International Journal of Infectious Diseases 104 (2021) 696–700

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Temporal profile of SARS-CoV-2 viral load in posterior nasopharyngeal samples: Analysis of 944 patients in Apulia, Italy

Moris Sangineto[a], Fabio Arena[b,c], Rosella De Nittis[d], Rosanna Villani[a], Crescenzio Gallo[b], Gaetano Serviddio[a,*]

[a] C.U.R.E. (University Center for Liver Disease Research and Treatment), Liver Unit, Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy
[b] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy
[c] IRCCS Don Carlo Gnocchi Foundation, Florence, Italy
[d] Microbiology Unit, Ospedali Riuniti University Hospital, Foggia, Italy

ARTICLE INFO

Article history:
Received 30 October 2020
Received in revised form 25 January 2021
Accepted 26 January 2021

Keywords:
COVID-19
Coronavirus
Pandemic
SARS-CoV-2
Viral load
RT-PCR
Region

ABSTRACT

Objectives: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has heavily impacted Italy. The government's restriction measures have attenuated the burden on hospitals. The association of high viral replication with disease severity suggests the potential for lower viral load in milder clinical presentations.
Methods: The reverse-transcription-polymerase-chain-reaction (RT-PCR) profile of 944 consecutive, non-replicate, positive retropharyngeal swabs was collected from 3 March to 8 June 2020 to investigate the temporal profile of SARS-CoV-2 viral load in the region of Capitanata, Apulia. Cycle threshold (Ct) values of 3 targets (N [nucleocapsid protein], E [envelope protein] and RdRP [RNA-dependent RNA-polymerase]) were analysed.
Results: The median Ct values of the 3 targets increased considerably over the study period, showing a progressive and constant weekly change. The negative detection rate of E and RdRP increased over time. These data suggest that SARS-CoV-2 viral load progressively decreased along the outbreak course. During the first epidemic peak (March and April) the viral load among patients >80-years was significantly higher than for younger subjects. However, in May this age-dependent difference disappeared, underlying viral load reduction in the elderly.
Conclusions: An attenuation of viral transmission or pathogenicity during the epidemic course is suggested, likely due to restriction measures, although viral factors might also be considered.

Introduction

From early 2020 a new Coronavirus disease named COVID-19 has spread worldwide with Italy being one of the most affected countries, albeit with substantial regional differences (IstitutoSuperiorediSanità; WHO). In the Apulia region (southern Italy) approximately 7900 cases of COVID-19 infection have been reported so far, with a peak at the end of April and a substantial decrease from May-June. The rate of hospitalization and number of severe cases also fell during this time (IstitutoSuperiorediSanità). The Italian Government's restriction measures have contributed to pandemic control (Prem et al., 2020). Social distancing, use of personal protective equipment (PPE) and active territorial surveillance may all have reduced SARS-CoV-2 transmission (Chan et al., 2020; Prather et al., 2020; Prem et al., 2020). However, viral factors, host-virus interaction, and the summer season are also potential contributing factors in the pandemic slowdown. It should also be considered that changes to diagnostic strategies occurred in the later phases of the pandemic in Italy.

Some studies have correlated viral load with disease severity, analysing different biological specimens (Huang et al., 2020; Shi et al., 2020; To et al., 2020; Yu et al., 2020; Zheng et al., 2020). An age-dependent effect on clinical outcome has been described, with elderly people usually having higher viral loads and worse prognosis (Davies et al., 2020; Liu et al., 2020a,b; Rockx et al., 2020; Shim et al., 2020). A trend of decreasing SARS-CoV-2 viral load in nasopharyngeal samples has been reported in a small study over the latest phase of the COVID-19 pandemic in Italy's northern regions (Clementi et al., 2020).

* Corresponding author at: C.U.R.E. (University Center for Liver Disease Research and Treatment), Liver Unit, Department of Medical and Surgical Sciences, University of Foggia, Viale Pinto 1, 71122 Foggia, Italy.
E-mail address: g.serviddio@unifg.it (G. Serviddio).

https://doi.org/10.1016/j.ijid.2021.01.068

In Italy, COVID-19 incidence and excess mortality data follow a clear north-south gradient, with southern regions being less affected (Michelozzi et al., 2020).

In this study, we collected the reverse transcription-polymerase chain reaction (RT-PCR) data of the first nasopharyngeal swab of all 944 patients consecutively diagnosed from 3 March to 8 June 2020 in the region of Capitanata, in Apulia, southern Italy. To investigate the viral load pattern in the general population between the different epidemic phases, the cycle threshold (Ct) values of RT-PCR targets were used as a surrogate for absolute viral load.

Methods

From 3 March to 8 June 2020, 944 subjects had SARS-CoV-2 positive tests at the hospital "Riuniti di Foggia". The patients came from the region of Capitanata, an area with approximately 600 000 inhabitants located in North Apulia. Replicate samples from the same patients were excluded since for this study only the diagnostic swab was needed.

COVID-19 testing was by RT-PCR analysis of a nasopharyngeal swab, which was performed by acceding into the nasopharynx from both nostrils with the same swab. The nasopharyngeal samples were collected in tubes with a standard viral transport medium accordingly to WHO guidelines (Organization, 2020). Viral RNA was extracted within 2 hours of sample collection using the STARMag 96×4 Universal Cartridge kit with the Microlab NIMBUS IVD instrument according to the manufacturer's instructions (Seegene Inc. Seoul, Korea). Amplification and detection of target genes N (nucleocapsid protein), E (envelope protein) and RdRP (RNA-dependent RNA-polymerase) was performed using the commercially available kit AllplexTM 2019-nCoV Assay (Seegene Inc. Seoul, Korea) with the CFX96TM instrument (Bio-Rad, Hercules, CA). The assay negative control consisted of RNase-free water added to the master mix before PCR. The assay positive control was constructed using plasmids encoding AllplexTM 2019-nCoV Assay target sequences and was included in each test run. The AllplexTM 2019-nCoV Assay included a full process Internal Control composed of the MS2 phage genome. The test is available as a CE-IVD test for countries accepting the CE-mark. A positive test for SARS-CoV-2 infection was defined as the detection of at least 1 gene target.

Interpretation of results and calculation of Ct values for each target was performed with the Seegene Viewer software (V 3.20). The cut-off Ct values were fixed at 40 for all targets.

The Ct values of each gene target at the time of diagnosis were collected and differences were analysed between time- and age-stratified cohorts. Ct values are used as viral load surrogate markers, Ct value and viral load are inversely correlated as the lower the viral RNA the higher the number of cycles needed for amplification.

Statistical analysis and graphs were elaborated with GraphPad 8. Differences between 2 groups of interest were analysed with the Mann-Whitney U test, while the Kruskal-Wallis test followed by post-hoc analysis (Dunn's test) for multiple comparisons was performed to analyse differences between 3 or more groups. Statistical significance was determined where $P < 0.05$. Ct values for new weekly infections and for different age categories were shown as the median figure with interquartile range (IQR).

Results

Ct values of the 3 molecular targets were analysed by stratifying the data week-by-week according to the time of nasopharyngeal swab collection. The median Ct values of 'N', the most sensitive gene target, significantly increased during the epidemic course, so that patients in the 1st week of March presented a median Ct of 21.7 (25th percentile 3.2; 75th percentile 27.0), while patients diagnosed in the 1st week of June presented a median Ct of 37.2 (25th percentile 33.2; 75th percentile 38.5) (Figure 1).

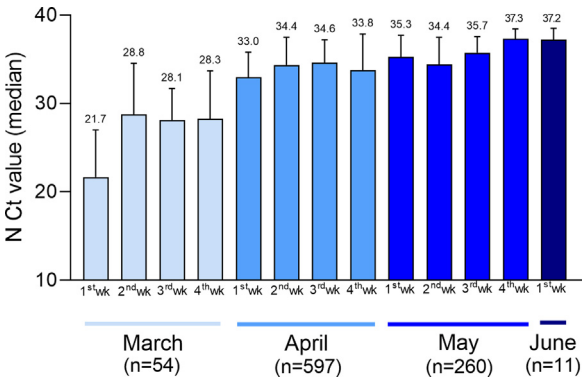

Figure 1. N Ct values increase over time. N target Ct values of all SARS-CoV-2 positive patients stratified week-by-week according to the swab collection time. Data are shown as median with interquartile range.

The increase in median 'N' Ct was constant and progressive (Figure 1). The 'E' and 'RdRP' gene expression also showed an increase in median Ct value, rising, respectively, from 23.5 (25th percentile 18.5; 75th percentile 26.8) in the 1st week of March to 28.6 (25th percentile 25.7; 75th percentile 32.3) in the 1st week of June, and from 25.5 (25th percentile 19.7; 75th percentile 30.3) to 35.3 (25th percentile 31.9; 75th percentile 37.6) (Figure 2A and B). These data indicated a constant decrease of the nasopharyngeal RNA amount of SARS-CoV-2 over time. This trend was more evident for N gene profile than for RdRP and E genes (Figure 1; Figure 2A and B). However, the negative detection rate with RT-PCR for E and RdRP targets was much higher in April compared to March (48.93% vs 8.93% for E; 36.17% vs 7.14% for RdRP) and even higher in May (60.53% for E; 45.49% for RdRP) (Figure 2C). The rate of 2 simultaneous negative targets significantly increased in May (45.49%) compared to March (8.93%) and April (9.50%) (Figure 2D). Therefore, in the latest outbreak phase, several SARS-CoV-2 patients presented low nasopharyngeal RNA quantity with the subsequent detection of only 1 or 2 targets.

To verify whether the Ct change was dependant on age, patients were stratified into 10-year classes; age distribution did not differ across the 3 months of the study (Figure 3).

It is known that elderly patients are particularly susceptible to high viral replication and development of severe COVID-19 (Davies et al., 2020; Liu et al., 2020a,b; Rockx et al., 2020; Shim et al., 2020). The Italian authorities reported that >50% of COVID-19 deaths were of people >80 years (IstitutoSuperiorediSanità). In March and April, patients >80 years showed significantly lower N Ct compared to younger subjects (March: 32.5 [IQR: 27.5, 35.7] in <80y vs 25.5 [IQR: 18.3, 30.5] in >80y, $P < 0.05$; April: 34.7 [IQR: 29.0, 37.4] in <80y vs 29.0 [IQR: 23.9, 34.7] in >80y, $P < 0.0001$) (Figure 4). Both groups of patients showed a significant increase in N Ct over time (<80y: 32.5 [IQR: 27.5, 35.7] in March vs 34.7 [IQR: 29.0, 37.4] in April vs 35.4 [IQR: 32.2, 37.7] in May, $P < 0.0001$; >80y: 25.5 [IQR: 18.3, 30.5] in March vs 29.0 [IQR: 23.9, 34.7] in April vs 35.1 [IQR: 32.8, 37.7] in May, $P < 0.0001$) (Figure 4) and, in May, no difference was observed between <80 and >80 year old patients (35.4 [IQR: 32.2, 37.7] in <80y vs 35.1 [IQR: 32.8, 37.7] in >80y, $P = 1.0$) (Figure 4).

The trend observed in the N gene was confirmed by the analysis of the E and RdRp genes, however, in May, E and RdRp were negative in more than 2/3 of patients (Supplementary Figure 1).

By contrast, the sex of the patient did not seem to impact gene expression.

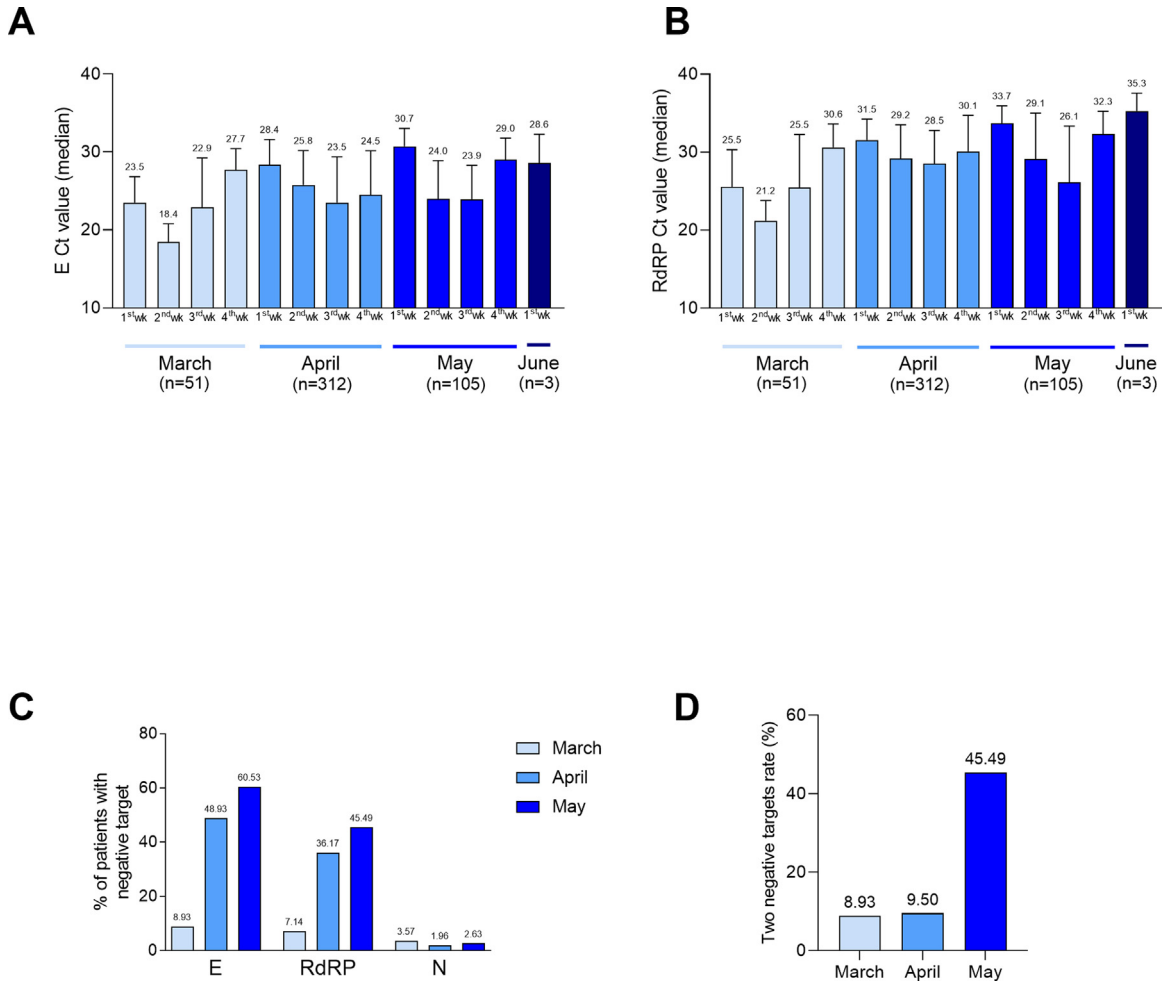

Figure 2. RT-PCR data of E and RdRP targets. (A and B) E and RdRP Ct values of all SARS-CoV-2 positive patients stratified week-by-week according to the swab collection time. Data are shown as median with interquartile range. (C) Rate of patients with negative detection of each target in each month. Data expressed in percentage. (D) Rate of patients with two negative targets in each month. Data expressed in percentage.

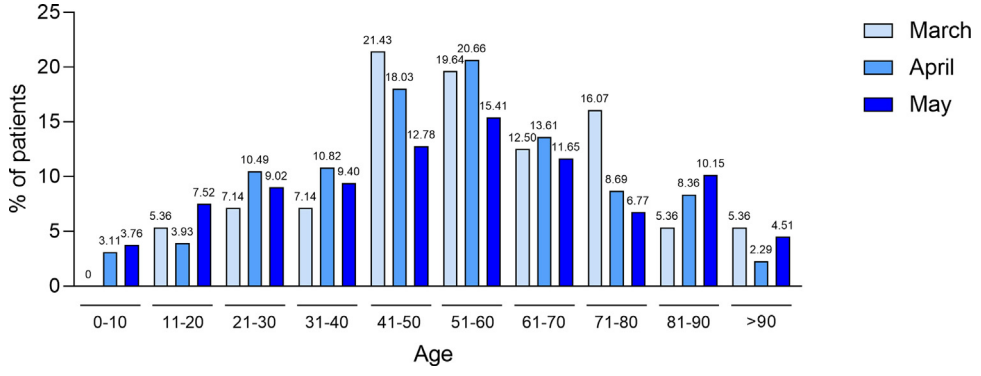

Figure 3. Age distribution. Age distribution (10-year categories) of SARS-CoV-2 positive subjects during 3 months of the epidemic (March, April and May). Data are shown as percentages.

Discussion

The number of new COVID-19 infections decreased in Italy after the adoption of restriction measures. Despite Italy's lockdown in May, the daily number of new COVID-19 cases remained stable at approximately 200 (IstitutoSuperiorediSanità). It is not clear whether the restriction measures and other conditions, such as climate and viral factors, concurred to attenuate SARS-CoV-2 spread and pathogenicity. The potential seasonality of SARS-CoV-2 has been recently described as similar to other seasonal respiratory tract infections and other coronaviruses (Moriyama et al., 2020; Paules et al., 2020; Sajadi et al., 2020). Recent broad genome analysis showed that SARS-CoV-2 is mutating and adapting to the human host (van Dorp et al., 2020), however, the authors were

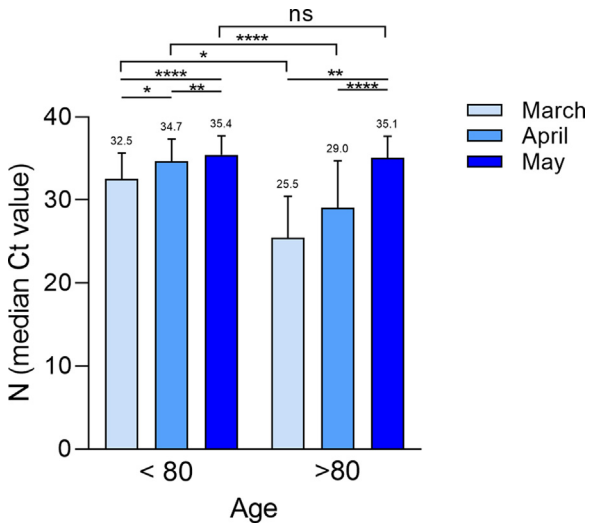

Figure 4. Distribution of Ct values resulting from the amplification of the N target of SARS-Cov-2 at time of diagnosis. Differences of median N Ct values in 2 different age categories (<80 years old, >80 years old), in each month. Data are shown as median with interquartile range. Differences between the 2 age cohorts within a month have been evaluated with the Mann–Whitney *U* test, while differences between the 3 months within 1 age cohort have been evaluated with the Kruskal–Wallis test followed by post-hoc analysis (Dunn's test) for multiple comparison. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

unable to conclude whether these mutations are attenuating virulence or transmissibility. A small study conducted in a Lombardy hospital at the end of the first epidemic phase reported that the viral load was significantly reducing (Clementi et al., 2020), however, results were not transferable due to the small sample size of the study population and the limited period of analysis. Recently, Piubelli et al. showed that COVID-19 disease severity decreased from March to May in parallel with a reduction in viral load, however, the study had a small sample size with single centre enrolment (Piubelli et al., 2020). In this study, we aimed to elucidate the viral spread in a broad population over time and in a different setting (lower incidence and excess mortality). During 3 months (3 March to 8 June 2020) we collected data from 944 RT-PCR-positive nasopharyngeal swab samples obtained from subjects diagnosed with COVID-19 from the region of Capitanata, in Apulia, Italy. Ct values were used as a viral load surrogate measure. Although there are some limitations when the viral load of SARS-CoV-2 is inferred from the RT-PCR Ct value, the method has been widely used in other studies (Clementi et al., 2020; Sethuraman et al., 2020; Shi et al., 2020; Yu et al., 2020; Zheng et al., 2020; Zou et al., 2020).

We observed that viral loads constantly decreased across the study period. This was particularly evident by analysing Ct values of N; when E and RdRP were used the viral load reduction was milder since in many cases they were undetectable. Therefore, in May diagnoses were mostly based only on 1 target, the N gene. We could therefore hypothesize that a viral factor might be involved in the recent mild clinical scenario of our geographical region, although the reason for this remains unclear. Lau S. et al. speculate that the genomic selective pressure on Coronavirus could generate attenuated variants with more efficient inter-human transmission and lower pathogenicity and mortality (Lau et al., 2020); further genome studies are needed to validate this hypothesis.

It is more likely that changes in the diagnostic strategies occurring in the later phases of the pandemic in Italy could have significantly influenced the epidemiological and clinical scenario. The increased diagnostic potential (linked to wider availability of diagnostic assays, reagents, and an increase in the number of active laboratories) has enabled earlier identification of suspected cases and an expansion of screening and tracing activities among mildly affected or asymptomatic subjects.

Furthermore, we cannot exclude that social distancing and PPE usage contributed to reducing the infectious dose, thus decreasing the viral particles shedding (Chan et al., 2020; He et al., 2020; Prather et al., 2020). Similar dynamics regarding influenza virus transmission have shown that the higher the infectious dose given to healthy subjects, the more attenuated the viral replication and clinical manifestations. (Memoli et al., 2015). Several studies report that patients with more severe disease and worse prognosis showed significantly higher viral loads (Liu et al., 2020a,b; Shi et al., 2020; Yu et al., 2020; Zheng et al., 2020). Elderly people are particularly susceptible to high viral replication and worse outcome (Davies et al., 2020; Liu et al., 2020a,b; Rockx et al., 2020; Shim et al., 2020). Italian official data confirm the high mortality rate in older people (IstitutoSuperiorediSanità; MinisterodellaSalute). In the first 2 months of the study, when the daily infections and number of severe cases were high, we observed that elderly people >80 years presented a significantly higher viral load. The median viral load of both age cohorts (<80 years old and >80 years old) significantly decreased month by month, and in May the difference between these 2 cohorts disappeared. We cannot exclude that the current less severe clinical situation in our geographical area is due to the low viral loads observed in the high-risk class of elderly subjects. This implies that maintaining social distancing and PPE, particularly in the older population, may dramatically impact disease severity and mortality.

In conclusion, we believe that monitoring the viral load trend in the general population might provide important information to predict the pandemic course and impact on the healthcare system, particularly when high-risk categories, such as elderly people, are analysed. Molecular studies are needed and should be combined with broad epidemiological data to identify potential modifications in SARS-CoV-2 behaviour.

Authors' contribution

MS contributed to data analysis, data interpretation, and writing of the manuscript; FA contributed to study design, conceptualization, and writing; RDN, RV, and EG contributed to data production and collection; GS designed and supervised the study and drafted the manuscript.

Declaration of interests

We declare no competing interests.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

The study was approved by the Institutional Review Board of the University of Foggia. All patients provided signed informed consent.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2021.01.068.

References

Chan JF, Yuan S, Zhang AJ, Poon VK, Chan CC, Lee AC, et al. Surgical mask partition reduces the risk of non-contact transmission in a golden Syrian hamster model for Coronavirus Disease 2019 (COVID-19). Clin Infect Dis 2020;.

Clementi N, Ferrarese R, Tonelli M, Amato V, Racca S, Locatelli M, et al. Lower nasopharyngeal viral load during the latest phase of COVID-19 pandemic in a Northern Italy University Hospital. Clin Chem Lab Med 2020;.

Davies NG, Klepac P, Liu Y, Prem K, Jit M, group CC-w, et al. Age-dependent effects in the transmission and control of COVID-19 epidemics. Nat Med 2020;.

He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. Nat Med 2020;26(5):672–5.

Huang JT, Ran RX, Lv ZH, Feng LN, Ran CY, Tong YQ, et al. Chronological Changes of Viral Shedding in Adult Inpatients with COVID-19 in Wuhan, China. Clin Infect Dis. 2020;.

IstitutoSuperiorediSanità. https://www.epicentro.iss.it/coronavirus/bollettino/Bollettino-sorveglianza-integrata-COVID-19_14-luglio-2020.pdf.

Lau SY, Wang P, Mok BW, Zhang AJ, Chu H, Lee AC, et al. Attenuated SARS-CoV-2 variants with deletions at the S1/S2 junction. Emerg Microbes Infect 2020;9(1):837–42.

Liu Y, Mao B, Liang S, Yang JW, Lu HW, Chai YH, et al. Association between age and clinical characteristics and outcomes of COVID-19. Eur Respir J 2020a;55(5).

Liu Y, Yan LM, Wan L, Xiang TX, Le A, Liu JM, et al. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis 2020b;20(6):656–7.

Memoli MJ, Czajkowski L, Reed S, Athota R, Bristol T, Proudfoot K, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. Clin Infect Dis 2015;60(5):693–702.

Michelozzi P, de'Donato F, Scortichini M, Pezzotti P, Stafoggia M, De Sario M, et al. Publisher Correction to: temporal dynamics in total excess mortality and COVID-19 deaths in Italian cities. BMC Public Health 2020;20(1):1325.

MinisterodellaSalute. https://www.salute.gov.it/portale/nuovocoronavirus/homeNuovoCoronavirus.jsp?lingua=english.

Moriyama M, Hugentobler WJ, Iwasaki A. Seasonality of respiratory viral infections. Annu Rev Virol 2020;.

Organization WH. Laboratory testing for coronavirus disease (COVID-19) in suspected human cases: interim guidance, 19 March 2020. World Health Organization. License: CC BY-NC-SA 3.0 IGO; 2020.

Paules CI, Marston HD, Fauci AS. Coronavirus infections-more than just the common cold. JAMA 2020;.

Piubelli C, Deiana M, Pomari E, Silva R, Bisoffi Z, Formenti F, et al. Overall decrease of SARS-CoV-2 viral load and reduction of clinical burden: the experience of a Northern Italy hospital. Clin Microbiol Infect 2020;.

Prather KA, Wang CC, Schooley RT. Reducing transmission of SARS-CoV-2. Science 2020;368(6498):1422–4.

Prem K, Liu Y, Russell TW, Kucharski AJ, Eggo RM, Davies N, et al. The effect of control strategies to reduce social mixing on outcomes of the COVID-19 epidemic in Wuhan, China: a modelling study. Lancet Public Health 2020;5(5):e261–70.

Rockx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Oude Munnink BB, et al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. Science 2020;368(6494):1012–5.

Sajadi MM, Habibzadeh P, Vintzileos A, Shokouhi S, Miralles-Wilhelm F, Amoroso A. Temperature humidity, and latitude analysis to estimate potential spread and seasonality of coronavirus disease 2019 (COVID-19). JAMA Netw Open 2020;3(6):e2011834.

Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA 2020;.

Shi F, Wu T, Zhu X, Ge Y, Zeng X, Chi Y, et al. Association of viral load with serum biomakers among COVID-19 cases. Virology 2020;546:122–6.

Shim E, Tariq A, Choi W, Lee Y, Chowell G. Transmission potential and severity of COVID-19 in South Korea. Int J Infect Dis 2020;93:339–44.

To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis 2020;20(5):565–74.

van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. Infect Genet Evol 2020;83:104351.

WHO, https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports.

Yu X, Sun S, Shi Y, Wang H, Zhao R, Sheng J. SARS-CoV-2 viral load in sputum correlates with risk of COVID-19 progression. Crit Care 2020;24(1):170.

Zheng S, Fan J, Yu F, Feng B, Lou B, Zou Q, et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. BMJ 2020;369:m1443.

Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Engl J Med 2020;382(12):1177–9.