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Version 4.4

STATENS SERUM INSTITUT

Methods of the SARS-CoV-2 wastewater surveillance data in Denmark

1. How is SARS-CoV-2 monitored in wastewater?

For the national wastewater surveillance of SARS-CoV-2, 29 wastewater samples are currently taken from 28 treatment plants across Denmark – two samples are taken from two inlets of one treatment plant (Lynetten).

1.1. Coverage of wastewater surveillance

- Since 04.02.2023 and onward, 29 samples are taken from 28 treatment plants twice a week.
- In the period from 09.07.2022 to 03.02.2023, 87 samples were taken from 83 treatment plants twice a week. In addition, two weekly samples from seven decentralized sampling sites were included.
- In the period from 01.01.2022 to 08.07.2022, 202 samples were taken from 198 treatment plants and 26 samples from decentralized sampling sites three times a week.

1.2. Sampling

- 29 samples are taken from 28 treatment plants twice a week as 24-hour composite samples by autosamplers at wastewater treatment plants.
- 4 wastewater treatment plants collect time-proportional samples, with the remaining collecting flow-proportionally.

- Samples are transported and stored at 4°C and analyzed within 24 hours of sampling.

1.3. Laboratory analysis

Since 01.04.2023, the laboratory analysis has been performed at Statens Serum Institut (SSI)[1]. 10mL for each sample is processed as follows: first viral particles are concentrated using NanoTrap® Microbiome A particles (Ceres Nanosciences) and subsequently total nucleic acid is purified using Maxwell® HT Environmateal TNA reagents (Promega). The entire workflow is conducted on a Hamilton VANTAGE (Hamilton Robotics) liquid handling platform. The workflow proceeds as follows:

1. Samples are mixed by inversion
2. 10mL raw wastewater per sample
3. Split sample into 2 wells of 5mL each in 24-well Whatman Uniplate plate
4. Add 50µl of Nanotrap® Enhancement Reagent 2 (ER2) to each well
5. Using a suitable pipette, mix thoroughly 10 times
6. Add 75µl of Nanotrap® Microbiome A Beads to each well
7. For a total incubation of 10min at room temperature: Using a suitable pipette, gently mix the particles 2-3 times every 2minutes
8. Place 24-well Whatman Uniplate plate on Alpaqua MagPlate 24
9. Leave for 15min or until completely cleared
10. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet.
11. Remove all remaining supernatant from cleared centre of pellet with smaller pipette
12. Add 250uL Cell Lysis Buffer to each well
13. Resuspend Beads by shaking for 10 min at 500 RPM
14. Place 24-well Whatman Uniplate plate on Alpaqua MagPlate 24
15. Leave for 3min or until completely cleared
16. Transfer supernatant to 96-well Nunc plate combining both 24-wells from each sample into one well
17. Add 50uL Alkaline protease to each well
18. Incubate at 56°C for 20min shaking at 1200 RPM
19. Add 435 uL Resin/Isopropanol mix to each well
20. Incubate at room temperature for 20min shaking at 1200 RPM
21. Place 96-Well Deep Well Plate on Magnabot® 96 Flex Separation Device
22. Leave for 5min to clear
23. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet

[1] Until 31.03.2023, the laboratory analysis was performed by the commercial sub-contracted laboratory Eurofins Environment Testing Denmark, Eurofins Miljø A/S.

24. Add 850uL Wash solution to each well
25. Resuspend the pellet by shaking for 30 sec at 1200RPM
26. Place 96-Well Plate on Magnabot® 96 Flex Separation Device
27. Leave for 5min to clear
28. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet.
29. Add 850uL Wash solution to each well
30. Resuspend pellet by shaking for 30 sec at 1200RPM
31. Place 96-Well Deep Well Plate on Magnabot® 96 Flex Separation Device
32. Leave for 3min to clear
33. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet.
34. Add 850uL Wash solution to each well
35. Resuspend pellet by shaking for 30 sec at 1200RPM
36. Place 96-Well Deep Well Plate on Magnabot® 96 Flex Separation Device
37. Leave for 3min to clear
38. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet.
39. Add 450uL 50% EtOH
40. Resuspend pellet by shaking for 30 sec at 1200RPM
41. Place 96-Well Deep Well Plate on Magnabot® 96 Flex Separation Device
42. Leave for 5min to clear
43. Carefully discard the supernatant using a suitable pipette, without disturbing the pellet.
44. Dry residual Ethanol by incubation at room temperature for 5min
45. Add 100uL 25mM Tris-HCl pH8
46. Elute resin by shaking for at 40C for 12min at 1200RPM
47. Place 96-Well Deep Well Plate on Magnabot® 96 Flex Separation Device
48. Leave for 5min to clear
49. Transfer supernatant to elution plate

Extracted nucleic acids are either immediately analyzed by RT-qPCR (as below), or stored at -80°C until subsequent analysis.
Three extraction controls are included in each analysis run: 1 negative and 2 positive controls. The negative control consists of 10ml RNAase/DNAase free water. Positive controls consist of pooled wastewater spiked with two different concentrations of cultured virus. Positive controls are prepared in bulk and stored at -80°C until use in analysis.

The concentration of SARS-CoV-2 in wastewater extracts are determined by RT-qPCR. For data normalization the concentration of 3 faecal markers (CrAssphage, PMMoV and human mitochondrial DNA) is also determined in a separate multiplex reaction.

1.4. RT-qPCR reactions

SARS-CoV-2 is quantified using the N1 and N2 markers with the following sequences:

Target	Forward primer	Reserve primer	Probe
2019-nCoV_N1	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	TEX615-ACCCCGCATTACGTTTGGTGGACC-IB-RQ
2019-nCoV_N2	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	FAM-ACAATTTGCCCCCAGCGCTTCAG-IB-FQ

RT-qPCR is performed in 1x Luna® Universal Probe One-Step Reaction mix (NEB) supplemented with 1x Luna WarmStart® RT Enzyme (NEB) and 500µg/µL UltraPure BSA (ThermoFisher) and 5µL template per reaction. The following PCR conditions are used:

1x 10min at 55°C

1x 3min at 95°C

45x 15sec at 95°C, 30 sec at 60°C

Faecal marker are quantified using markers against human mitochondrial DNA (hCytB), pepper mild mottle virus (PMMoV) and CrAssphage (CPQ056 and CPPol) with the following sequences:

Target	Forward primer	Reserve primer	Probe
hCytB	CAATGAATCTGAGGAGGCTAC	CGTGCAAGAATAGGAGGTG	FAM-ACCCTCACACGATTCTTTACCTTTCACT -IB-FQ
PMMoV	GAGTGGTTTGACCTTAACGTTTGA	TTGTCGGTTGCAATGCAAGT	HEX-CCTACCGAAGCAAATG-MGB-EDQ
CPQ_056	CAGAAGTACAAACTCCTAAAAAACGTAGAG	GATGACCAATAAACAAGCCATTAGC	TEX615-AAT+AACGA+TTTA+CGT+GAT+GTAA+CTC-IB-RQ
CPPol	ATGTWGGTARACAATTTCATGTAGAAG	TCATCAAGACTATTAATAACDGTNACAACA	Cy5-ACCAGCMGCCATTCTACTACGAGHAC-IB-RQ

RT-qPCR is performed in 1x Luna® Universal Probe One-Step Reaction mix (NEB) supplemented with 1x Luna WarmStart® RT Enzyme (NEB) and 500µg/µL UltraPure BSA (ThermoFisher) and 5µL template per reaction. The following PCR conditions are used:

1x 10min at 55°C

1x 3min at 95°C

45x 15sec at 95°C, 30 sec at 58°C

A 5 log10 dilution of a quantification standard with a concentration of 200.000 copies / PCR reaction to 20 copies / PCR reaction for each target is included on every RT-qPCR run and also analysed in technical triplicate. The concentration of the quantification standard is independently verified using digital PCR. Finally, a DNA sample of known concentration is included on each RT-qPCR run and used monitor the stability of the quantification standard.

2. How is the normalised concentration of SARS-CoV-2 in wastewater calculated?

The result of the PCR tests is calculated as the number of SARS-CoV-2 copies or the number of PMMoV copies per well, i.e. per the amount of purified material that has been PCR tested. The PCR test has a signal if RNA is detected in the PCR well. A missing signal in one or more of the three PCR tests is assigned the value of zero copies per well. Since the analysis is based on triplicates, it is estimated that both the detection and quantification limits for the average of the triplicates is 3 copies per well. See background in Annex 1.

For the SARS-CoV-2 genes, the average number of copies measured in the three PCR tests is calculated. In the event that results are available for fewer than three of the triplicates, a decision is made together with the laboratory as to whether the result for the gene in question can be used. For approved samples, the average number of copies is determined as follows:

- With a signal in at least two of the three triplicates and an average number of copies ≥3, the average is directly used in the subsequent calculations.
- With a signal in at least two of the three triplicates and an average number of copies <3, the average number of copies is determined to be 1.5.
- With a signal in just one of the three PCR tests, the average number of copies is determined to be 0.5.
- With no signal in all the three PCT tests, the average number of copies is determined to be 0.25[2].

The number of RNA copies per well is then converted to the number of copies per litre of wastewater using the expression

$$RNA_{copies/litre} = RNA_{copies/well} * 20 * 100$$

The factor 20 corrects for the fact that 0.005 mL out of 0.1 purified mL is used in the PCR reaction. The factor 100 corrects for using 0.1 mL from 10 purified mL of wastewater.

[2] As the values in the subsequent calculations are logarithm-transformed, the average number of copies in case of missing a signal in all the tests is set to 0.25 instead of 0.

The number of RNA copies per litre of wastewater is $\log_{10}$ transformed. Then the following calculations and analyses are carried out.

For each sample, the target is the faecal-normalized concentration of SARS-CoV-2 in the wastewater. The concentration is calculated as an average of SARS-CoV-2 RNA copies per litre of wastewater, measured with the two SARS-CoV-2 genes. Then, the SARS-CoV-2 average is normalized with the faecal indicator PMMoV. This gives that

$$\log_{10}(RNA_{faeces\ mean\ normalised}) = \frac{\log_{10}(N1) + \log_{10}(N2)}{2} - \log_{10}(PMMoV).$$

Before the average normalized values are presented in graphs, the values are back-transformed from $\log_{10}$ scale to normal scale. The back transformation also uses the scaling factor 10^7, which was introduced to make the results easier to read and understand. This gives that

$$\widehat{RNA}_{faeces\ mean\ normalised} = 10^{\log_{10}(RNA_{faeces\ mean\ normalised})} * 10^7 = RNA_{faeces\ mean\ normalised} * 10^7$$

3. How are the final wastewater results calculated?

The wastewater results are presented aggregated for the whole country, for the five regions and for each sampling location. All calculations related to the wastewater measurements are made on the basis of logarithm-transformed values ($\log_{10}$). There are two general categories of the wastewater results, which are described below.

3.1. Weekly weighted average

The virus concentration of SARS-CoV-2 in the wastewater is calculated as the average weekly number of SARS-CoV-2 RNA copies, in relation to the aforementioned faecal indicator PMMoV (see sections 2 and 3). In the overall graphs (national and regional), the results from each treatment plant are weighted according to the number of inhabitants in the catchment area ($\log_{10}$ inhabitants).

3.2. Trends (growth rate)

At the national level, a growth rate is calculated to describe the trend in the SARS-CoV-2 concentration based on the last three weeks of data. The growth rate is thus the average weekly change in concentration over the past three weeks. The growth rate is calculated by using a mixed-effects model with sewage treatment plant as a random effect.

The estimate from the model is converted to an average weekly percentage change as

$$average\ weekly\ growth = (10^{growth\ rate * 7} - 1) * 100\%.$$

The growth rate is classified as one of the seven categories below.

Very strong increase	≥50%	
Strong increase	25% to 49%	
Increase	10% to 24%	
Stable	-9% to 9%	
Decrease	-24% to -10%	
Strong decrease	-49% to -25%	
Very strong decrease	≤-50%	

The growth rate is shown in the figure of the normalized concentrations of SARS-CoV-2 in the wastewater. The most recent three weeks, that the estimate for the growth rate is based on, are coloured according to the category that the growth rate falls within, and is shown along with the actual numerical estimate of the growth rate. This can be seen in the weekly publication of wastewater results on the homepage.

3.3. Level assessment

Based on the highest and lowest observed values of the weighted weekly average of SARS-CoV-2 concentrations in the period from 29.06.23 to 07.12.23, five levels have been established. The boundaries between these levels are evenly distributed on a logarithmic scale and calculated so that the lowest and highest concentrations fall into the categories 'Very low level' and 'Very high level', respectively.

The levels are marked as horizontal bands in the graph of the normalized concentrations of SARS-CoV-2 in the wastewater, which is updated weekly.

Annex

4. Laboratory determination of detection and quantification limits

To determine the detection and quantification limits, three independent dilution series are made for each SARS-CoV-2 gene. For each dilution, 24 technical replicates are made, so that there is a total of 72 data points per concentration for each gene.
The detection limit is set so that detection is expected in at least 95% of the wells and the quantification limit is set so that the coefficient of variation is below 35%.
In an experiment, dilutions were made where 1, 2, 4, 8, 16, 32, 64 and 128 copies per well were expected. On this basis, the detection and quantification limits have been chosen as the lowest concentrations that meet the criteria.

Table 1 shows the limits found for the two SARS-CoV-2 genes that are included in the analysis.

Table 1: Detection and quantification limits for the two SARS-CoV-2 genes in single wells

	Detection limit (copies per well)	Quantification limit (copies per well)
N1 gene	4	8
N2 gene	2	16

5. Data processing

Based on the above laboratory determination of the detection and quantification limits for individual wells, here we describe the background for the selected limits based on the data analysis of the technical triplicates.

5.1. Detection Limit

The starting point for choosing a detection limit is that one wants to be sure of obtaining a signal in 95% of the tested samples. The number of copies in a well follows a Poisson distribution. Given the Poisson variation, the theoretical limit is that on average there must be at least 3 copies per well to obtain a signal in 95% of the analysed samples. If one looks at technical triplicates, instead of looking at the individual well, then, with a 95% probability of detection in each well, one will have >99% probability of detection in at least two wells. In case there is a signal in less than two wells, the estimated number of copies per well is based on the number of wells with a signal.

5.2. Quantification limit

As described above, the quantification limit is chosen so that the coefficient of variation (CoV) must be below 35% - the reason is again because of a Poisson variation. By using the average of the three triplicates, this variation is reduced. Figure 1 shows the result of a simulation experiment, which was made to investigate this. For a number of concentrations, expressed as the expected number of copies per well, 100 simulations of the calculation of the CoV were made using single measurements and triplicates, respectively. The CoV was determined as the standard deviation divided by the mean of 30 determinations.

Figure 1 shows boxplots for the 100 simulations for each concentration and number of replicates. The blue boxes correspond to the above experiments. In the experiment with single wells, the CoV was determined to be 8 or 16 for the three genes. No tests were carried out with intermediate concentrations. Figure 1 shows that the median at 8 copies per well is close to a CoV < 35%. If the experiment is repeated, CoV will therefore be determined to be 8 in half of the cases and 16 otherwise, since intermediate concentrations were not investigated. The experiment is therefore in full accordance with the theoretical expectations. If the calculations of the CoV are instead based on the average of triplicates (red boxes), the quantification limit becomes 3, as the median here is below 35%.

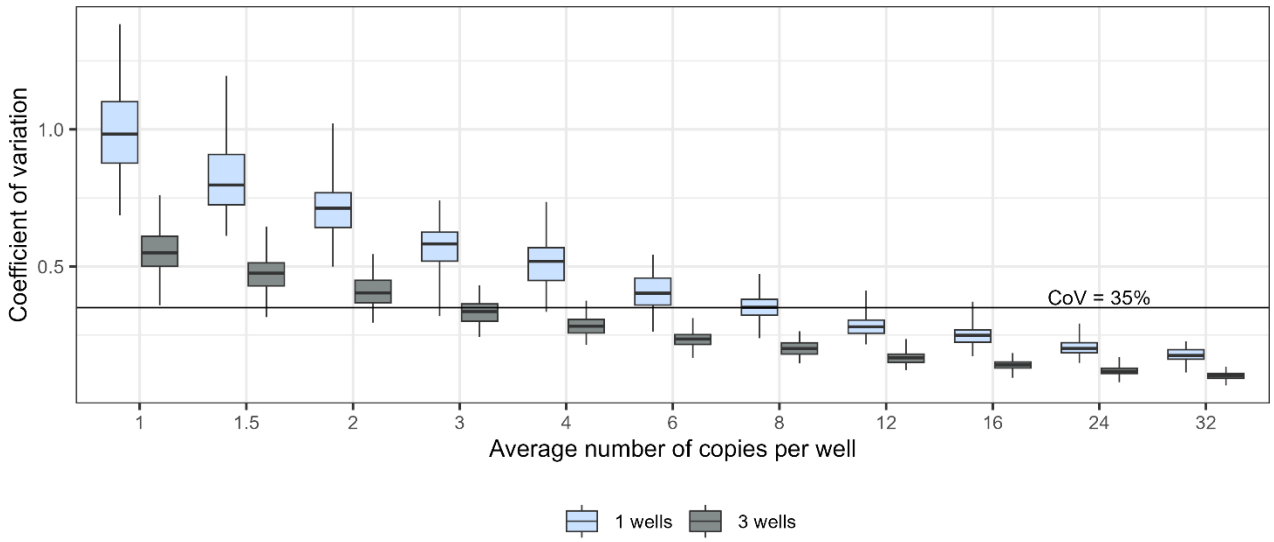

Figure 1: Simulated coefficients of variation (CoV) for different concentrations and for single wells and average of three technical triplicates.

Based on the above, it has been chosen to accept levels of quantification when the average of the triplicates is above 3. In practice, there can be several reasons why detection does not occur in a well. Therefore, the average is not used if there is only detection in one of the triplicates, nor if the average is over 6, when the probability of observing zero copies in a well is very small. In these cases, the observation is omitted until a discussion occurs with the laboratory about what is best to do.