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Determination of SARS-CoV-2 RNA in different particulate matter size fractions of outdoor air samples in Madrid during the lockdown

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ABSTRACT

Background: Previous studies described the presence of SARS-CoV-2 in outdoor air particulate matter (PM) in urban areas of northern Italy and USA. The city of Madrid was heavily affected by COVID-19 during March–June 2020. Also, this city usually displays high concentrations of PM under several atmospheric situations. This is mandatory to assess the presence of viral RNA in PM, as an indicator of epidemic recurrence. Our study was aimed at investigating the presence of SARS-CoV-2 RNA in outdoor air samples (on PM10, PM2.5 and PM1).
Methods: Six samples of PM10, PM2.5 and PM1 were collected between the May 4th and 22nd 2020 in Madrid, on quartz fiber filters by using MCV high volume samplers (30 m^3 h^{-1} flow) with three inlets (Digitel DHA-80) for sampling PM10, PM2.5 and PM1. RNA extraction and amplification was performed according to the protocol recently set by Setti et al.2020c in Italy. Up to three highly specific molecular marker genes (N1, N2, and RP) were used to test the presence of SARS-CoV-2 RNA.
Results: After RNA extraction and expression measurements of N1, N2 and RP genes from all the collected filters, no presence of SARS-CoV-2 RNA was observed. Control tests to exclude false positive results were successfully accomplished.
Conclusions: No presence of SARS-CoV-2 in quartz fiber filters samplers for PM10, PM2.5 and PM1 fractions was observed in our study carried out in Madrid during the month of May 2020. Nevertheless, the absence of viral genomes could be due to different factors including: limited social interactions and economic activities resulting in reduced circulation of the coronavirus, lower daily PM concentration in outdoor air, as well as to meteorological stability and higher temperature that characterize spring season. Further research should be carried out during winter, in presence of higher viral circulation and daily PM exceedances.

Funding

1. Introduction

On December 31, 2019, the World Health Organization was informed of a disease (COVID-19) caused by the SARS-CoV-2 virus in China, which has spread from country to country, becoming a global pandemic (Lu et al., 2020) during the year 2020.

As a viral respiratory infectious disease, the possible ways of transmission between humans are described through direct (person-to-person) and indirect (via fomites) contact (Asadi et al., 2020; Morawska and Cao, 2020). Setti et al. (2020d) hypothesised that the SARS-CoV-2 virus could be present in particulate matter (PM), with the airway being the third transmission mechanism (World Health Organization, 2020). It seems that high levels of urban air pollution, weather and specific climate conditions have a significant impact on the increased rates of confirmed COVID-19 total number, daily new and total death cases, possibly attributed not only to indoor but also to outdoor airborne bioaerosols distribution (Zoran et al., 2020).

In the case of other viruses (Ma et al., 2017; Sedlmaier et al., 2009; Srensen et al., 2000; Zhao et al., 2019), it has been shown that

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particulate matter can act as a 'carrier' for viral droplet nuclei, causing further spread of viral infection. Qing et al. (2016) showed that RSV (respiratory syncytial virus) infection was favoured by particle transport after a positive correlation between infection rate and PM2.5 (particles with aerodynamic diameter < 2.5 μm) and PM10 (particles with aerodynamic diameter < 10 μm) particle fractions. On the other hand, Cheng et al. (2020) showed that an increase in PM2.5 equal to 10 μg/m^3 was significantly associated with a higher incidence of measles in 21 Chinese cities. Also, Peng et al. (2020) showed that high levels of PM concentrations significantly affected the spread of measles in Lanzhou, China.

Previous studies considered an important role of airborne transmission causing the abnormal outbreaks of COVID-19 observed in northern Italy and the United States (Setti et al., 2020a). The Harvard Wu School of Public Health and others (Wu et al., 2020), have suggested a strong association in the United States between the increase in PM concentration and mortality rates from COVID-19. The Italian Society of Environmental Medicine (SIMA) described a possible relationship between the high mortality rates observed in northern Italy due to COVID-19 and PM concentrations (Setti et al., 2020b). A significant correlation was also found between the geographical distribution of daily PM10 exceedances in 110 Italian provinces (number of days

exceeding the PM10 limit value of 50 μg/m^3) and the spread of COVID-19 infection before the lockdown imposed by the Italian Government.

The presence of SARS-CoV-2 RNA in 34 PM10 samples in the ambient air of an industrial site in the province of Bergamo, which in March 2020 was the epicentre of the Italian epidemic, seems to confirm (at least in the case of atmospheric stability and high PM concentrations) that the virus can create clusters with the particles and be transported and detected in PM10 samples (Setti et al., 2020c, 2020d).

Spain has been a very affected country since the beginning of the pandemic. In the study period, from the 4th to the 22nd of May, 2020, were reported 250,287 confirmed cases of COVID-19. Its capital, Madrid, accounted for 28.5% of the total cases in Spain (ISCIII, 2020). However, a daily decrease in the number of confirmed cases and the number of deaths was observed in Madrid in that same period (Fig. 1) (Dirección General de Salud Pública y Sanidad Mortuoria. Comunidad de Madrid).

The aim of this study was to determine the presence of SARS-CoV-2 RNA in outdoor air samples (PM10, PM2.5 and PM1) taken in May 2020, as a possible indicator of COVID-19 diffusion in Madrid and assessing the hypothesis of air transmission due to the high number of observed

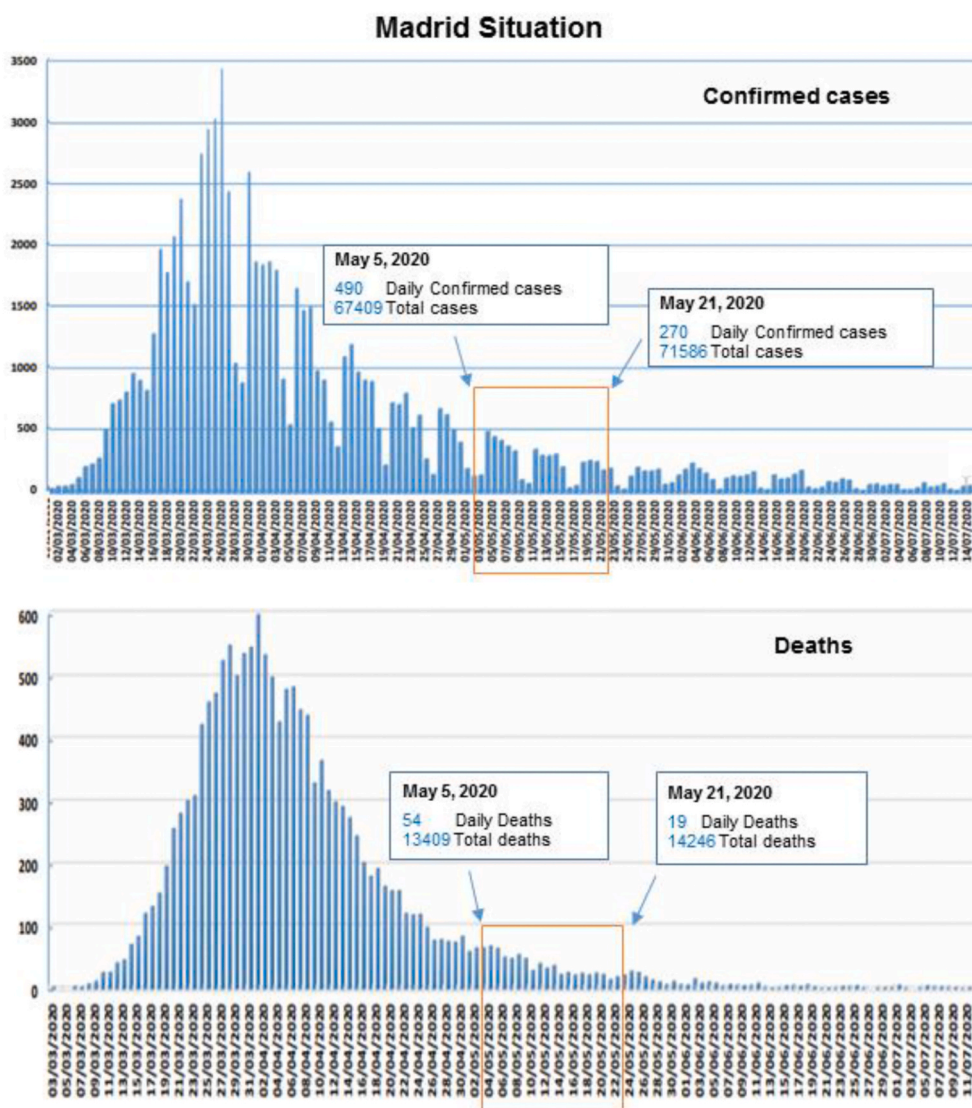
Fig. 1. Graphic representation of the evolution of confirmed cases per day and the daily number of deaths by COVID-19 in Madrid. It also shows the cumulative absolute number of confirmed cases and deaths in the study period. Modified from (Dirección General de Salud Pública y Sanidad Mortuoria, 2020). Comunidad de Madrid. https://www.comunidad.madrid/servicios/salud/2019-nuevo-coronavirus.

daily cases.

2. Materials and methods

Sampling of PM10, PM2.5 and PM1 (particles with aerodynamic diameter < 1 μm) particle matter in the ambient air was simultaneously performed during the study period at the CIEMAT research center facilities located in the UCM University area (coordinates: 40.456417 N,-3.7257242 W), belonging to the district 09 of Madrid capital, called, Moncloa-Aravaca. At this site, gaseous pollutants and PM concentrations and other properties are continuously monitored. Real time particle monitors TEOM 1405DF (™Tapered Element Oscillating Microbalance) and GRIMM™ 1107, validated against the gravimetric reference method, recorded PM10 and PM2.5 and PM1 mass concentration, respectively. Meteorological parameters were also recorded at the same site. Three MCV high volume ($30 \, m^3 \, h^{-1}$ flow) samplers were collocated with different inlets (Digitel DHA-80) for sampling the PM10, PM2.5 and PM1 specific size fractions. These instruments are considered equivalent to the reference samplers for PM10 and PM2.5 particles according to Annex B of BS EN 12341:2014 standard (BS EN 12341: 2014 standard) in Europe (Commission Directive (EU) 2015/1480). The sampling activities were planned in the frame of an international scientific collaboration (RESCOP - Research Group on COVID-19 and Particulate Matter) involving different European cities (Madrid, Barcelona, Milan, Bergamo, Bruxelles, Naples, London).

Sampling dates and periods are shown in Table 1. Sampling protocol was made according to standard for PM measurement in ambient air with special ad-hoc features. Before starting with the sampling process, Whatman Quartz fiber filters (150 mm diameter and QMA quality) were baked at 450 °C for 24 h. Filters were then placed in the inlet directly from the oven. Finally, six samples of each PM10, PM2.5 and PM1, were collected from the 4th to the 22nd of May, 2020. After sampling, filters were, directly from the inlet, ultrafrozen with liquid nitrogen and dry ice in Falcon tubes to be transported to the laboratory for analysis (Department of Biochemistry and Molecular Biology, UCM). Field blank filters were collected and analyzed with each batch of samples in order to establish the artifacts due to adsorption of constituents into quartz filters during and after sampling. The described procedure was used for the blank filters, which were placed in the inlets, but no sampling was performed in these cases.

Inlets were dismounted and cleaned with 70% ethanol before each

sampling, taking care of specific cleaning of the inlet holes and pins. Personnel involved in the sampling were wearing personal protective equipment (PPE): robes, gloves and masks, during sampling.

In the laboratory, RNA was extracted following the protocol described by Setti et al. (2020c). Given the 'environmental' nature of the sample, presumably rich in DNA and RNA polymerase inhibitors, we proceeded to extract RNA using the rapid RNA fecal soil microbe kit adapted to the type of filters (Zymoresearch Ldt, 2020 Cat #R2040).

Half filter was rolled, with the top side facing inward, in a 5 ml polypropylene tube, together with the beads provided in the kit. From the initial 1 ml of lysis buffer, we were able to get about 400 μl of solution, which was then processed as defined by the standard protocols, resulting in a final eluate of 15 μl. Subsequently, 6 μl were used for the SARS-CoV-2 testing using Efficient 2019-nCOV detection kit, one step RT-qPCR (AnyGenes, Paris Cat·#19nCoVd-100) and following the manufacturer's instructions for our amplification system (7500 Fast, Applied Biosystems). The probes and primers included in the detection kit specifically recognize two regions of the COVID-19 nucleocapsid (N1 and N2 genes) and also control human RNase P (RP gene) (species control, recommended by CDC, Centers for disease control and prevention). It also includes a positive control CTR-POS for quality control and a second positive control (CTR-HSC), as a control of RNA purification. Therefore, up to three highly specific molecular marker genes (N1, N2, and RP) were used to test the presence of SARS-CoV-2 RNA on particulate matter.

3. Results

Meteorological conditions in Madrid during the 2-months-period (April–May 2020) were analyzed from the station located at the sampling site in the CIEMAT facilities. Table 1 shows mean values of these concentrations and meteorological parameters during the samplings.

The month of April, was in general rainy and windy, typical of the Madrid spring weather, normally affected by the frequent pass of frontal systems. There were some precipitation events and relative humidity was high during this period, whereas temperature was in the range of the normal climatological values (Fig. 2 a). This atmospheric situation produced a natural ventilation and local cleaning of the atmospheric pollution during this month and therefore ambient concentrations of pollutants, both gaseous and particulate (PM10, PM2.5 and PM1) (Fig. 2 b) were low.

Table 1
Mean PM10, PM2.5 and PM1 concentrations and meteorological parameters during the sampling periods.

| Sample start time (UTC)	Samble end time (UTC)	Sampled hours	Particle Fraction PM10[a] μg/m³	PM2.5[a] μg/m³	PM1[b] μg/m³	Temp. °C	Rel. Hum. %	Wind Direction °	Vector	Speed m/s	Precip. mm	Atm. Press hPa	Sol. Rad. W/m²
04/05/2020 16:30	05/05/2020 10:00	17:30	17.3	9.0	4.0	18.5	52.9	222	SW	4.6	0.0	936.8	122.1
05/05/2020 12:30	06/05/2020 09:00	20:30	12.3	7.4	2.0	16.1	37.2	310	NW	1.9	0.0	938.9	193.0
07/05/2020 11:30	08/05/2020 10:30	23:00	21.1	10.0	3.7	20.6	45.2	69	ENE	4.3	0.0	937.8	194.4
19/05/2020 09:00	20/05/2020 08:00	23:00	11.5	10.1	2.6	21.8	41.7	157	SSE	1.2	0.0	940.2	302.4
20/05/2020 10:01	21/05/2020 08:00	21:59	13.5	10.5	3.1	22.6	31.8	237	WSW	1.0	0.0	939.7	290.6
21/05/2020 10:01	22/05/2020 10:00	23:59	18.0	16.1	8.0	23.7	39.5	306	NW	1.1	0.0	943.2	312.6

[a] TEOM 1405 DF.
[b] Grimm 1107.
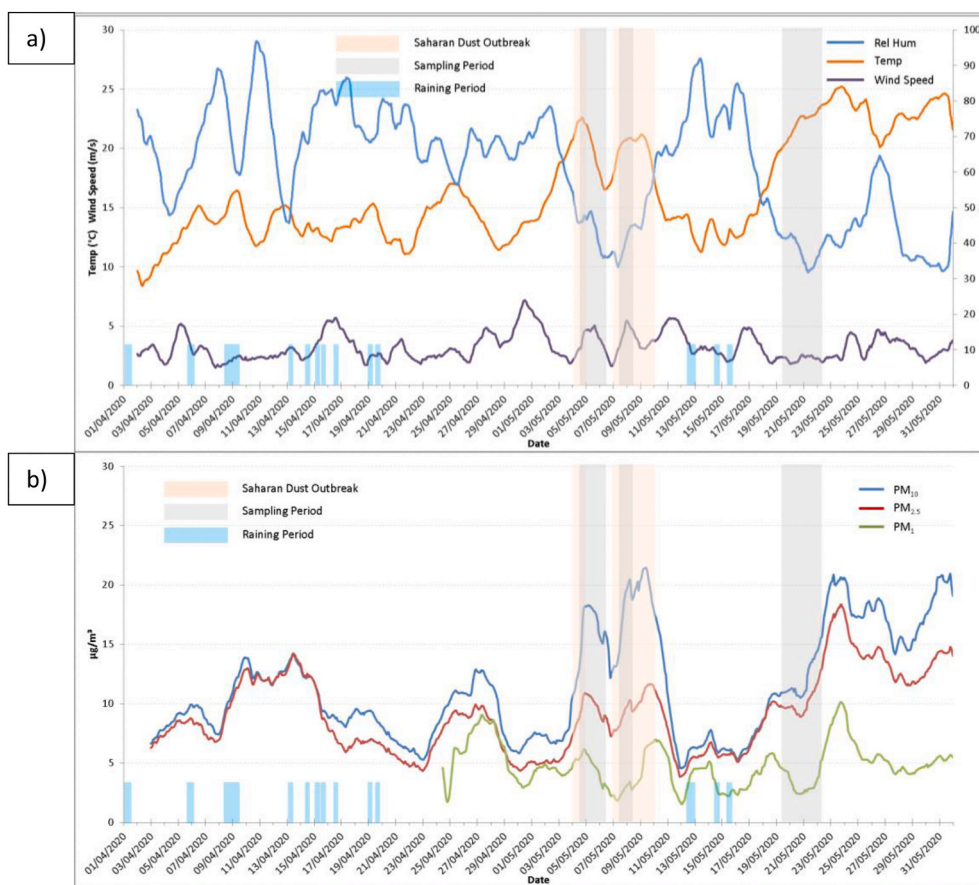
Fig. 2. a) Meteorology (Temperature, Precipitation, Wind speed, relative humidity) and b) particulate matter (PM10, PM2.5 and PM1) concentrations during April and May in Madrid. Natural processes like Saharan dust outbreaks and sampling periods are shown.

On the other hand, in May, two events of high particulate matter concentration associated to Saharan dust outbreaks, affected the Madrid area. This is a common and well documented phenomenon that, under specific synoptic patterns, contributes to increase the ambient levels of particulate matter in Spain, including the Madrid area (Salvador et al, 2013, 2014). These two events took place on the 4th of May and during the period between the 7th and the 9th of May. Consequently, the coarse fraction of particulate matter (PM10) concentrations experienced a visible increase during these periods (Fig. 2 b), although the daily PM mean values were in general not as high as in other times of the year in the Madrid metropolitan area.

At the sampling site, located in the Moncloa-Aravaca district, a reduction in the cumulative incidence rate was observed as shown in Fig. 3 (Red de Vigilancia Epidemiológica de la Comunidad de Madrid. Dirección General de Salud Pública. Consejería de Sanidad. Comunidad de Madrid). The map presented an interval between 51 and 100 confirmed cases by COVID-19 per 100,000 inhabitants in early May (Fig. 3 a). While at the end of the month, the incidence rate was reduced by half, with the number of confirmed cases falling from 26 to 50 per 100,000 inhabitants in this district (Fig. 3 b).

Regarding the molecular analysis of the PM10, PM2.5 and PM1 filters, we first carried out the control for the quality controls. The expected results for the quality controls are shown in the following Table 2, according manufacturer's instructions.

In our study we observed the following results for quality controls, shown in Table 3 where all Ct meet the manufacturer's criteria. N1 and N2 are specific to regions of the virus nucleocapsid (N) gene (two different sites) and human RNase P gene (RP) as control, according to CDC recommendations (Centers for Disease Control and Prevention).

Confronting the results of the controls with those expected, we could

affirm that the experiment went well and that the technique was carried out correctly.

Regarding the results of the samples, interpretation following the manufacturer's instructions is shown in Table 4.

It should be noted that Table 4 refers to respiratory samples in which the RP must always be present in large quantities (Ct < 35), while in our samples, being environmental samples, that value can be Ct > 35.

The results obtained from the 18 filters of PM10, PM2.5 and PM1 were negative in all cases (Table 5).

4. Discussion

Lombardy and the northern regions of Italy have been very affected by COVID-19 due to the rapid spread of the virus (Distante et al., 2020). These were some of the most contaminated areas in Europe at the beginning of the pandemic. In addition, these areas experience high concentrations of particles (PM10 and PM2.5) with frequent severe pollution episodes which, in 2018, exceeded the PM10 daily limit value and the annual PM2.5 limit value (EEA, 2020). Setti et al. showed that in Bergamo, between February 21st and March 13th, 2020, it was possible to confirm the presence of SARS-CoV-2 viral RNA in 8 of the 34 ambient air PM10 filters studied for the three relevant genes (Setti et al., 2020c). They collected the PM10 filters before the Italian government declared total confinement to its population. The filters were then stored for at least four weeks before the molecular genetic analysis (Setti et al., 2020c).

A similar situation occurred in Madrid, where high concentrations of PM particles exceeded for several days the PM10 daily limit value of 50 μg/m³ before the pandemic lockdown (even if, in contrast with the north of Italy, the limit values were never exceeded during 2018) and where a
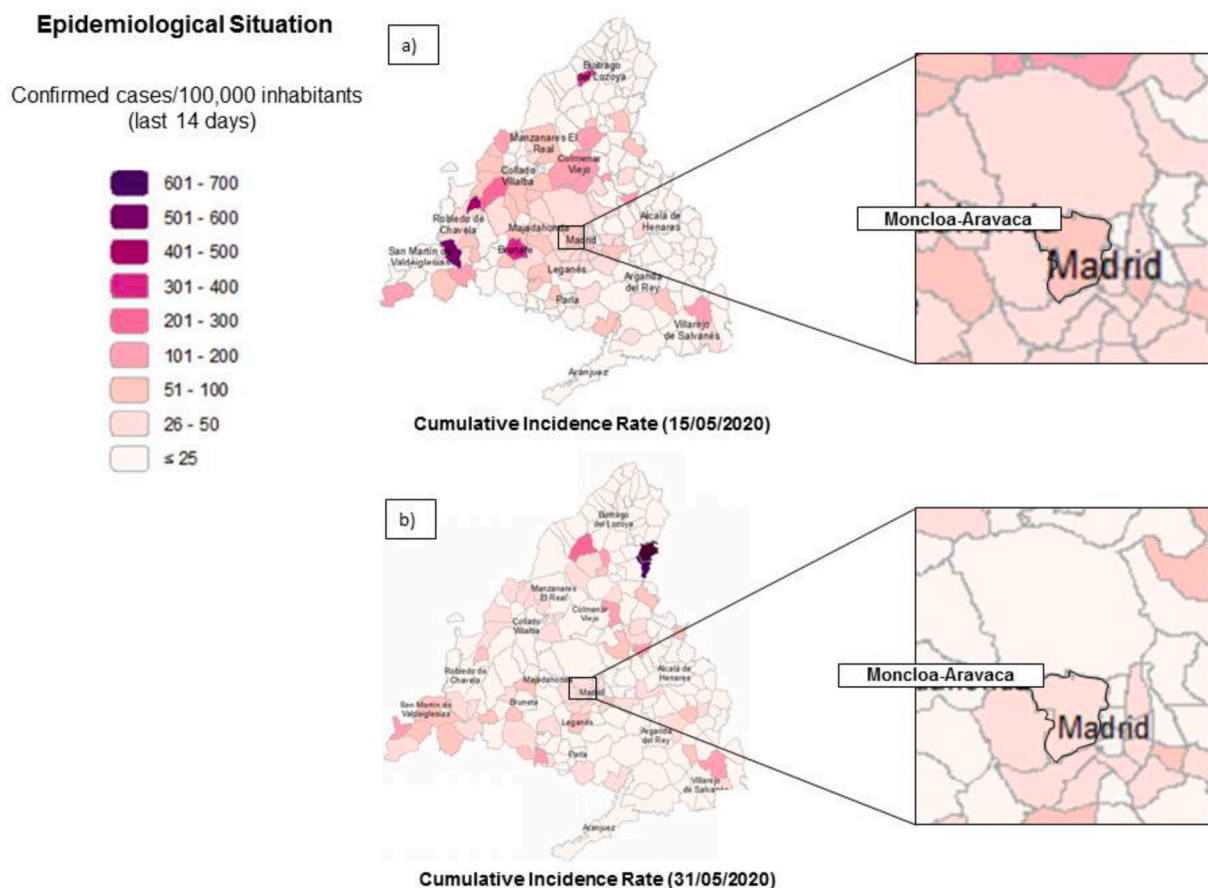
Fig. 3. Map of the accumulated incidence rate in the last fourteen days in Madrid, highlighting the study district Moncloa-Aravaca a) number of confirmed cases per 100,000 inhabitants on 15/05/2020 and b) incidence rate on 31/05/2020. Modified from (Red de Vigilancia Epidemiológica de la Comunidad de Madrid, 2021). Consejería de Sanidad. Comunidad de Madrid. https://www.comunidad.madrid/servicios/salud/2019-nuevo-coronavirus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Expected results for Quality Controls.

Quality control	Control of	Expected N1 result	Expected N2 result	Expected RP result
Positive control (CTR POS)	RT-qPCR efficiency & primers/probe integrity	Cq < 35	Cq < 35	Cq < 35
Human Specimen Control (CTR HSC)	RNA isolation & potential contamination	Cq > 35	Cq > 35	Cq < 35
Negative Control (CTR NEG)	RT-qPCR mix or reagent contamination	Cq > 35	Cq > 35	Cq > 35

Table 3
Our results for controls.

Quality control	N1 result	N2 result	RP result
Positive control (CTR POS)	26.95	32.97	22.73
Human Specimen Control (CTR HSC)	Undet	Undet	18.99
Negative Control (CTR NEG)	Undet	Undet	Undet

Table 4
Results interpretation (only possible if the results with quality controls are optimal).

N1 result	N2 result	RP result	Results interpretation
Cq < 35	Cq < 35	Cq < 35 or Cq > 35	2019-nCov amplification - > positive sample
Cq > 35	Cq > 35	Cq < 35	2019-nCov not detected
Cq > 35	Cq > 35	Cq > 35	Invalid results (possible reasons: insufficient RNA material, poor RNA quality, error in RT-qPCR mixes)

of May, Madrid was in phase 0 (beginning phase towards the new normality and relaxation of the isolation measures). During this period, the activity in some workplaces was restored, the stores began to open by previous appointment and people were allowed to go out with time limitation and following a time schedule. These activities began to increase the levels of atmospheric pollutants, being noticed a difference between the first days of May and the end of the month (Fig. 2a and b), also enhanced by the precipitation events and general unstable meteorological conditions.

Once the atmospheric conditions became more stable, the study began. Hence, the filters were obtained from May 4th to May 21st. The filters were collected at the beginning of phase 0 and were analyzed simultaneously to their collection.

It should be mentioned that in the filters collected from the 4th and the period between the 7th and 9th of May, there was presence of

high COVID-19 presence was observed too. However, in Madrid, during the pandemic lockdown, the limit values of pollution were never exceeded. On March 14th, Spain declared a state of alarm and a lockdown situation until April 28th. In these conditions, only essential and primordial activities could be carried out. From April 28th until the end

Table 5
Samples collected (filter code and fraction of PM) with the results obtained.

Sample start time	Filter code	Fraction	N1	N2	RP	Result
04/05/2020 16:30	HV-QZ-016/2020	PM10	undet	undet	30,93	negative
04/05/2020 16:30	HV-QZ-017/2020	PM2,5	undet	undet	32,89	negative
04/05/2020 16:30	HV-QZ-018/2020	PM1	undet	undet	34,85	negative
05/05/2020 12:30	HV-QZ-020/2020	PM10	undet	undet	35,68	negative
05/05/2020 12:30	HV-QZ-021/2020	PM2,5	undet	undet	undet	negative
05/05/2020 12:30	HV-QZ-022/2020	PM1	undet	undet	34,57	negative
07/05/2020 11:30	HV-QZ-024/2020	PM10	undet	undet	33,3	negative
07/05/2020 11:30	HV-QZ-025/2020	PM2,5	undet	undet	35,05	negative
07/05/2020 11:30	HV-QZ-026/2020	PM1	undet	undet	undet	negative
19/05/2020 9:00	HV-QZ-028/2020	PM10	undet	undet	32,53	negative
19/05/2020 9:00	HV-QZ-029/2020	PM2,5	undet	undet	undet	negative
19/05/2020 9:00	HV-QZ-030/2020	PM1	undet	undet	36,7	negative
20/05/2020 10:01	HV-QZ-032/2020	PM10	undet	undet	undet	negative
20/05/2020 10:01	HV-QZ-033/2020	PM2,5	undet	undet	31,42	negative
20/05/2020 10:01	HV-QZ-034/2020	PM1	undet	undet	43,75	negative
21/05/2020 10:01	HV-QZ-036/2020	PM10	undet	undet	33,95	negative
21/05/2020 10:01	HV-QZ-037/2020	PM2,5	undet	undet	undet	negative
21/05/2020 10:01	HV-QZ-038/2020	PM1	undet	undet	undet	negative

Saharan dust. This is an important event since dust storms are very effective for the propagation of several kind of viruses. In the case of the influenza A virus, it was shown that when the levels of Asian dust particles were significantly higher than the average ones, there was greater spread (Chen et al., 2016). However, in our study, during this period, the three investigated genes were negative suggesting that there was no presence of SARS-CoV-2 RNA.

It should be taken into account that some factors could also have affected the RNA identification from the virus in the PM10, PM2.5 and PM1 filters collected in May (Table 5) in Madrid. First, the limitations of socio-economic activity which led to the low atmospheric pollution contamination levels (lower daily particulate matter means values in outdoor air). This is also reinforced by the sampling site, which is located at an urban background area near the university with no academic activity during the sampling period and therefore low pedestrian flow. This was reflected in a fifty percent reduction in the cumulative incidence rate since the beginning of the study compared to the end of May in the Moncloa-Aravaca district (Fig. 3a and b). Finally, the meteorological conditions that cleaned up the atmosphere in the period before the sampling could have helped reducing the PM as well as the virus concentrations. This could be enhanced by the meteorological stability and higher temperature that characterize spring season.

Our results support the idea that, to be able to identify the SARS-CoV-2 RNA in outdoor samples both high PM levels and high COVID-19 presence have to occur at the same time. This is in accordance with previous studies that identified the presence of SARS-CoV-2 RNA in PM10 from outdoor air samples under conditions of atmospheric stability and high PM concentrations together with high COVID-19 presence. In fact, these studies suggested that the identification of the SARS-CoV-2 RNA in outdoor samples could be considered as an indicator of

the severity of the COVID-19 infection, in terms of diffusion and health outcomes observed, as occurred in northern Italy and the USA (Setti et al., 2020c; Tung et al., 2021; Zoran et al., 2020).

On this regard, the search for viral genomes in particles could be considered as a preventive strategy for future epidemics and strategies for the reduction of PM emitted by some anthropogenic sources. This would allow to mitigate the exposure of citizens to PM and uncontrolled aerosols, which is well known that cause many negative health effects. In relation with COVID-19, air pollution could influence its progression by increasing host susceptibility to viral infections and by independently increasing the risk of cardiovascular complications, chronic obstructive pulmonary disease (EPOC), and other conditions that increase the severity of viral infections (Setti et al., 2020b; Zoran et al., 2020). However, more research needs to be done to know the role of ambient air pollution in the spread of this virus.

Particularly in Madrid, it would be interesting in the near future to measure again the presence of SARS-CoV-2 RNA in the PM filters. This is due to the increase in the number of COVID-19 cases observed in the city after the partial recovery of the socio-economic activity that also led to an increase of contamination under different atmospheric and meteorological situations (Tellier et al., 2019).

An additional step would be to investigate not only the presence but the possible virulence of SARS-CoV-2 present in the particles to see if the virus can remain vital and infectious for a defined time in the outside particles (Ficetola et al., 2020.; van Doremalen et al., 2020).

5. Conclusions

No presence of SARS-CoV-2 in quartz fiber filters samplers for PM10, PM2.5 and PM1 fractions was observed in our study conducted in Madrid during May 2020. Nevertheless, the absence of viral genomes could be due to different factors, including the limitations of socio-economic activity in the country that result in reduced circulation of the SARS-CoV-2 in general, and around the experimental site, in particular. Atmospheric and meteorological situation could have also affected the dispersion capacity of the atmosphere and the state of pollution concentration level. In addition, this study was performed in spring season, which is characterized by higher temperature and meteorological stability. Further research should be carried out during winter, in presence of higher viral circulation and daily PM exceedances. Finally, as there is no standardized method for sampling the virus in PM10, PM2.5 and PM1, this has to be developed and carefully checked in every stage of the sampling and the analytical procedure. For these reasons, further research needs to be performed to investigate the influence of these factors and discerning the role of pollution in the aerial transmission of the virus.

Author contributions

"Conceptualization, E.V., B.A and J.A; methodology, E.DR. and L.R.; investigation, B.LP., L.R and E.DR; data curation, B.A. and E.V.; writing—original draft preparation, B.LP., L.R and E.DR.; writing—review and editing, E.V., B.A. and J.A; supervision, B.A. and E.V.; project administration J.A. All authors have read and agreed to the published version of the manuscript."

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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