrity was verified by checking again the viral load of known virus-positive samples by real-time PCR before carrying out SARS-Coronavirus RT-PCR. Samples chosen were positive for other RNA-viruses relevant for respiratory diseases, such as HRV, RSV and Influenza A.

Table 1. Demographic characteristics of the population initially included in the study. For evaluation of the presence of SARS-associated Coronavirus only patients with acute exacerbation of COPD (AE-COPD) were included (n = 141).

	AE-COPD (n=141)	*Stable COPD (n=70)*
Gender male n (%)	111 (79)	59 (84)
Age years mean (SD)	67 (9)	65 (11)
Packyears smoked mean (SD)	39 (28)	38 (29)
Inhaled corticosteroids n (%)	78 (55)	48 (69)
Systemic corticosteroids n (%)	74 (53)	39 (56)
FEV1 %predicted mean (SD)	39 (14)	48 (17)

This renewed examination confirmed the viral load results of former PCR examinations without significant deviations compared to former values.

Specificity of the newly combined primers/probe system for SARS-Coronavirus detection was analysed by performing one-step RT-PCR amplifications of different concentrations (10^5 copies/µl – 10^{-2} copies/µl) 21.14 (10^5 copies/reaction) to 39.91 (10^{-1} copies/reaction). To determine precision and reproducibility of the assay mean intra-assay and inter-assay standard deviations (SD) were calculated. The mean SD-value was 0.38 for intra-assay runs (range 0.05 to 0.92) and 1.12 for inter-assay runs (range 0.23 to 1.76). Intra-assay and inter-assay coefficients of variation (CVs) were 1.17% (range 0.25% to 2.42%) and 3.84% (range 0.58% to 6.39%), respectively on average. For absolute quantification of viral genomes a standard curve was designed from Ct values plotted versus the log of standard concentrations (Fig. 1). Based on standard curve and CV values the reliable limit of detection was 10 copies/reaction (Table 2). Analysis of the SARS-Coronavirus RT-PCR-amplification product by agarose gel electrophoresis showed a clear single band corresponding to the predictive length of 190 bp.

Sensitivity of SARS-Coronavirus real-time RT-PCR was tested by analysing ribonucleic acid of sputum samples of different patients, which had been spiked with different concentrations of SARS-Coronavirus standard RNA corresponding to 4000 and 4 SARS-Coronavirus RNA copies/µl, respectively (10^5 copies/reaction and 10^2 copies/reaction). RT-PCR amplification plots of these samples were typical (Fig. 2) and the determination of SARS-Coronavirus standard RNA copy numbers did not show any statistically significant deviation compared with corresponding standard RNA dilutions. Moreover the SD values of these measurements turned out to be very low (1.3% and 0.7%), so that PCR-inhibiting component parts in ribonucleic

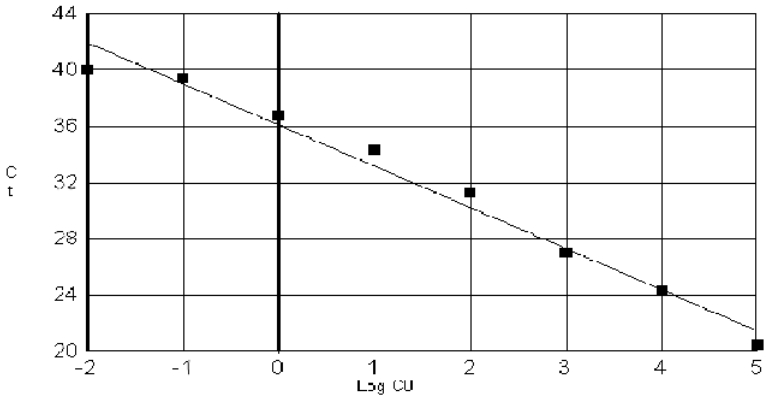

Fig. 1. Standard curve of SARS-Coronavirus standard RNA (CT = cycle threshold).

SARS-Coronavirus standard RNA copies/reaction	Ct values of 7 replicates (mean	±	SD)	CV %
10^5	21,14	±	1,35	6,39
10^4	25,29	±	1,37	5,42
10^3	28,63	±	1,76	6,15
10^2	32,21	±	1,49	4,63
10^1	35,25	±	1,39	3,94
10^0	38,60	±	1,40	3,63
10^{-1}	39,91	±	0,23	0,58
10^{-2}	40,00	±	0,00	0,00

Table 2. Interassay precision of SARS-Coronavirus one-step real-time RT-PCR for standard RNA.

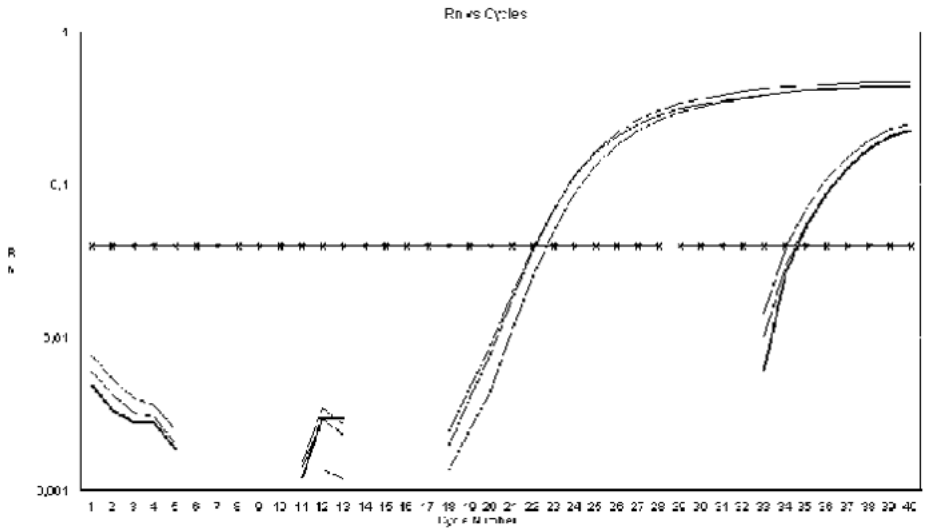

Fig. 2. Amplification plots of SARS-Coronavirus RNA spiked ribonucleic acid of respiratory tract specimens.

and to exclude cross-reactivity we investigated whether other human respiratory RNA-viruses (HRV, Respiratory Syncytial virus (RSV), PIV-3 and Influenza A virus) were detected by SARS-Coronavirus real-time RT-PCR. For this purpose sputum samples (n = 79) with known virus contamination were studied. Neither HRV-, RSV-, PIV-3- nor Influenza A virus-positive specimens or multiple infected specimens yielded any PCR-amplification with SARS-Coronavirus specific real-time RT-PCR primers and probe. RT-PCR for SARS-coronavirus was negative in all patients (0/141, 0%).

Discussion

RT-PCR testing of respiratory tract specimens is an accepted method of confirmation of SARS. In regions like central Europe, where the awareness of SARS is disproportionally high, the disease may be suspected even in patients presenting with respiratory symptoms but no infiltrations in initial chest X-ray examination. Since the suspicion of SARS prompts various health care procedures potentially disabling entire outpatient clinics, physicians will find themselves more often in the position to exclude rather than to confirm SARS.

We therefore evaluated the specificity of real-time RT-PCR for SARS-associated coronavirus in a cohort of hospitalised patients with exacerbation of COPD. No respiratory sample of this cohort showed positive results even in the presence of other respiratory viruses. This demonstrates that SARS coronavirus was not present in a well defined population of patients with AE-COPD. A possible limitation of this study may be the fact that the majority of samples were drawn before the outbreak and the clinical appearance of the cohort was distinct to that postulated for SARS. However, asymptomatic carriage has not yet been described for SARS-associated coronavirus and the sensitivity of the recommended PCR method has been confirmed elsewhere(Drosten, Gunther et al., 2003). Furthermore, respiratory samples of patients with SARS may be negative during the first days of the disease but patients in this study presented to the emergency room several days after onset of symptoms, a time range associated with positive PCR in SARS patients(Peiris, Chu et al., 2003).

We conclude that asymptomatic carriage is unlikely in patients with exacerbation of COPD and RT-PCR for SARS associated coronavirus is probably a valid and practicable method to further exclude SARS in X-ray negative patients with respiratory symptoms, even in the presence of other respiratory RNA viruses.

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