

SARS-CoV-2 in Municipal Wastewater Treatment Plant, Collection Network and Hospital Wastewater

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Abstract

The current outbreak of coronavirus disease (COVID-19) has led to creating a public health emergency conditions since 2019. COVID-19, which is caused by SARS-CoV-2, is spread via human-to-human transmission by direct contact or droplets. Through conducting this study, we were looking for detecting SARS-CoV-2 in wastewater produced in Iran country (Ardabil, Nir, Khalkhal and Givi) (wastewater collection network, wastewater treatment plant and hospital wastewater). In this research, samples ($n = 76$) were collected from influent and effluent of municipal and hospital wastewater treatment plants and some samples were also collected from Ardabil municipal wastewater manholes. The sampling duration included the white (lower risk of covid-19) and red (high risk of covid-19) conditions. Samples were stored at -20°C for further diagnostic tests. Out of 76 samples, a total of 16 samples (21.05%) collected from wastewater in Ardabil province (Ardabil, Nir, Khalkhal, and Givi), were positive in terms of SARS-CoV-2. Wastewater epidemiology can facilitate detection of the incidence of pathogens through metropolises, measurement of population prevalence without direct testing, and provision of information to public health system about the efficiency of intervening efforts.

Highlights

- Double-membrane vesicles may play a role in SARS-COV-2 resistance in harsh conditions.
- 05 % of wastewater samples were positive for coronavirus.
- When the area was a white condition for coronavirus, the results of all the samples examined from different parts of the wastewater treatment plant were negative.
- In the case of the red condition, the results were positive.
- Two of the studied samples in the hospital wastewater were positive for the presence of coronavirus.
- All the samples of Ardabil province wastewater collection network except 4 cases were affirmed to be negative in terms of the presence of coronavirus.

1. Introduction

Coronaviruses are single-stranded positive-sense RNA viruses, which are a member of the family *Coronaviridae* and the order of *Nidovirales* and are widely distributed in human and mammalian species (Chekol and Melesse 2020; Nghiemet al. 2020; Sarailooet al. 2021). Despite the mildness of most coronavirus infections, outbreaks of both beta-coronaviruses has been associated with acute respiratory syndrome coronavirus (SARS-CoV) (Drostenet al. 2003; Ksiazeket al. 2003; Kuikenet al. 2003) and Middle East acute respiratory syndrome coronavirus (MERS-CoV) (Dargahiet al. 2021; de Grootet al. 2013; Zaki et al. 2012). These two diseases have led to death of a large number of people ($> 10,000$) in the last two decades. Their death rates are reported to be 10% and 37%, respectively. In December 2019, a series of unknown cases of pneumonia, which had medical symptoms similar to viral pneumonia appeared in Wuhan, China. According to advanced sequencing analysis of lower respiratory tract samples, a new coronavirus known as the New Coronavirus 2019 was detected (Dargahi Abdollahet al. 2021; Munsteret

al. 2020). In the early stages of this pneumonia, symptoms of severe acute respiratory infection appeared, which in some patients developed acute respiratory distress syndrome (ARDS), severe respiratory failure, and other complications (Chenet al. 2020). As reports have indicated, clinical symptoms of patients with COVID-19 include cough, fever, sore throat, diarrhea, fatigue, and shortness of breath (Chenet al. 2020; Michael-Kordatouet al. 2020).

The occurrence of current epidemics around the world has been associated with growing knowledge about the risk of exposure to emerging pathogens during the collection and treatment of wastewaters. The entrance of emerging pathogens into wastewater systems may occur through the following ways including pathogen shedding in human waste, release of contaminated wastewater, illegal activity, animal farming, and hospital effluents, or surface water runoff from a wide-area following a biological incident. Some emerging pathogens (e.g., Ebola [EBOV] virus and SARS-CoV-2) are serious health threats, so that serious health consequences may be rised due to their discharge to the sewage system (Lahrichet al. 2020; Mallapaty 2020). Therefore, the assessment of the possibility of exposure to virus and its transmission through sanitation systems is of great importance. Previous studies have been indicative of the detection of SARS-CoV-2 and its RNA in sewage excreted from people's saliva, sputum, and feces (Waggoner et al. 2020). The inhalation by person-to-person transmission and aerosol/droplet, as well as fomite and hand contamination have been mentioned as foremost pathways for transmission of virus (Manojet al. 2020). Nonetheless, current data suggest that a better understanding of the role of wastewater, which is considered as a possible source of epidemiological data and as a risk factor for public health is an important subject. Detecting the SARS-CoV-2 in feces has led to conducting multiple research projects throughout the world to encourage the examination of wastewater for evaluating its circulation in populations (Lodder and de Roda Husman 2020; Mallapaty 2020).

Scientific evidence of SARS-CoV-2 ribonucleic acid (RNA) has also been perceived not only in the feces of patients with symptoms but also in asymptomatic patients with COVID-19 and its transfer to wastewater has been confirmed (Caiet al. 2020; Gaoet al. 2020; Holshueet al. 2020; Wuet al. 2020). These studies have shown the possibility of presence of virus in municipal sewage of communities infected by virus. Wastewater-Based Epidemiology (WBE) has been introduced as a auspicious way for realizing the prevalence of viruses for a population at wastewater treatment plants (WWTPs) because the wastewater contains the viruses that enter the wastewater treatment plant and are excreted in the environment from individual with and without symptoms (Sinclairet al. 2008; Xagoraraki and O'Brien 2020). According to previous reports provided by the studies conducted on intestinal viruses (e.g., norovirus, hepatitis A virus, and poliovirus), the early warning of disease outbreaks and awareness of the effectiveness of public health interventions is possible using mentioned approach, i.e., WEB (Asghar et al. 2014; Hellmeret al. 2014). In Iran, this disease was first announced in February 2019. During the conducting this study and its completion (February 12, 2021), Iran with over 1,496,455 confirmed COVID-19 infections and more than 58,751 deaths was ranked 15th in the world in terms of coronavirus (<https://www.worldometers.info/coronavirus/>). Due to the impossibility of simultaneous testing of all members of the community, dozens of research groups around the world are examining wastewater analysis methods as a way for estimating the total number of individuals infected with the new

coronavirus. Scientists believe that these methods can be of great value, especially if COVID-19 returns to communities. In addition, the virus in the sewage can be transmitted to humans in various ways and cause disease again in exposed individuals. The studies showed that coronavirus were able to survive in faces for 3 days and in raw chlorine-free water and hospital wastewater for 2 days at 20°C. If wastewater is not treated, the virus can enter surface and groundwater and thereby cause environmental pollution and disease in humans (Gundyet al. 2009; Lodder and de Roda Husman 2020; Medemaet al. 2020a; Naddeo and Liu 2020).

Although several studies in different countries of the world (such as the Netherlands, the United States and Australia) have reported molecular detection of SARS-CoV-2 in wastewater treatment plant samples (Lodder and de Roda Husman 2020; Medemaet al. 2020a; Waggoner et al. 2020; Wu et al. 2020), no reports for detecting SARS-CoV-2 in Iranian wastewater have been provided. As no study has so far been conducted on the identification of SARS-CoV-2 in wastewater collection network, wastewater treatment plants and hospital wastewater, the aim of conducting this study was the identification of SARS-CoV-2 in wastewater produced in Ardabil, Nir, Khalkhal and Givi (wastewater collection network, wastewater treatment plant and hospitals wastewater).

2. Material And Method

2.1. Approval statement

This study was **confirmed** to by the ethical code IR.ARUMS.REC.1399.249 in agreement to the ethical concept and the general custom and standards for accompanying medical research in Iran.

2.2. Specimen collection, storage, and transfer

In this project, **wastewater samples were collected** from influent and effluent of municipal and hospital wastewater treatment plants and some samples were also collected from Ardabil metropolitan wastewater manholes. Ardabil province is one of the northwestern Iran with a human population of about 625000 people. Some parts of the province are affected by the corona virus, and are often considered as red zones alert. Ardabil province has 10 cities, which are listed in Fig. 1.

The sampling period included the white (lower risk of covid-19) and red (high risk of covid-19) situations. The location of sampled points in the present study are presented in Fig. 1. To collect samples, staffs used standard personal protective equipment (PPE) (such as long pants, security boots, hardhat, goggles, and protection gloves) in agreement with the health procedure for sampling from sewage (Waggoner et al. 2020). The sample size for each infected manhole was 250 ml that was dispensed and preserved in special closed glass bottles. It should be noted that all sampling steps were accomplished according to the standard technique (Federation and Association 2005). The sample size was selected based on the number of Ardabil towns, hospitals and wastewater treatment plants. We also followed to the sampling procedures of similar studies to determine the number of samples needed. In the present study, 76 samples were taken from the main manholes and important points of all Ardabil cities. Samples were

collected using the combined sampling method (by sampling from a specific point at different times 10 am to 2 pm and combining them together). The Biochemical oxygen demand (BOD), Chemical oxygen demand (COD) ratio and temperature in influent 0.56, 0.62, 0.59, 0.55 and 11.8 were respectively. The samples were transferred to the laboratory on ice and stored at the -20° C in order to deactivate the bacterial activity and subsequently the degradation for further analyses. A 24-hour sample of raw wastewater was taken from the wastewater and pasteurized at 60 ° C for 90 minutes to inactivate the virus.

2.3. RNA extraction method

The sample was then filtered through 0.45-μm-pore-size (Millipore Sigma) to remove bacterial cells and supernatant of pre-centrifugation is applied to the filter devise. Pre-centrifugation that is commonly used in concentration, to remove larger particles and fragments, could affect the recovery. To concentrate the samples isolated from the wastewater, 100–200 ml of the sample were centrifuged (Universal 320R) at 4750 g for 30 minutes and then the supernatant was carefully removed without disturbing the stagnation of the material. Supernatant were then centrifuged at 3500 g for 15 minutes along with the centrifugal filter with a cut-off of 10 kDa. The containers including the concentrated sample were placed upside down on top of the sample filter cup and centrifuged at 1000 rpm for 2 minutes. We took 250 μL of the concentrated sample from the collection cup with a pipette and extracted the sample directly using the RNA extraction kit using the (high pure viral nucleic acid kit). Bacteriophage φ6 used as external control in order to determinate the effectiveness of RNA extraction. Appropriate primers and probes were designed for N and RdRP genes to perform Realtime PCR. The following steps were performed for virus identification: At the first stage, the appropriate concentrations of reagents, temperature cycle and sufficient number of replication cycles were observed for initial screening stage. The specific primer and probe real-time reverse transcriptase- polymerase chain reaction (RealTime PCR) targeting ORF1ab and N genes (Nucleoprotein gene) were applied to detect viral genomes of the SARS-CoV-2 virus in the wastewater samples. Applied Biosystems™ Real-Time PCR System 7500 with software v2.0.5 was used to run Real TimePCR. Appropriate concentrations of the synthesis reaction for Real time PCR MasterMix are as follows: H₂O (RNase free) 1.1 μl, 2x Reaction mix 12.5 μl, MgSO₄(50mM) 0.4 μl, BSA (1 mg/ml) 1 μl, Primer RdRP_SARSr-F and 2019-nCoV_N F (10 μM stock solution) 1.5 μl, Primer RdRP_SARSr-R and 2019-nCoV_NR (10 μM stock solution) 2 μl, Probe RdRP_SARSr-P and 2019-nCoV_N P (10 μM stock solution) 0.5 μl, SSIII/Taq EnzymeMix 1 μl, Total reaction mix 20 μl, Template RNA, add 5 μl, Total volume 25 μl. Thermal Cycler: 55°C 10' 94°C 3' 94°C 15" 58°C 30", 45x (Table 1). (Cormenet al. 2020; Waggoner et al. 2020).

2.4. Positive and negative control

Positive Control 2019-nCoV-qPCR was used to monitor whether the qRT-PCR process worked properly in each detection run. Negative Control 2019-nCoVqPCR was used to monitor whether there was any

contamination for the rRT-PCR course in each detection run. An internal control in extraction step was used by Wuhan Corona virus N-gene kit (TIB Molbiol, Berlin, Germany) to authorize the extraction and PCR amplification procedure.

2.5. Running real-time PCR and data analysis

The micro tube containing extracted genome along with other reaction components to detect the SARS-CoV-2 virus was placed in the real time thermal cycler, Then the real-time PCR results were interpreted based on data analysis criteria by operator .

3. Results And Discussion

Table 1 shows the specifications of all samples of SARS-CoV-2 collected in the wastewater treatment plants. SARS-CoV-2 samples collected in the wastewater treatment plants in two different white and red conditions were examined. According to the results presented in Table 1, when the area was a white zone for corona virus, the results of all the samples examined from different parts of the wastewater treatment plant were negative and in the case of the red alert condition, the results of the samples were also positive. The results taken from hospitals wastewater are summarized in Table 2. A total of 15 contaminated samples of SARS-CoV-2 were collected from hospitals wastewater in Ardabil province (Ardabil, Nir, Khalkhal, and Givi) (Table 3). Samples taken from hospital wastewater in white condition in terms of coronavirus were examined. According to the presented results, two of the studied samples (Imam Khomeini Corona wards and Ardabil City Health Center) in the hospital wastewater were positive for the presence of corona virus.

The results obtained from identification of SARS-CO-2 virus in wastewater collection network are summarized in Table 4. A total of 4 contamination samples of SARS-CoV-2 were collected from wastewater collection network in Ardabil province (Ardabil, Nir, Khalkhal, and Givi) (Table 4). In fact, the samples collected from the sewage collection network were examined in white condition of coronavirus threat. According to the presented results, all the studied samples of Ardabil province wastewater collection network (Ardabil, Nir, Khalkhal and Givi cities) (except 4 cases) were affirmed to be negative in terms of the presence of coronavirus.

When the prevalence of coronavirus in the community is low, if the virus sample become positive in the community sewage, surveying the sewage contamination with the virus can be a means to monitor the spread of the virus in the community (Medema et al. 2020b). Despite the fact that RT-PCR detects SARS-CoV-2 in a large proportion of fecal samples, there are only two researches that have recorded infectious virus culture from stool. (Wanget al. 2020). As a result, sewage does not appear to be a major transmission route for SARS-CoV-2. These findings have significant implications to determine how WBE may help identify diseases across communities and predict community incidence without the need for separate tests, as well as educate public health officials on the effectiveness of treatments. According to the records available, SARS-CoV-2 seems to have a poor level of environmental stability. These viruses

can be inactivated or removed using traditional wastewater treatment methods. The viral RNA, on the other hand, can still be present in the treated wastewater. (Amoahet al. 2020).

Since viral envelopes are susceptible to disconnection by detergents including bile salts, maximum enveloped viruses do not recruit infection in the gastrointestinal tract. While enteric coronaviruses are noteworthy exceptions, it is uncertain if these enveloped viruses will survive the harsh conditions of viral pathogenesis in the gastrointestinal tract. CoVs' RNA-producing machinery interacts with altered host membranes, such as double-membrane vesicles (DMVs), which are produced from membranes of Coronavirus replication factories in the secretory pathway, which could contribute to coronavirus resistance in harsh environments. Convolved membranes and DMVs, which are hallmarks of beta-CoV infections, as well as DMSs, which have not previously been identified for beta-Corona viruses, are among the interconnected modified membrane structures induced (Fig 2). Replication factories for Coronavirus SARS-CoV-2 appears to be deactivated in water earlier than non-enveloped human enteric viruses that have been linked to waterborne transmission. Temperature has been shown to be a key factor in viral survival in studies. While SARS-CoV-2 survives in medical and domestic wastewater for two days at 20°C and 14 days at 4°C, the future of SARS-CoV-2 in wastewater still seems to be unknown. (Alygizakiset al. 2020). Recovery rates for enveloped viruses including SARS-CoV-2 are also acceptable. According to the findings of this research, all the different parts of Ardabil and Khalkhal wastewater treatment plants were red in corona red conditions. The main outputs of both cities were positive, which shows that following coronavirus through sewage is one of the best and most economical ways to detect coronavirus. Because through sewage, people with coronavirus can be identified with and without symptoms in different areas. Necessary precautions should be taken before different outbreaks in different areas. The lack of ideal external control criteria that could have the same characteristics as the SARS-CoV-2 was a significant shortcoming of the current protocol as defined.

As a limitation of this study, the lack of equipment such as ultra-centrifuge and DNA sequencer can be further evaluated. More study is needed to adapt to enveloped viruses, as well as the sampling and concentration methods commonly carried out in aquatic media..

4. Conclusion

Our findings show that testing SARS-CoV-2 in wastewater is possible.

These results can help decision makers to make appropriate decisions on the main prevention measures such as social distancing and quarantine efforts based on wastewater catchment-level estimations of prevalence.. WBE is a suitable way to recognize the prevalence of viruses among the population based on wastewater treatment plants, because sewages contain the viruses from individuals with and without symptoms that enter the wastewater treatment plant.

Declarations

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Conflict of interests:

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.

Ethical approval:

No ethical approval was required as all the data analyzed were publicly available.

Consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and material:

The dataset and analyzed during the current study are available from the corresponding authors on realistic demand.

Code availability:

Not applicable

Author Contributions statement:

Chiman karami: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Supervision, Funding acquisition. Abdollah Dargahi & Mehdi Vosoughi: Methodology, Validation, Formal analysis, Writing - original draft. Farhad Jeddi: Methodology. Hamed Zandian Validation, Formal analysis, Supervision. Ali Normohammadi and Vahid Asghariazar: Methodology. Ahamad Mokhtari: Writing - original draft. Morteza Alighadri: Formal analysis, Investigation. Anoshirvan sedigh: Formal analysis, Investigation.

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Tables

Table 1

Primers and probes used in this study

Organisms	Target gene	Sequence (5'-3')	Cycling parameters
SARS-CoV-2	Probe & Primer ORF1a/b	FACAGGTGGAACCTCATCAGGAGATGC-BBQ F-GT GARATGGTCATGTGTGGCGG R-CARATGTTAAASACACTATTAGCATA	55°C 10' 94°C 3' 94°C 15'' 58°C 30'' 45x.
	Primer & Probe N gene	F-AAATTTGGGACCAGGAAC R-TGGCAGCTGTGTAGGTCAA PFAM-ATGTCGCGCATTGGCATGGA-BHQ	55°C 10' 94°C 3' 94°C 15'' 58°C 30'' 45x.

Table 2

Identification of Covid 19 virus in wastewater treatment plants

wastewater treatment plant	Corona virus status				
	White		Red		
	Results	Results	Ct- ORF1ab gene	Ct- N gene	
Ardabil and Nir	Inlet wastewater	Negative	Positive	35.12	32.21
	Aeration basin	Negative	Positive	36.31	32.68
	Secondary sedimentation basin 1 (before chlorination)	Negative	Positive	35.95	33.5
	Secondary sedimentation basin 2 (before chlorination)	Negative	Positive	35.72	32.3
	Outlet wastewater (after chlorination)	Negative	Positive	37.8	34.01
Khalkhal and Givi	Inlet wastewater	Negative	Positive	36.31	33.17
	Secondary sedimentation basin	Negative	Positive	35.46	31.61
	Sludge drained into the bed	Negative	Positive	36.73	32.48
	Outlet wastewater (after chlorination)	Negative	Positive	34.66	30.57

Table 3

Identification of Covid 19 virus in hospitals wastewater

City	Hospitals	Results	Ct- ORF1ab gene	Ct- N gene
Ardabil and Nir	Imam Khomeini (Corona wards)	Positive	38.45	33.68
	Imam Khomeini (Public wards)	Negative	-	-
	Imam Reza	Negative	-	-
	Ghaem	Negative	-	-
	Alavi	Negative	-	-
	Bouali	Negative	-	-
	Fatemi	Negative	-	-
	Isar	Negative	-	-
	Sabalani	Negative	-	-
	Ardabil City Health Center	Positive	38.19	34.4
	Nir City Health Center	Negative	-	-
Khalkhal and Givi	Imam Khomeini Khalkhal	Negative	-	-
	Imam Khomeini Givi (Infectious)	Negative	-	-
	Imam Khomeini Givi (Public wards)	Negative	-	-

Table 4

Identification of Covid 19 virus in wastewater collection network

City	Sample code	Results	Ct-ORFlab	Ct- N gene	City	Sample code	Results	Ct-ORFla	Ct- N gene
Ardabil	1	Negative	-	-	Nir	3	Negative	-	-
	2	Negative	-	-		4	Negative	-	-
	3	Negative	-	-		5	Negative	-	-
	4	Positive	38.51	36.71		6	Negative	-	-
	5	Negative	-	-		7	Negative	-	-
	6	Negative	-	-		8	Positive	37.69	33.4
	7	Negative	-	-	Khalkhal	1	Negative	-	-
	8	Negative	-	-		2	Negative	-	-
	9	Negative	-	-		3	Negative	-	-
	10	Negative	-	-		4	Negative	-	-
	11	Positive	39.47	36.57		5	Negative	-	-
	12	Negative	-	-		6	Negative	-	-
	13	Negative	-	-		7	Negative	-	-
	14	Negative	-	-		8	Negative	-	-
	15	Negative	-	-		9	Negative	-	-
	16	Negative	-	-		10	Negative	-	-
	17	Negative	-	-	Givi	1	Negative	-	-
	18	Negative	-	-		2	Negative	-	-
	19	Negative	-	-		3	Negative	-	-
	20	Negative	-	-		4	Negative	-	-
	---	---	---	---		5	Negative	-	-
	Nir	1	Negative	-		6	Negative	-	-
	2	Positive	37.85	36.26					

Figures

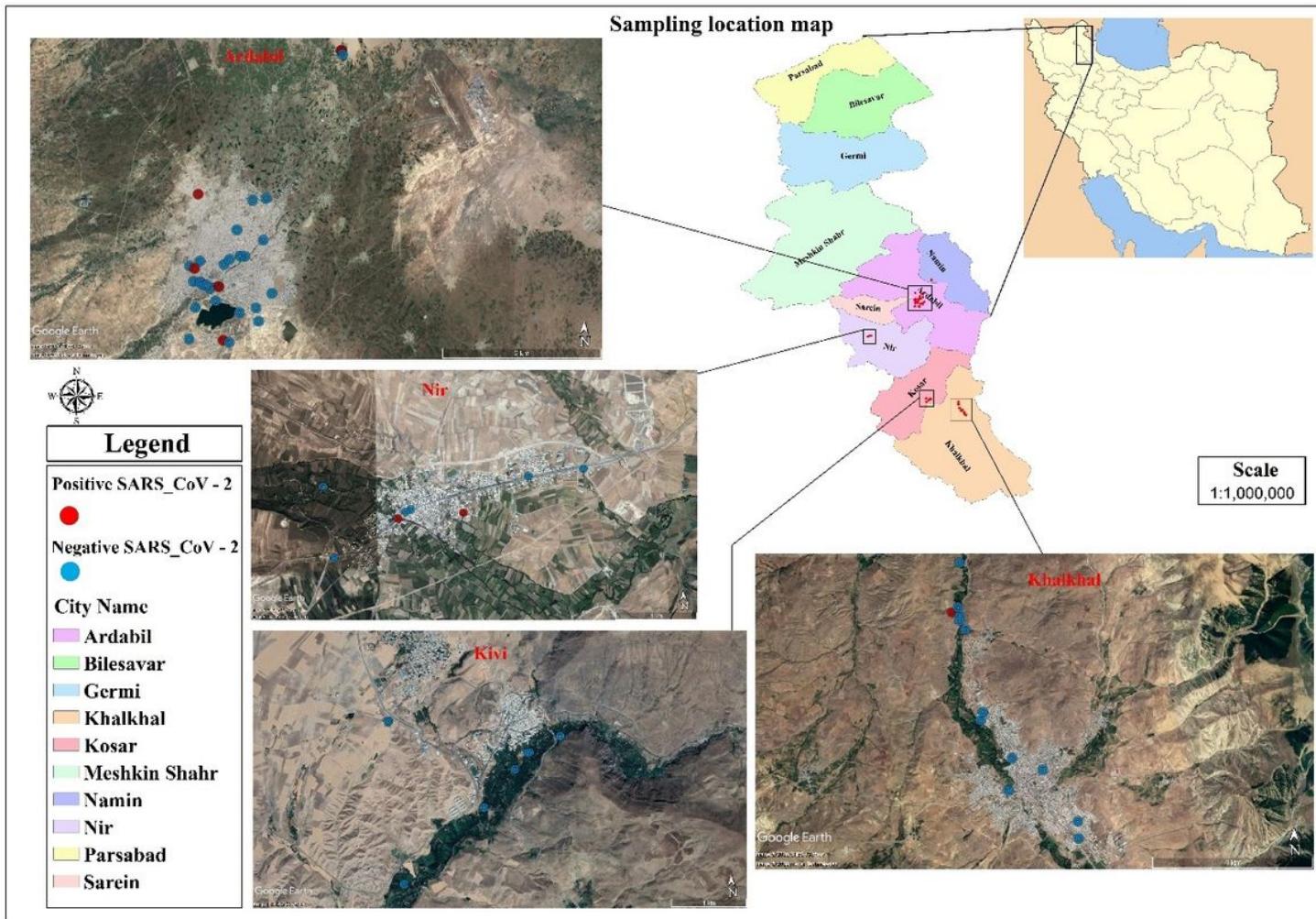


Figure 1

Position of different sampling points in this study

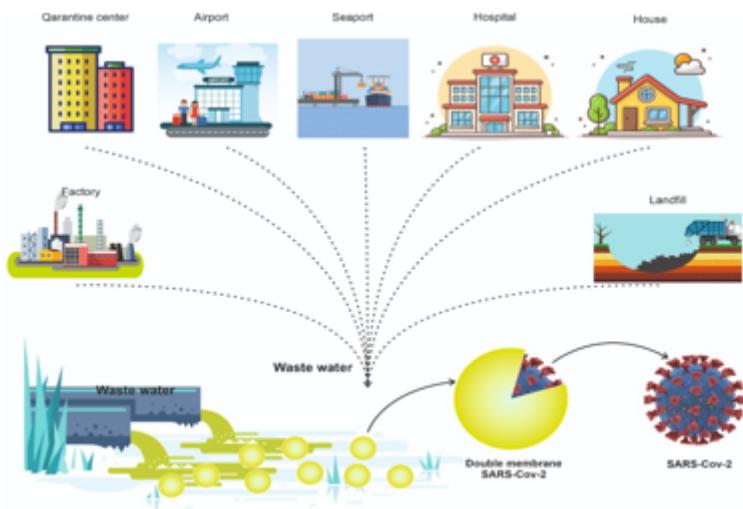


Figure 2

Please see the Manuscript file for the complete figure caption

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