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Viral loads in clinical specimens and SARS manifestations

Key Messages

1. A high viral load in nasopharyngeal aspirate (with or without a high viral load in serum) is a useful prognostic indicator of respiratory failure or mortality. The presence of viral RNA in multiple body sites is also indicative of poor prognosis.
2. Early treatment with an effective antiviral agent before day 10 may decrease the peak viral load, and thus ameliorate the clinical symptoms and mortality, and reduce viral shedding and the risk of transmission.

Introduction

The SARS pandemic affected 8098 people with 774 fatalities in 2002-2003.[1] A novel coronavirus was isolated from SARS patients who had specific seroconversion to this virus.[2-4] Animal models using macaque monkeys, ferrets and domestic cats were established. However, no extrapulmonary lesions could be identified in these animals though virus isolation and real-time polymerase chain reaction (RT-PCR) for viral RNA were positive from their pharyngeal secretions, tracheobronchial secretions, urine, rectal swabs or stool, kidney or lung tissues. We reported the use of RT-PCR to detect SARS-CoV RNA from nasopharyngeal aspirate (NPA), throat swab, urine and stool specimens. We also developed RT-qPCR assays using the LightCycler System (Idaho Technology, Idaho Falls [ID], US) to augment the sensitivity of detection. The serial viral load in NPA was used for monitoring the clinical progress and the response to anti-viral therapy, whereas the admission viral load in serum was used as a marker of prognosis. Unlike the animal models, extrapulmonary manifestations such as haematological changes, diarrhoea, and liver derangement were common in SARS patients. In this study, we assayed and analysed the viral load of clinical specimens from different anatomic sites between days 10 to 15 after the onset of symptoms to understand the role of this virus in the pathogenesis of the clinical manifestations and abnormal laboratory tests in SARS patients.

Aims and objectives

To correlate SARS-CoV viral load in different clinical specimens with the clinical manifestations of SARS.

Methods

Patients who fulfilled the modified World Health Organization definition of SARS (n=154), managed in the United Christian Hospital and Caritas Medical Centre were included in this quantitative virological study. All patients were either serologically confirmed by demonstrating a four-fold rise of indirect immunofluoresent antibody titre against SARS-CoV in the serum taken on admission and within day 28 after symptoms onset, or had positive RT-PCR for SARS-CoV RNA confirmed from their clinical specimens (for those who died or failed to seroconvert before day 28). The case definition included fever of 38ºC or higher, cough or shortness of breath, and new pulmonary infiltrates on chest radiography or high-resolution computed tomography in the absence of an alternative diagnosis to explain the clinical presentation. During the first 15 days, patients were prospectively monitored for occurrence of diarrhoea, oxygen desaturation, mechanical ventilation, and laboratory evidence of lymphopaenia, renal impairment, liver dysfunction, abnormal urinalysis, and mortality. For the diagnosis of SARS-CoV infection, NPA and acute sera were taken on admission. Convalescent sera were taken between days 7 and 28 after the onset of symptoms. In all patients, RT-PCR for SARS-CoV was performed on the NPA collected on admission. RT-qPCR was performed for patients who had their NPA, sera, stool and urine specimens collected on days 10 to 15 after the onset of symptoms. All virological diagnostic laboratory tests including viral culture, RT-PCR, RT-qPCR and immunofluorescent antibody detection for IgG seroconversion against SARS-

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CoV were performed according to our protocols. From clinical samples, RNA was extracted using the QIAamp virus RNA mini kit (Qiagen) as instructed by the manufacturer. For all specimens, 140 μL of the sample were used for RNA extraction and extracted RNA was finally eluted in 30 μL of RNase-free water and stored at -20ºC. For the RT-qPCR assay, RNA and cDNA was generated as described; cDNA was amplified in a 7000 Sequence Detection System (Applied Biosystems) by using TaqMan PCR Core Reagent kit (Applied Biosystems). In a typical reaction, 2 μL of cDNA was amplified in a 25 μL reaction containing 0.625 U AmpliTaq Gold polymerase (Applied Biosystems), 2.5 μL of 10x TaqMan buffer A, 0.2 mM of dNTPs, 5.5 mM of MgCl2, 2.5 U of AmpErase UNG, and 1x primers-probe mixture (Assays by Design, Applied Biosystems). The forward primer was 5'-CAGAACGCTGTAGCTTCAAAAATCT-3' (corresponding to nt 17718 to 17742 of SARS-CoV genome) and the reverse primer was 5'-TCAGAACCCTGTGATGAATCAACAG-3' (complementary to nt 17761 to 17785). The sequence of the reporter probe was 5'-(FAM)TCTGCGTAGGCAA TCC(NFQ)-3' (FAM, 6-carboxyfluorescein; NFQ, non-fluorescent quencher; complementary to nt 17745 to 17760). Reactions were first incubated at 50ºC for 2 min, followed by 95ºC for 10 min. Reactions were then thermal-cycled for 40 cycles (95ºC for 15 sec, 60ºC for 1 min). Plasmids containing the target sequences were used as standard controls. To monitor the integrity of RNA extraction for each sample, a housekeeping gene, beta-actin was detected by RT-PCR using two primers: beta-actin forward, 5'-CCCAAGGCCAACCGCGAGAAGAT-3' and reverse, 5'-GTCCCGGCCAGCCAGGTCCAG-3'. All samples were found to contain detectable beta-actin RNA.

Statistical analysis

All timed data were calculated from the onset of symptoms. We compared the viral load in these specimens with the presence or absence of diarrhoea, oxygen desaturation, mechanical ventilation, lymphopaenia, hepatic dysfunction, abnormal urinalysis and mortality by Chi squared or Fisher's exact test for categorical variables and Mann-Whitney *U* test for continuous variables. A two-tailed P value of less than 0.05 was taken to be significant. Correlation between the number of anatomical sites with detectable viral load by RT-qPCR and mortality was calculated by linear regression.

Results

Viral load in NPA (n=142) between days 10 and 15 after the onset of symptoms was associated with oxygen desaturation (odds ratio [OR]=3.1; 95% confidence interval [CI], 1.6-6.2), mechanical ventilation (OR=11.3; 95% CI, 3.6-35.1), diarrhoea (OR=2.5; 95% CI, 1.3-5), hepatic dysfunction (OR=2.5; 95% CI, 1.2-5.2) and mortality (OR=54; 95% CI, 7-415). Serum viral load (n=53) was associated with oxygen desaturation (OR=5; 95% CI, 1.5-16.4), mechanical ventilation (OR=1.5; 95% CI, 1.1-2) and mortality (OR=17.1; 95% CI, 2.0-151). Stool viral load (n=94) was associated with diarrhoea (OR=14.1; 95% CI, 1.7-114), as was urine viral load (n=111) with abnormal urinalysis (OR=7.2; 95% CI, 1.6-32.9).

Discussion

The viral load reflects the dynamic interaction between viral replication and viral clearance by body defence mechanisms. Viral load study in SARS has been used for virological diagnosis and monitoring of progress or response to anti-viral therapy. In our study, the viral load in the NPA peaked around day 10 and was immediately followed by a decrease with a concomitant normalisation of the lymphocyte count and a corresponding rise of serum antibodies specific for the SARS-CoV. The presence of the virus and the viral load in different body fluids may have a bearing on the possible modes of transmission. The infectivity at day 10 as reflected by a mean peak viral load of 5.8 and 7.0 $\log_{10}$ copies/mL in positive specimens of NPA and stool respectively suggested that respiratory droplets and indirect contact with faeces might be an important mechanism of transmission. Previous viral load study centred on NPA and serum at the time of admission as a diagnostic tool and a prognostic indicator. Viral load study in various body fluids in addition to NPA and serum has not been performed to determine the transmission and pathogenesis of the pulmonary and extra-pulmonary manifestations of SARS.

The SARS is predominantly a viral pneumonia with a rapid tempo of deterioration. The importance of SARS-CoV as a respiratory pathogen is supported by the strong association of the viral load in the NPA with oxygen desaturation, mechanical ventilation and mortality as evident by odds ratios of 3.1, 11.3 and 54 respectively. Unexpectedly it was also associated with diarrhoea (OR=2.5) and hepatic dysfunction (OR=2.5). Anecdotal reports of the usefulness of steroids in the treatment of SARS suggest these extra-pulmonary manifestations could just be part of an inflammatory spill-over from a process of virus induced immuno-dysregulation or excessive cytokine activation in the lungs. However, our findings suggest that viral replication in these extra-pulmonary sites may be as important since the viral load in the stool correlated strongly with diarrhoea. Moreover, electron microscopy of the ileal and colonic biopsy from SARS patients showed numerous viral particles intra- and extra-cellularly.

The serum viral load also correlated with oxygen desaturation, mechanical ventilation, and mortality. This was not surprising, as viraemia has also been reported in adenovirus, respiratory syncytial virus and rotavirus infections.[5-7] However, viraemia even if present is very short lasting in these mucosal infections. In one study, five out of 41 neonates with positive respiratory syncytial virus (RSV) antigen in nasal washes were positive for RSV-RNA in blood. High levels of adenovirus DNA in serum was also associated with fatal outcome in children who developed adenovirus

infection after allogeneic stem-cell transplantation. Six (86%) of seven children who died of adenovirus infection, compared with only two (7%) of 29 other patients, had high serum levels of adenoviral DNA (P<0.0001). The absence of an association between viral load in any specimens with lymphopaenia at day 10 could be explained by the routine use of steroids which induces apoptosis of lymphocytes. The apparent inferior performance of serum viral load as a prognostic indicator could be related to a lower number of available serum samples in this cohort. However, the proportion who had oxygen desaturation in these 53 (38%) patients was not significantly different from the 142 (46%) patients who had submitted nasopharyngeal samples between days 10 and 15.

Compared with other common viral respiratory diseases, the onset of peak viral load in the nasopharynx appeared to be delayed. In a prospective study of viral shedding in nasopharyngeal secretions in experimental adult infections as enumerated by $TCID_{50}$ (median tissue culture infective dose) viral titre or RT-qPCR, RSV was detected between days 2 and 12, with a plateau phase between days 3 and 8 at a peak viral load of 5 $\log_{10}$ copies/mL. In the case of experimental adult influenza, viral replication in NPA peaked at about 48 hours after the onset of symptoms and declined sharply thereafter, with an insignificant degree of viral shedding after days 6 to 8. The peak virus titres in symptomatic volunteers inoculated with influenza A H3N2 ranged from $10^{2.5}$ to $10^{7.0}$ $TCID_{50}$/mL of nasopharyngeal wash. The viral load correlated positively with the clinical symptoms of fever and malaise, as well as the degree of viral shedding. However, the reported low incidence of viraemia and the early peak nasopharyngeal viral load in these two conditions could be accounted by the inherent behaviour of viral replication, background IgG and IgA antibodies with cross-reactivity against homologous antigens (due to previous infections or innate immunity of the host). In many of these experimental infections where the profile of the viral load in NPA was documented, the volunteers were adults and had a low level of background antibodies and therefore concomitant cell mediated immunity against influenza or RSV.

One limitation of the present study was its retrospective nature. Only those who had sent the specimen at around day 10 could be tested and analysed. Changes of lymphocyte subset were also not analysed due to the retrospective nature of this study. Nonetheless, lymphocytes changes in SARS patients were well reported by two other groups who showed a consistent decrease in the peripheral blood level of dendritic cell subsets, natural killer cells, CD4+ and CD8+ T lymphocytes and B lymphocytes in SARS patients.[8,9]

Conclusions

SARS is predominantly a respiratory infection with spread through viraemia to extrapulmonary sites where viral replication leads to non-respiratory manifestations. There could be concomitant immuno-dysregulation and associated inflammatory damage that accentuates its morbidity and mortality. A high viral load in NPA with or without a high viral load in serum is a useful prognostic indicator of respiratory failure or mortality. The presence of viral RNA in multiple body sites is also indicative of a poor prognosis. Early treatment with an effective antiviral agent before day 10 may decrease the peak viral load, and thus ameliorate clinical symptoms and mortality, and reduce viral shedding and the risk of transmission.

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