Virological characterization of the first two COVID-19 patients diagnosed in Italy: phylogenetic analysis, virus shedding profile from different body sites and antibody response kinetics.

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Summary: The daily monitoring of viral RNA loads and antibody titres of two COVID-19 cases showed that SARS-CoV-2 RNA can be detected in different body samples even with the presence of IgM, IgA and IgG apprearing within the first week of infection.

Abstract

Background: The pathogenesis of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection remains unclear. We report the detection of viral RNA from different anatomical districts and the antibody profile in the first two COVID-19 cases diagnosed in Italy.

Methods: We tested for SARS-CoV-2 RNA clinical samples, either respiratory and non-respiratory (i.e. saliva, serum, urine, vomit, rectal, ocular, cutaneous, and cervico-vaginal swabs), longitudinally collected from both patients throughout the hospitalization. Serological analysis was carried out on serial serum samples to evaluate IgM, IgA, IgG and neutralizing antibodies levels.

Results: SARS-CoV-2 RNA was detected since the early phase of illness lasting over two weeks in both upper and lower respiratory tract samples. Virus isolate was obtained from acute respiratory sample, while no infectious virus was rescued from late respiratory samples with low viral RNA load, collected when serum antibodies had been developed. Several other specimens resulted positive including saliva, vomit, rectal, cutaneous, cervico-vaginal, and ocular swabs. Specific IgM, IgA and IgG were detected within the first week since diagnosis, with IgG appearing earlier and at higher titres. Neutralizing antibodies developed during the second week, reaching high titres 32 days since diagnosis.

Conclusions: Our longitudinally analysis showed that SARS-CoV-2 RNA can be detected in different body samples which may be associated with broad tropism and different spectra of clinical manifestations and modes of transmission. Profiling antibody response and neutralizing activity can assist laboratory diagnosis and surveillance actions.

Key words: SARS-CoV-2; COVID-19; phylogenesis; virus shedding; viral culture; antibody response; Italy.

Introduction

In January 2020, a novel coronavirus was identified as the cause of pneumonia cases, with the first cases reported in December 2019 in Wuhan City, Hubei Province of China [1,2]. The new pathogen belongs to Betacoronaviruses genus lineage B and due to its close phylogenetic relation to other bat severe acute respiratory syndrome (SARS)-like coronaviruses was named SARS coronavirus 2 (SARS-CoV-2). Initially linked to the possible exposure to infected wildlife animals, human-to-human transmission was identified and the outbreak rapidly spread to other parts of China and outside the country [2]. As of 20 August 2020, 22 431 929 COVID-19 cases (corona virus disease 2019, the illness caused by SARS-CoV-2), with 787 773 deaths have been reported worldwide [3].

Transmission is mainly through respiratory droplet, but other routes cannot be excluded and are under investigation as SARS-CoV-2 was detected in several body fluids (i.e. saliva, stool, ocular fluid) [1,4–7]. Much still needs to be learned about this infection, and researches are under way worldwide to better understand the clinical features and the extent of inter-human transmission. A better knowledge of viral RNA shedding kinetics from different body districts could help to understand SARS-CoV-2 transmission and pathogenesis, supporting surveillance and clinical management. In addition, due to the current emergency context, very few data about the antibody response are available in literature.

Here, we report the kinetics of viral RNA shedding from different body sites and the concomitant antibody profile (IgM, IgA, IgG and neutralizing Ig) along the disease course in the first two COVID-19 confirmed cases reported in Italy and hospitalized at the National Institute for Infectious Diseases “Lazzaro Spallanzani” (INMI) in Rome.

Methods

Clinical samples

Clinical samples from the first two COVID-19 patients were longitudinally collected for diagnostic purposes starting from the first day of hospitalization (corresponding to day 1 from symptoms onset (DSO), as declared by the patients at admission) up to DSO 32. These samples included upper (URT, i.e. nasopharyngeal swab, nasal swab, throat swab) and lower (LRT, i.e. sputum and bronchoalveolar lavage, BAL) respiratory tract specimens, and non-respiratory specimens (i.e., saliva, serum, urine, rectal swab, ocular swab, cervico-vaginal swab, cutaneous swab).

Patient Consent Statement

The patients's written consent was obtained. This study was approved by the INMI Ethical Board.

Nucleic acid extraction and molecular tests

Viral RNA was extracted by QIAsymphony (QIAgen, Germany) and Real-time reverse-transcription PCR (RT-PCR) targeting E and RdRp viral genes was used to assess the presence of SARS-CoV-2 RNA [8]. Confirmation of diagnosis was performed by in-house RT-PCR targeting the viral membrane protein (M) gene, followed by Sanger sequencing (327 bp). The follow-up of the infection course was then performed using E gene Real-time RT-PCR only. Other respiratory tract infections were investigated using multiplex nucleic-acid test (QIAstat-Dx® Respiratory Panel, QIAgen, Germany).

Virus isolation

Viral culture was performed in BSL-3 laboratory, clinical samples (i.e. nasopharyngeal swab, sputum, BAL, and ocular swab) were diluted in MEM plus viral inoculating broth (VIB) 1X containing antibiotics. The mixture was kept at room temperature for 1 hour and then inoculated on Vero E6 cells for 1 hour, finally complete medium was replaced with MEM

containing 2% FBS and 0.5X VIB. Cytopathic effect (CPE) appearance was observed by light microscope and Cytation 5 reader, Biotek. First samples collected at diagnosis (nasopharyngeal swabs on both patients and sputum of Pt1) were immediately inoculated into the cell culture for isolation purpose. The follow-up samples (nasopharyngeal swabs, BAL, and ocular secretions) were stored at -80°C and never thawed before inoculation for viral culture, that was performed after three months from samples collection.

Next generation sequencing (NGS) and bioinformatics analysis

NGS was performed using Ion Torrent S5 platform as described in [9]. Reads were de-novo assembled and consensus sequences were manually controlled and confirmed by Sanger Sequencing.

Bayesian phylogenetic tree was inferred using Markov chain Monte Carlo (MCMC) approach in BEAST v1.10.4, with BEAGLE v2.1.2 library program. HKY mutation model was used as nucleotide substitution model and independent MCMC runs for strict evolutionary rate model, along with constant population size coalescent priors, as reported in previous study [10]. Chains were conducted for at least 100 x 106 generations with sampling every 10000 steps and burn-in 10 x 106 generations. The convergence of the MCMC was assessed by calculating for each parameter the ESS (accepted if ESS>250). Maximum clade credibility tree was obtained from the trees' posterior distributions with the Tree-Annotator software v 1.10.4.

Serological tests

Indirect immunofluorescence assay (IFA) was used to detect specific IgM, IgA and IgG on slides prepared in-house with Vero E6 cells infected with SARS-CoV-2 isolate, as described elsewhere [11]. All sera were depleted of IgG using Eurosorb reagent (Euroimmun, Germany) and tested using 1:20 screening dilution with titration by limiting dilution. FITC-

conjugated anti-human IgM, IgA and IgG antibodies (Euroimmun, Germany) were used as secondary antibody and Evans Blue as cell counterstain.

For neutralizing antibodies evaluation, sera were heat-inactivated, diluted 1:10 and titrated in duplicate in two-fold dilutions. Equal volumes of 100 TCID50/well SARS-CoV-2 and serum dilutions were mixed and incubated at 37°C for 30 minutes. Subsequently, 96-wells plates with sub-confluent Vero E6 cells were incubated with 100 µl/well of virus-serum mixtures at 37 °C, 5% CO2. Neutralizing antibody titers were calculated as the highest serum dilution not presenting CPE at day 6 post-infection.

Results

On January 29th, 2020, two spouses, a 66-year-old woman (Patient 1, Pt1) and a 67-year-old man (Patient 2, Pt2) visiting Rome for vacation, were admitted at INMI, as possible COVID-19 cases. Both patients arrived in Italy on January 23rd from Wuhan, Hubei Province, China, and since January 28th presented relevant respiratory symptoms. Pt1 had history of hypertension, whereas Pt2 had no other comorbidities and milder illness at presentation, as recently reported [12].

Diagnosis of SARS-CoV-2 infection was confirmed by Real Time RT-PCR on nasopharyngeal swab and sputum for Pt1 (Ct: 14.28 and 16.12, respectively) and on nasopharyngeal swab for Pt2 (Ct: 24.58), followed by viral M gene sequencing. Nasopharyngeal swabs at admission were negative for all other respiratory pathogens tested. Virus was isolated from Pt1 acute-phase sputum (named 2019-nCoV/Italy-INMI1) **(Fig. 1)**.

Both patients developed progressive respiratory failure on DSO 4, and required mechanical ventilation support in intensive care unit (ICU) on DSO 6 for Pt2 and DSO 7 for Pt1. During their stay in ICU, both patients received 3 days lopinavir/ritonavir therapy, followed by intra-venous administration of remdesivir for 13 days. At the time of writing, both patients were discharged.

Full genome sequences of Pt1 were obtained by NGS from both virus isolate and clinical sample (nasopharyngeal swab). As described in [9], the analysis of consensus sequences from the clinical sample showed two non-synonymous changes with respect to the Wuhan-Hu-1 NCBI Reference Genome (Accession number: MN908947.3) leading to change in Orf1a, and in Orf3a. One additional synonymous substitution in Orf1a (A2269T) was detected in the isolate only [9]. The partial Pt2 sequence was very similar to the sequence of Pt1, and consistent with the full genome sequence of the strain isolated by the national reference center (GISAID accession ID: EPI_ISL_412974) from Pt2 nasopharyngeal swab [13].

Bayesian phylogenetic analysis places Pt1 sequence (referred as INMI1) in the V clade, characterized by G251V substitution in ORF3a gene, according to the NJ tree in the GISAID EpiCov portal. In this analysis, the origin of entire clade V appears to date back to January 14th (95 % HPD: January 5th – January 23rd) (node 2), which is highly consistent with travel history of patients from China. This analysis inferred a mutation frequency of 1.824 * 10-3 (95% HPD: 1.01*10-3 – 2.73*10-3) **(Fig.2)**.

Several body fluids, including non-respiratory specimens, were tested daily for SARS-CoV-2 RNA for 32 DSO. For Pt1, 148 samples, including 54 from the URT and LRT, and 94 from other body sites (i.e. saliva, vomit, serum, ocular swab, urine, rectal swab, cervico-vaginal swab, cutaneous swab) were tested. For Pt2, 119 samples were analysed, including 48 from the URT and LRT and 71 from other body sites (i.e. saliva, serum, ocular swab, urine, rectal swab, cutaneous swab).

Dynamics of viral RNA levels in different specimens are shown in **Fig. 3**. Since DSO 1, for both patients, high viral loads were detected in respiratory samples. Compared to Pt2, Pt1 presented higher viral RNA levels in the URT at admission (difference of ~10 Ct) and along their hospitalization. During the progression of diseases, for both patients, specimens obtained from the LRT (i.e. sputum and BAL) had higher SARS-CoV-2 RNA levels than

those from the URT **(Fig. 3a** and **3d)**. Last positive-testing result from respiratory sample was at DSO 26 for Pt1 (nasopharyngeal swab) and at DSO 17 for Pt2 (BAL). Viral culture was attempted on late follow-up respiratory samples collected at DSO 14 (nasopharyngeal swab, Ct: 27.5; and BAL, Ct: 23.3) and DSO 25 (nasopharyngeal swab, Ct: 34.1) from Pt1, and at DSO 14 (BAL, Ct: 30.3) from Pt2. No replication competent virus was recovered from any of these samples. None of the urine samples from both patients tested positive for SARS-CoV-2 RNA. Serial saliva specimens resulted negative for Pt2, but highly positive for Pt1 with a discontinuous and fluctuant trend of viral loads **(Fig. 3b** and **3e)**. SARS-CoV-2 RNA was detected only in one out of 7 serum samples from Pt1 at DSO 5 (Ct: 35.5), and in none of the sera from Pt2 (not shown). All ocular swabs from Pt2 resulted negative for SARS-CoV-2 **(Fig. 3e)**; on the contrary, viral RNA was detected in sequential ocular swabs collected from Pt1, who presented persistent bilateral conjunctivitis which improved from 15 DSO and resolved at DSO 20. In fact, as we described elsewhere [14], SARS-CoV-2 RNA was detected in Pt1 ocular swabs starting from DSO 3 up to DSO 21 with declining viral RNA levels (Ct values from 21.66 to 36.56, respectively); a relapse was observed after 5 days of negative results, with a new positive result in the ocular swab sample collected at 27 DSO **(Fig. 3b)**. Notably, infectious virus was cultured from the first ocular sample, as detailed elsewere [13]. Rectal swabs resulted positive at DSO 5 (Ct: 30.10), 7 (Ct: 36.31) and 15 (Ct: 36.21) for Pt1 **(Fig. 3b)**, and at DSO 16 (Ct: 35.21) and 17 (Ct: 38.59) for Pt2 **(Fig. 3e)**. The unique vomit specimen collected at DSO 5 from Pt1 tested positive at high RNA load (Ct: 19.49) (not shown). SARS-CoV-2 RNA was detected also in cervico-vaginal swabs (Ct: 32.9 and 37.23) collected from Pt1 at DSO 7 and 20, respectively. Cutaneous swabs collected from the back of Pt2 resulted positive for SARS-CoV-2 at DSO 5 (Ct: 35,77), and negative at DSO 18; all cutaneous swabs available for Pt1 (at DSO 5 and 6) were negative (data not shown).

Kinetics of specific IgG, IgM and IgA response was evaluated on serial serum samples in a timeframe between DSO 1 and 32 **(Fig. 3c and 3f)**. For both patients, IgG were detected earlier and at higher titers than IgM and IgA, starting since DSO 6 for Pt1 and DSO 3 for Pt2. The titer of all antibody classes steadily increased since the second week of illness, mirroring an inverse trend toward decreasing levels of viral RNA in respiratory tract samples. Within the timeframe considered for the serological investigation, a four-fold increase in IgM titers, six-fold increase in IgA and eight-fold increase in IgG was observed in Pt1; for Pt2, a four-fold increase in IgM and IgA titers, and nine-fold increase in IgG titers was found. Both patients developed neutralizing antibodies, which were first detected at DSO 10 for Pt2 and DSO 17 for Pt1. The neutralization titer steadily increased in both patients, reaching 1:320 in Pt1 and 1:80 in Pt2 at 32 DSO. As shown in Fig. 3, an earlier and more robust seroconversion occurred in Pt2 in comparison to Pt1; inversely, along the entire disease course, the virus RNA levels in virtually all body sites were lower in Pt2 as compared to Pt1.

Conclusions

Here, we report virological and serological characterization of the first two COVID-19 cases diagnosed in Italy during the 2020 pandemic. The two patients travelled from Whuan, China, to Italy on January 23rd, developed symptoms in Rome on January 28th and were hospitalized the following day. A detailed description of the clinical presentation has been published [12].

The patients harbored the same virus strain, clustering with clade V, characterized by a non-synonymous mutation in ORF3a gene (G251V), according to GISAID EpiCov portal [9,13]. Bayesian phylogenetic analysis is consistent with the plausible date of exposure that presumably occurred in China before the travel start.

Sequential sampling in a wide range of body fluids was performed to monitor viral dissemination and shedding profile as well as antibodies kinetics throughout the illness.

SARS-CoV-2 RNA was detected both in URT and LRT samples since initial phases of disease. Similarly to what was observed in MERS and SARS patients, our analysis showed that LRT samples presented higher levels of SARS-CoV-2 RNA than those in paired samples form the URT [15–17]. The results are consistent with the expression of the candidate SARS-CoV-2 cell entry receptor, human angiotensin-converting enzyme 2 (ACE2), which is found primarily in the LRT [18,19].

Nevertheless, the presence of high levels viral RNA in the URT samples during the early phase of illness, coupled with the isolation of infectious virus obtained by others groups [20] and by us on different patients (authors' unpublished data), strongly suggest an high potential for SARS-CoV-2 transmission [20,21]. Duration of viral shedding in respiratory samples was 26 DSO for Pt1 and 17 for Pt2, in line with observation reporting 20 days as median shedding duration for survivors [22, 23]. Live virus was isolated from the respiratory samples collected from at presentation the two patients [13]. In line with previous reports [20, 23], despite numerous attempts in Vero E6 cell culture, no replication competent virus was recovered from later respiratory samples, when antibodies were detected. Several factors, among which suboptimal sensitivity of the virus culture system especially for low viral load samples and storage at -80°C, as well as the presence of antibodies against SARS-CoV-2, may have contributed to unsuccessful virus culture attempts. However, the difficulties in SARS-CoV-2 isolation from late samples support the idea that despite the long duration of viral RNA shedding, the transmission of the infection is likely limited to the early of infection, when viral load is high and antibody response has not yet been developed [23]. Shorter duration of viral shedding was observed for non-respiratory samples, which showed lower viral loads since the early phase of illness and a more discontinuous trend.

Among the non-respiratory samples, saliva resulted positive from the early to the late phase of disease (up to DSO 26) for Pt1, supporting the idea of transmission via saliva droplets [4].

Stool represents another specimen of clinical and epidemiological interest, and stool samples testing for follow-up monitoring and patients discharge has been suggested. In fact, the presence of SARS-CoV-2 RNA in faecal samples was reported even after viral clearance from the respiratory tract [5,24], and our results on rectal swabs partially support these literature data, as fluctuant positivity was found during illness. However, to our knowledgethere have been no reports of faecal−oral transmission yet, and this issue is still under debate, although several authors provided data suggesting that, at least in some cases, gastrointestinal tract may harbor SARS-CoV-2 replication [25-26]. SARS-CoV-2 presence in blood is still controversial: some reports on COVID-19 patients found no viral RNA, in other studies occasional (10%) positivity was reported, possibly associated with severe manifestations [27-31]. In this study, only one serum sample from Pt1 tested positive for SARS-CoV-2 RNA with high Real Time RT-PCR Ct values; this was collected at DSO 5 that corresponded to the worsening of clinical picture and ICU admission. However, the detection of low level viral genome fragments in blood is not to be taken as definitive evidence of bloodstream dissemination of the virus, from our and other existing data it seems that blood does not play a major role in virus transmission [23, 28, 32].

We did not find viral RNA in urine samples of both patients. However, urinary shedding of viral RNA has been occasionally reported with evidence of renal tropism [28, 31-33], and infectious virus was cultured from urine of a COVID-19 case in China [34]. The limited number of patients included in our study may account for the apparent discrepancy and does not allow to establish a definitive role of urinary shedding in viral diagnosis. Attention on possible involvement of conjunctiva, either as site of virus entry and source of contagion, has been claimed [35, 36]. Ocular samples collected from Pt1 (who presented conjunctivitis at admission and up to DSO 20) resulted positive for SARS-CoV-2 RNA from very early phase of infection up to DSO 27. Surprisingly, as we described elsewhere [14], infectious virus was

cultured from the first acute ocular sample, supporting the evidence of persistent sustained viral replication in conjunctiva and viral shedding from this site [14]. No virus detection was instead observed for Pt2, who did not present any ocular symptoms. These findings indicate that contact with conjunctival secretion from COVID-19 patients with ocular symptoms may represent a potential risk of infection, therefore eye protection represents an important measures to prevent virus transmission especially in health-care settings. SARS-CoV-2 RNA was recovered from several additional non-respiratory samples. Although with low viral RNA levels we found positive cutaneous swab from Pt2, and vomit samples and cervico-vaginal swabs in Pt1. These findings are unique so far [37], and need to be confirmed in further studies in order to define the transmission potential linked to this wide RNA shedding. To date, knowledge on the antibody response during SARS-CoV-2 infection is still limited. We monitored kinetics of IgM, IgA, IgG and neutralizing antibodies in the two patients using IFA based on whole-virus on serial samples collected during the hospitalization. In line with other reports on COVID-19 cases, we observed seroconversion for all antibody classes within the first week since diagnosis which corresponds to the date of symptoms onset based on the recorded anamnestic data provided by the patients [31, 38, 39]. Surprisingly, in both patients IgG were detected already at 3 (Pt1) and 6 (Pt2) DSO at high titres, when IgM and IgA were still low or undetectable [40,41]. We cannot exclude the occurrence of a pauci-symptomatic phase that may have prolonged the effective time lapse from the initial infection and IgG appearance. The early appearance of high titer IgG in contrast to IgM could be also due to an anamnestic response to past infection with other endemic coronaviruses, as reported [20, 42]. Increasing antibodies levels were observed during the second week, with high titres of IgG and IgA. In addition, according with earlier findings, both patients developed neutralizing antibodies during the second week of illness, reaching high levels at DSO 32 [31]. IgA are predominantly present in mucosal tissues, including the URT, providing the first line of

defense in mucosal immunity. As shown in this study and others published, detecting seroconversion of IgA as well as IgG and IgM, can be useful to fully evaluate the humoral response in COVID-19 cases [38, 39].

Although our study examined two patients which represents a limitation of the present results, the description of virus dynamics based on daily monitoring of both virological and serological aspects during the course of disease can give important insight into the pathogenesis and host response. Overall, the results show that, on one side, SARS-CoV-2 shedding and its duration may involve sevaral body sites associated on different spectrum of clinical manifestation. Further studies are needed for a better understanding of this aspect that is important to inform clinical management and public health decision making. On the other side, the detection and profile of specific antibodies can assist diagnosis, provide valuable information for screening of suspect cases (including in subclinical cases) and evaluate the disease course. Furthermore, the evaluation of antibody response will be crucial for surveillance and epidemiological studies of this novel disease and may be informative in vaccine development for SARS-CoV-2. Further investigation should clarify the level and duration of protection following the infection.

Conflict of interest

The authors declare that no conflicting financial interests or other competing relationships exist.

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Figure 1.

SARS-CoV-2 isolation in cell culture. Mock-infected Vero E6 cells (left) and cells inoculated with sputum from Pt1 (right) observed after 24 hours post-seed. Magnification insets (100X) of selected regions are shown. Virus-induced cytopathic effect is evident in inoculated Vero E6 cells; Real-time RT-PCR test on spent cell growth medium confirmed SARS-CoV-2 replication (inoculum Ct value= 16.73 vs 24 hours post-inoculum Ct value=8.15). Images captured by Cytation 5, Biotek.

Figure 2.

Estimated Bayesian maximum-clade-credibility tree of SARS-CoV-2 whole genomes sequences. Red dots correspond to nodes with >85% posterior probability. The INMI-1 Pt1sequence is highlighted in red. The nodes leading to the INMI-1 sequence segregation are shown in red. Chains were conducted for at least $100x10^6$ generations with sampling every 10000 steps and burn-in $10x10^6$ generations. The convergence of the MCMC was assessed by calculating for each parameter the ESS (accepted if ESS>250). Maximum clade credibility tree was obtained from the trees' posterior distributions with the Tree-Annotator software v 1.10.4.

Figure 3.

Kinetics of SARS-CoV-2 RNA in different clinical samples and of antibody response in the first two COVID-19 patients diagnosed in Italy. Viral RNA levels detected in respiratory tract secretions **(a)** and in non-respiratory tract samples **(b)**, and the antibody titers **(c)**. Pt1 is shown on the left; pt2 is shown on the right. Antibody titers for IgM, IgG, IgA and neutralizing antibodies (NT Ab) are expressed as reciprocal of serum dilution and are shown in log2 scale; viral RNA levels are expressed as cycle threshold values (Ct) of E gene amplification. Dashed-lines represent the limits of detection of IFA (1:20, in **c** and **f**) and of Real Time RT-PCR (Ct: 45, in **a**, **b**, **d** and **e**).

Figure 1

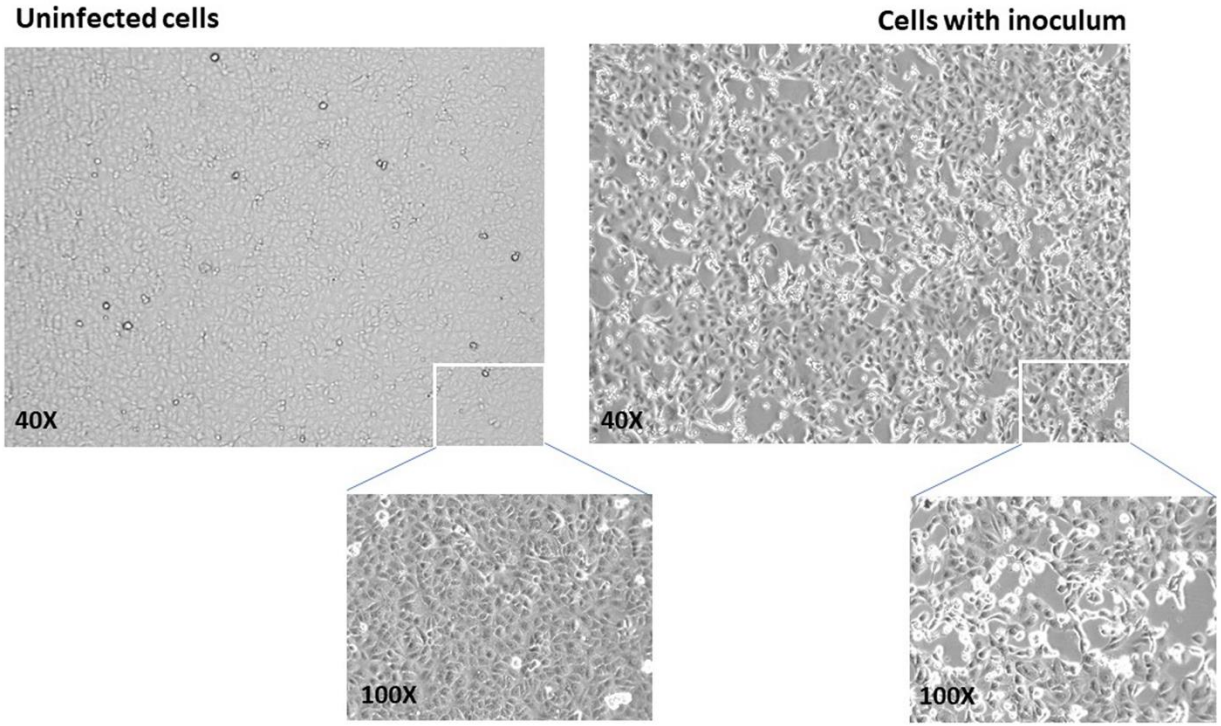

Figure 2

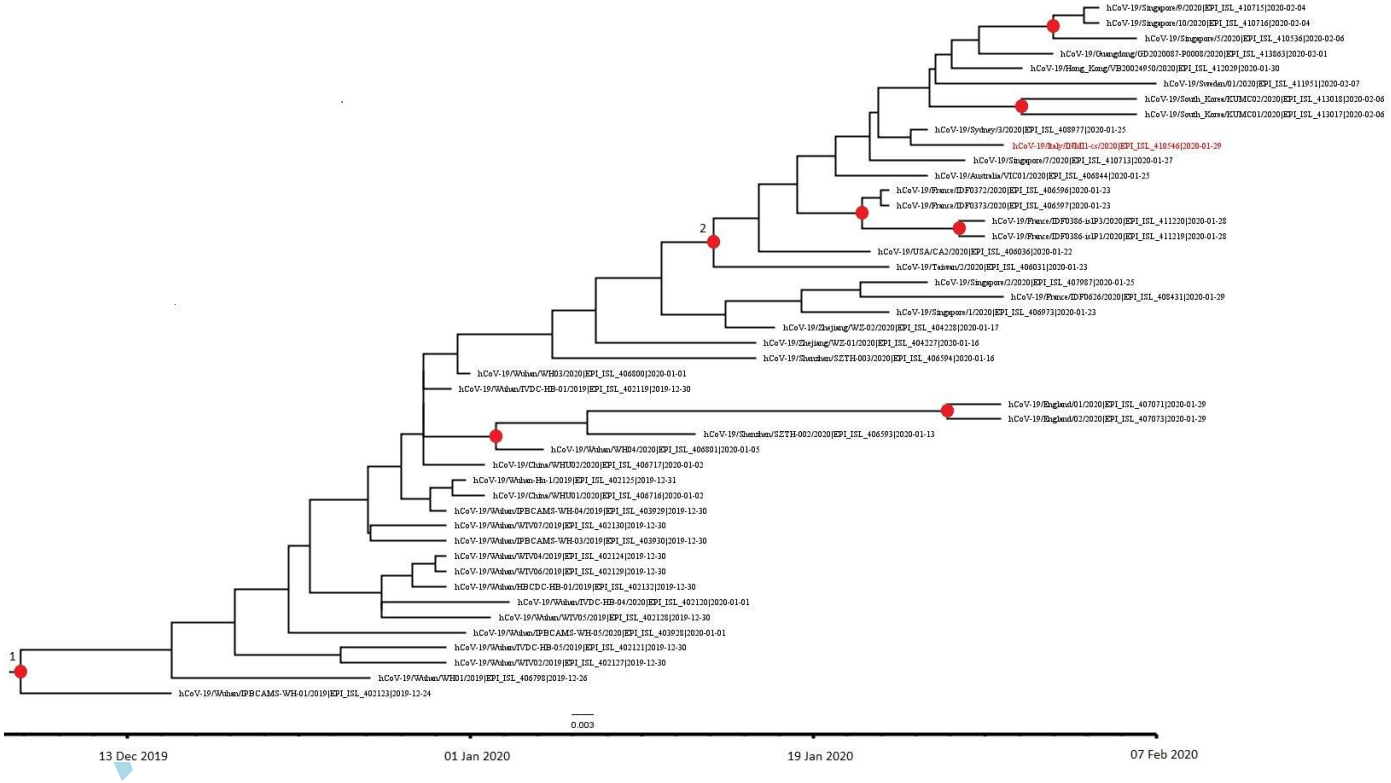

Figure 3