Supplementary Information

Molecular monitoring of SARS-CoV-2 in different sewage plants in Venice and the implications for genetic surveillance

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Supplementary Figure S1. Localization of the wastewater treatment plants investigated in the current study. CH indicates the Civil Hospital. SF indicates Sacca Fisola.
Optimization of the analytical method for SARS-CoV-2 RNA detection in liquid samples

Procedures for aqueous samples concentration, RNA isolation and viral genome quantification by qRT-PCR were optimized stepwise, under controlled laboratory conditions. All tests were run in duplicate. IN, OUT and EF negative samples were artificially spiked with two different doses of a thermally inactivated SARS-CoV-2 strain (IZSVe20VIR1935, derived from cell culture supernatant), yielding a final concentration of approximately $10^8$ and $10^5$ genome copies per liter (GC/L) to mimic the level of virus contamination typically recorded in field samples $^{(9,13)}$. Infectious bronchitis virus (IBV; strain QX/IBV/Chicken/Italy/2000), an avian gamma-coronavirus, was seeded to negative samples to double check the efficiency of the viral concentration phase and to assess the possibility of using this virus as process control during field samples analysis (5 µl of virus with hemagglutination titer of 1:256 per 50 ml sample). The absence of cross-reactivity between the IBV genome and SARS-CoV-2 oligonucleotides was preliminarily verified in silico and in vitro. Centricon Plus-70 Centrifugal Filter 100 KDa and Amicon Ultra-15 (Millipore) with different molecular cut-offs (i.e. 10 KDa, 30 KDa, 50 KDa, 100KDa) were initially compared for their efficiency. Amicon Ultra-15 with a nominal molecular weight limit of 30 kDa, resulted in slightly better concentration efficiency for both SARS-CoV-2 and IBV, and thus were used for all subsequent steps. For the optimization of the viral concentration phase the following conditions were evaluated: (i) processing of 45 mL of material through sequential centrifugation rounds of 15 mL each using the same column; (ii) addition of glycine 1M to the Amicon Ultra-15 columns before material processing; (iii) washing-out by reconstituting the concentrate to a volume of 15 mL with nuclease-free water followed by a centrifugation step; and (iv) filter washing with 100 µL of nuclease-free water. Two sample fractions were analyzed by qRT-PCR (see below) to quantify both the virus recovered in the concentrated sample in the filter device as well as the virus lost in the filtered fraction. For each matrix, a non-concentrated sample was used as reference to calculate the recovery concentrate with the following formula:

$$\text{Recovery concentrate (SARS-CoV-2)} = \frac{\text{GC/L concentrated sample}}{\text{GC/L non concentrated sample}}$$
For RNA isolation from the concentrated IN, OUT, EF and PT spiked samples, the Quick Viral RNA kit (Zymo research) and the QIAamp Viral RNA kit (QIAGEN) were compared. To further optimize this step, different ratios of the concentrated sample volume and RNA elution volume were tested. To monitor any issue occurring during the extraction phase and the presence of residual PCR inhibitors in the purified RNA, an exogenous internal control (IC-RNA intype, Indical Bioscience) was added to the lysis buffer for each sample in a quantity equal to 1/10 of sample elution volume.

Quantitative real-time RT-PCR (qRT-PCR) targeting the E (39) and N (i.e. N1 and N2) genes (40) were assembled using the TaqMan™ Fast Virus 1-Step Master Mix (Thermofisher Scientific). Briefly, 5 µl of RNA were added to the reaction mix containing 5 µL of 4X RT-PCR Buffer, 1 µL 20X RT-PCR Enzyme mix, 0.4 µM primers, and 0.2 µM probe, 2 µL of 4 mg/mL BSA (Bovine Serum Albumin, Thermofisher Scientific) (for IN and OUT, only) and nuclease-free water up to 20 µL. For IN, PT, OUT and EF, qRT-PCR was optimized to co-amplify in a duplex format the IC-RNA, as well (final concentration of primers and probe equal to 2.5 µM and 1.875 µM, respectively). For the SL samples, process conformity was verified through the amplification of RNAse P in a separate reaction. qPCRs were run on a LightCycler 480 instrument (Roche Diagnostics), under the following cycling conditions: 50˚ C for 5 min, 45 cycles of 95˚ C for 10 sec and 60˚ C for 30 sec. The oligonucleotide sets used are reported in Supplementary Table S1.

For each viral target, the analytical limit of detection (LoD) (2 GC/µl of total RNA) and the limit of quantification (LoQ) (6.32 GC/µl of total RNA for E gene, 20 GC/µl of total RNA for N1/N2 gene) were assessed using serial dilutions of plasmids containing the target region (i.e. 2019-nCoV_E and 2019-nCoV_N plasmid containing both N1 and N2; Integrated DNA Technologies). Calibration curves were verified to fulfill optimal values under the experimental settings adopted in this study (amplification efficiency=2.02, slope=3.3 and Y intercept=41.64, expressed as mean value of all experiments run). The optimized laboratory workflow based on the experimental conditions that showed the best performances was applied for the analysis of field samples, as reported in the main text.
**Supplementary Table S1**: Real-time RT-PCR oligonucleotides used in the study.

| Oligos            | Sequence                          | Reference |
|-------------------|-----------------------------------|-----------|
| E_Sarbeco_For     | ACAGGTACGTAAATAGTTAATAGCGT        | (39)      |
| E_Sarbeco_Rev     | ATATTGCAGCAGTACGCACACA            |           |
| E_Sarbeco_Probe   | FAM-ACACTAGCCATCTCTTACTCGCGCTTCG-BHQ1 |           |
| 2019-nCoV_N1-For  | GACCCAAAAATCAGCGAAA              | (40)      |
| 2019-nCoV_N1-Rev  | TCTGGTTACTGCGAGTTGAAATCTG         |           |
| 2019-nCoV_N1-Probe| FAM-ACCCGCATTACGGTTGGAGACC-BHQ1’ |           |
| 2019-nCoV_N2-For  | TTACAAACATTTGGCCGCAAA            | (40)      |
| 2019-nCoV_N2-Rev  | GCCCGCACATTCCGAAGAA              |           |
| 2019-nCoV_N2-Probe| FAM-ACAATTTGCCCCAGCGCTCAG-BHQ1   |           |
| RNAseP_For        | AGATTTGGACCTGCGAGCG              | (40)      |
| RNAseP_Rev        | GAGCGGCTGTCTCCAAAGT              |           |
| RNAseP_Probe      | FAM-TTCTGAACCTGAAGGCTCGCG-BHQ1   |           |
| IC-11F            | CAGCCACAAGCTCTATATCATG           | (41)      |
| IC-2R             | GAACCTGATCGGACGACCAGT            |           |
| IC- Probe         | CYS-AGCACCGAGTCGCGGTGAGC-BHQ2    |           |
| IBV_For           | GCTTTGAGCGGCTATG                 | modified from 42 |
| IBV_Rev           | GCCATGTCTGCTGCATTG               |           |
| IBV_Probe         | FAM 5’-CACCACAGAGTCTGACAATG-3’BHQ1 |           |
Performances of optimized laboratory procedures

The efficiency of the concentration step for IN, OUT and EF artificially contaminated samples was assessed by qRT-PCR, measuring viral concentration in the original spiked matrices and in the concentrate samples. Quantification data for the E and N1 target genes showed that ultrafiltration with Amicon Ultra-15 (30 KDa) resulted in a concentration between 33- and 723-fold. A comparison with the viral load measured in the flowthrough (filtrate sample) indicates that the amount of virus lost along the concentration process was negligible (Supplementary Table S2). Similar results were observed for IBV (Supplementary Table S3), employed as an internal process control for the concentration step that demonstrated to be suitable as an internal process control for the concentration phase. Overall, the mean recovery efficiency for spiked SARS-CoV-2 RNA (expressed in percentage as the ratio between GC/L seeded and GC/L obtained after concentration) for all the matrices and the gene targets investigated was 79 ± 25 %. Notably, no sample showed inhibition of the amplification reaction despite the concentration process as assessed by IC-RNA conformity (Cq < 28 for all analyzed samples).
**Supplementary Table S2:** Performance of the concentration step of spiked wastewater matrices IN, OUT and EF.

| Matrix | Spiked Dose | GC/L original spiked sample | GC/L concentrate sample | GC/L filtrate | Recovery Concentrate (fold) |
|--------|-------------|-----------------------------|-------------------------|--------------|-----------------------------|
|        | E gene      | N1 gene                     | E gene                  | N1 gene      | E gene                      | N1 gene |                     |
| IN     | High        | 5.58E+07                    | 1.76E+09                | 3.12E+09     | 2.22E+11                    | 1.35E+0 | 3.63E+0             | 56      | 126             |
|        | Low         | 5.03E+05                    | 2.74E+06                | 1.66E+07     | 4.59E+08                    | Negative| 1.61E+03           | 33      | 168             |
| OUT    | High        | 3.11E+06                    | 1.18E+07                | 9.56E+08     | 2.38E+09                    | 2.94E+04| 1.40E+05           | 307     | 203             |
|        | Low         | 1.01E+04                    | 1.66E+05                | 7.31E+06     | 1.83E+07                    | Negative| 5.91E+03           | 723     | 110             |
| EF     | High        | 2.15E+06                    | 2.06E+06                | 1.33E+08     | 2.85E+08                    | 9.11E+03| 1.62E+04           | 62      | 138             |
|        | Low         | 1.37E+04                    | 9.19E+03                | 9.27E+05     | 2.24E+06                    | 8.96E+01| Negative            | 68      | 244             |
**Supplementary Table S3:** Recovery concentration of IBV QX artificially spiked into liquid matrices calculated as the difference between the Ct values of the concentrate sample and the original spiked sample.

| Matrix | Ct original spiked sample | Ct concentrate sample | Ct Filtrate | Recovery Concentrate (DCt) |
|--------|---------------------------|-----------------------|-------------|---------------------------|
| IN     | 25.4                      | 21.2                  | 35          | 4.2                       |
| OUT    | 24.8                      | 19.2                  | Not analyzed | 5.6                       |
| EF     | 29                        | 24.3                  | 34.9        | 4.7                       |
**Supplementary Table S4.** Time course detection of SARS-CoV-2 RNA in different sample matrices. Results are described separately for CH (a) and SF (b). Values are reported as GC/L. Mean estimates of GC/L per matrix were calculated to assess overall changes in viral load across different sample types. The number of positive cases, based on daily regional and hospital bulletins are also reported.

a.

| Date       | CH N. | IN E | N1   | N2   | PT E | N1   | N2   | SL E | N1   | N2   | OUT E | N1   | N2   | EF   |
|------------|-------|------|------|------|------|------|------|------|------|------|-------|------|------|------|
| 07Sep2020  | 1     |      |      |      |      |      |      |      |      |      |       |      |      |      |
| 21Sep2020  | 3     |      |      |      |      |      |      |      |      |      |       |      |      |      |
| 05Oct2020  | 13    |      | 2.91E+03 | 7.83E+03 | 6.36E+03 | 5.01E+04 | 2.77E+04 | 1.47E+05 | 1.89E+07 | 9.07E+05 | 3.47E+04 |      |      | 1.05E+04 |
| 17Nov2020  | 42    |      | 1.68E+04 | 1.71E+04 | 7.77E+03 | 1.25E+05 | 1.28E+04 | 5.55E+04 |      |      |      |      |      |      |
| 30Nov2020  | 53    |      | 6.88E+04 | 1.99E+05 | 1.23E+05 | 2.84E+06 | 5.56E+06 | 3.66E+06 | 1.18E+06 | 1.07E+06 | 3.09E+05 |      |      |      |
| 14Dec2020  | 73    |      | 2.47E+03 | 5.81E+03 | 9.91E+03 | 3.96E+04 | 2.78E+04 | 9.31E+04 | 2.21E+06 | 8.76E+06 | 1.47E+06 |      |      |      |
| 29Dec2020  | 66    |      | 1.04E+05 | 2.32E+05 | 1.09E+05 | 1.78E+06 | 5.53E+04 | 2.29E+05 | 1.03E+06 | 1.78E+06 | 1.47E+06 |      |      |      |
| 02Feb2021  | 22    |      | 1.50E+05 | 7.02E+03 | 3.77E+04 | 8.42E+05 | 2.60E+06 | 9.08E+04 |      |      |      |      |      |      |
| 16Feb2021  | 2     |      | 1.75E+05 | 2.77E+04 | 9.57E+05 | 3.17E+06 | 6.90E+05 |      |      |      |      |      |      |
| Mean       |       | 6.09E+04 | 7.55E+05 |      | 2.64E+06 |      |      |      |      |      | 1.05E+04 |      |      |      |
### b.

| Date       | SF  | N.  | IN     | E   | N1   | N2   | PT     | E   | N1   | N2   | SL     | E   | N1   | N2   | OUT    | E   | N1   | N2   | EF     |
|------------|-----|-----|--------|-----|------|------|--------|-----|------|------|--------|-----|------|------|--------|-----|------|------|--------|
| 08Sep2020  | 380 |     |        |     |      |      |        |     |      |      |        |     |      |      |        |     |      |      |        |
| 21Sep2020  | 388 |     |        |     |      |      |        |     |      |      |        |     |      |      |        |     |      |      |        |
| 05Oct2020  | 813 | 6.91E+03 | 5.97E+03 | 6.39E+04 | 3.07E+05 | 1.48E+05 |  |     |      |      |        |     |      |      |        |     |      |      |        |
| 19Oct2020  | 1549 |   |        |     |      | 5.68E+04 |        | 1.13E+05 | 3.90E+04 | 8.29E+03 | 6.66E+03 |     |      |      |        |     |      |      |        |
| 02Nov2020  | 4594 | 4.26E+03 | 9.60E+04 | 2.18E+05 | 3.16E+05 | 2.51E+06 | 2.26E+06 | 3.54E+05 | 5.06E+05 | 5.69E+03 |     |      |      |        |     |      |      |        |
| 16Nov2020  | 8862 | 9.35E+03 | 3.50E+04 | 3.51E+03 | 3.67E+04 | 6.15E+04 | 1.14E+05 | 1.16E+06 | 2.72E+06 | 1.99E+05 | 1.87E+03 |     |      |      |        |     |      |      |        |
| 30Nov2020  | 11267 |     |        |     |      | 1.28E+05 |        | 1.73E+04 | 2.74E+06 | 3.55E+06 | 1.83E+06 |     |      |      |        |     |      |      |        |
| 14Dec2020  | 11515 |     |        |     |      | 4.57E+04 | 9.63E+03 | 7.72E+03 | 3.26E+05 | 1.42E+06 | 5.03E+05 | 1.30E+04 | 3.62E+03 |     |      |      |        |     |      |      |        |
| 29Dec2020  | 12505 | 3.83E+03 |    |     |          | 7.05E+03 | 2.29E+04 | 4.28E+06 | 8.40E+06 | 3.88E+06 |     |      |      |        |     |      |      |        |
| Mean       |     |      |        |     |      | 1.05E+04 |          | 8.91E+04 | 2.02E+06 | 1.87E+03 | 9.05E+04 |     |      |      |        |     |      |      |        |
Supplementary Table S5. Values of standard deviation (SD GC/L) calculated for (a) the estimated viral load for the different matrices from the CH (related to quantification data reported in Table S4a) and (b) the estimated viral load for the different matrices from SF (related to quantification data reported in Table S4b). Table (c) reports the SD values based on the viral loads for matrices IN + PT for comparison with number of hospitalized cases as per regional bulletin (see Table S6). The symbol (+) refers to samples that yielded only one positive replicate in more than one gene target.

a.

| Date       | N. | IN   | PT   | SL   | OUT  | EF   |
|------------|----|------|------|------|------|------|
|            |    | E    | N1   | N2   | E    | N1   | N2   | E    | N1   | N2   | E    | N1   | N2   |
| 07Sep2020  | 1  |      |      |      |      |      |      |      |      |      |      |      |      |
| 21Sep2020  | 3  |      |      |      |      |      |      |      |      |      |      |      |      |
| 05Oct2020  | 13 | +    | 8.12E+03 | 3.41E+03 | 5.77E+04 | 1.09E+04 | 1.74E+05 | +    | 1.41E+05 | 6.76E+05 |      |      |      |
| 17Nov2020  | 42 | 1.94E+04 | +    | 4.98E+03 | +    | 2.95E+03 | 2.10E+04 |      |      |      |      |      |      |
| 30Nov2020  | 53 | 3.06E+03 | 6.04E+04 | 5.89E+04 | 3.30E+04 | 2.45E+06 | 1.42E+06 | 3.73E+05 | 4.77E+05 | 6.31E+05 |      |      |      |
| 14Dec2020  | 73 | +    | 2.67E+03 | 7.42E+03 | 3.84E+04 | 1.09E+04 | 4.82E+04 | 9.28E+05 | 2.87E+06 | 5.90E+06 |      |      |      |
| 29Dec2020  | 66 | 5.68E+04 | 1.29E+05 | 7.47E+04 | 1.14E+05 | 1.56E+04 | 8.66E+04 | 1.60E+05 | 1.43E+06 | 1.64E+06 |      |      |      |
| 02Feb2021  | 22 |      |      |      |      |      |      |      |      |      |      |      |      |
| 16Feb2021  | 2  |      |      |      |      |      |      |      |      |      |      |      |      |
| SF (SD GL/L) | N.  | IN     | PT     | SL     | OUT    | EF    |
|-------------|-----|--------|--------|--------|--------|-------|
|             |     | E  | N1  | N2  | E  | N1  | N2  | E  | N1  | N2  | E  | N1  | N2  |
| Date        |     | E  | N1  | N2  | E  | N1  | N2  | E  | N1  | N2  | E  | N1  | N2  |
| 08Sep2020   | 380 | 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 21Sep2020   | 388 | 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 05Oct2020   | 813 | 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 19Oct2020   | 1549| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 02Nov2020   | 4594| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 16Nov2020   | 8862| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 30Nov2020   | 11267| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 14Dec2020   | 11515| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| 29Dec2020   | 12505| 1,02E+03 | 3,00E+03 | 2.64E+04 | 1.66E+05 | 4.06E+04 | |
| Sampling (IN+PT from the CH) | Cases number | SD GC/L - Estimated Infected Cases (EIC) |
|-----------------------------|--------------|-----------------------------------------|
|                             |              | E | N1 | N2 |              |              |              |              |
|                             |              | N | J  | N  | J   | N  | J   |
| 07Sep2020                   | 1            | 6,09E+01 | 5,48E+00 | 1,92E+01 | 1,72E+00 | 0,00E+00 | 0,00E+00 |
| 21Sep2020                   | 3            | 0,00E+00 | 0,00E+00 | 5,18E+00 | 4,70E-01 | 0,00E+00 | 0,00E+00 |
| 05Oct2020                   | 13           | 6,02E+01 | 5,42E+00 | 4,15E+03 | 3,73E+02 | 1,58E+02 | 1,42E+01 |
| 17Nov2020                   | 42           | 5,49E+01 | 4,94E+00 | 1,39E+01 | 1,25E+00 | 3,37E+01 | 3,03E+00 |
| 30Nov2020                   | 53           | 1,43E+03 | 1,29E+02 | 2,83E+03 | 2,54E+02 | 1,30E+03 | 1,17E+02 |
| 14Dec2020                   | 73           | 4,13E+01 | 3,72E+00 | 1,60E+01 | 1,44E+00 | 4,72E+01 | 4,25E+00 |
| 29Dec2020                   | 66           | 1,93E+02 | 1,74E+01 | 2,09E+02 | 1,88E+01 | 3,62E+01 | 3,26E+00 |
| 02Feb2021                   | 22           | 8,00E+01 | 7,20E+00 | 4,50E+00 | 4,00E-01 | 2,01E+01 | 1,81E+00 |
| 16Feb2021                   | 2            | 9,33E+01 | 8,40E+00 | 0,00E+00 | 0,00E+00 | 1,47E+01 | 1,33E+00 |
**Supplementary Table S6.** Inference of the number of infected cases based on the viral load as assessed by E, N1 and N2 targets quantification in the influent matrices (IN + PL). For this purpose two different formulas (denoted N and J) were used and compared to the real number of hospitalized people at the CH. Mean Δ(EIC) indicates (as an absolute value) the mean value of the difference between real cases number and the number of Estimated Infected Cases calculated with the two formulas for all the genes analyzed.

| Sampling (IN+PT from the CH) | Cases number | Estimated Infected Cases (EIC) |   |   |   |   |   |   |
|-----------------------------|-------------|--------------------------------|---|---|---|---|---|---|
|                             |             | E                              | N | J | N | J | N | J |
| 07Sep2020                   | 1           | 0.00                           | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 21Sep2020                   | 3           | 0.00                           | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 05Oct2020                   | 13          | 48.87                          | 4.40 | 32.76 | 2.95 | 141.05 | 12.69 |
| 17Nov2020                   | 42          | 130.73                         | 11.77 | 27.57 | 2.48 | 58.33 | 5.25 |
| 30Nov2020                   | 53          | 2681.74                        | 241.37 | 5309.26 | 477.86 | 3486.58 | 313.81 |
| 14Dec2020                   | 73          | 38.79                          | 3.49 | 30.99 | 2.79 | 94.98 | 8.55 |
| 29Dec2020                   | 66          | 1736.94                        | 156.33 | 265.11 | 23.86 | 311.51 | 28.04 |
| 02Feb2021                   | 22          | 138.29                         | 12.45 | 6.47 | 0.58 | 34.73 | 3.13 |
| 16Feb2021                   | 2           | 161.34                         | 14.52 | 0.00 | 0.00 | 25.54 | 2.30 |

| Mean Δ(EIC) | 517.97 | 18.81 | 599.68 | 26.16 | 430.86 | 10.97 |
Supplementary Figure S2. The regions with coverage >10x in NGS sequenced samples are shown in blue. (A) VEN-IZSVe-21RS1497_VE_CH-SL collected on October 05, 2020; (B) VEN-IZSVe-21RS1495_VE_CH-IN collected on November 30, 2020; (C) VEN-IZSVe-21RS2156-3_VE_CH-PT collected on November 30, 2020; (D) VEN-IZSVe-21RS1496_VE_CH-IN collected on December 29, 2020; (E) VEN-IZSVe-21RS1498_VE_CH-PT collected on December 29, 2020; (F) VEN-IZSVe-21RS1499_VE_SF-SL November 02, 2020; (G) VEN-IZSVe-21RS2156-4_VE_SF-IN November 16, 2020; (H) VEN-IZSVe-21RS2156-6_VE_SF-PT November 16, 2020. Genome annotation is reported below.
**Supplementary Figure S3.** SARS-CoV-2 lineage distribution in the Veneto region, Italy, from October 2020 to January 2021, based on complete sequences available in GISAID (60) on December 1, 2021. Dashed lines indicate the collection dates of WW samples to which the lineage could be attributed (November 30, 2020 and December 29, 2020).